

## Identification of CD93 expression on hematopoietic stem cells in human neonatal umbilical cord blood cells

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### Abstract

Human CD93 is a heavily *O*-glycosylated type I transmembrane protein consisting of unique C-type lectin domains (CTLDs) containing glycoprotein. CD93 is mainly expressed on myeloid cells (monocytes and granulocytes) and endothelial cells. However, the expression patterns of CD93 on various other kinds of cells are not well understood. In this study, we found that CD93 was recognized by a CD93 monoclonal antibody (mAb) (mNI-11) that was established in our laboratories and was expressed on a broad hematopoietic stem cell population (CD34<sup>+</sup> cells) from human neonatal umbilical cord blood cells (UCBCs), as shown using a two-color flow cytometric analysis. In addition, the CD93 recognized by mNI-11 was also expressed on a narrow hematopoietic stem cell population (CD34<sup>+</sup>CD45<sup>dim+</sup> cells) in which the non-specific reactivity of CD34 mAb from human neonatal UCBCs was excluded using a three-color flow cytometric analysis. Taken together, these results provide the first evidence concerning the identification of CD93 expression on hematopoietic stem cells. These cell populations (CD34<sup>+</sup>CD93<sup>+</sup> and CD34<sup>+</sup>CD45<sup>dim+</sup>CD93<sup>+</sup> cells) in human neonatal UCBCs are thought to have an important role in cell biology, transplantation, and immature/mature immune responses.

**Key words** : CD93, flow cytometric analysis, hematopoietic stem cells, umbilical cord blood cells (UCBCs).

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### Introduction

Human CD93 has a molecular weight of about 100 - kDa. CD93 belongs to the Group XIV family of transmembrane glycoproteins. This molecule is a heavily *O*-glycosylated type I transmembrane protein consisting of C-type lectin-like domains (CTLDs) containing glycoprotein, followed by a series of epidermal growth factor (EGF)-like repeats.<sup>1)</sup> Although CD93 was previously identified as a receptor for complement component 1, subcomponent q phagocytosis (C1qRp) that interacts with defense collagens such as C1q, mannose-binding

lectin (MBL), and pulmonary surfactant protein A (PS-A) and is involved in the C1q-mediated enhancement of phagocytosis for various antigens,<sup>2, 3)</sup> several recent studies have reported that CD93 is not a C1q receptor involved in the C1q-mediated enhancement of phagocytosis against various antigens and the clearance of apoptotic cells.<sup>4, 5)</sup> However, the detailed immunological functions of CD93 remain uncertain.

On the other hand, CD93 is mainly expressed on myeloid cells (monocytes and granulocytes) and endothelial cells.<sup>5)</sup> CD93 is strongly upregulated after the exposure of human monocyte-like cell line (U937) to a

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protein kinase C (PKC) activator, phorbol myristate acetate (PMA), and this upregulation is controlled by a PKC delta isoenzyme inhibitor, Rottlerin.<sup>6)</sup> However, the pattern and regulation of CD93 expression on various kinds of cells other than those with a myeloid cell lineage, especially hematopoietic stem cells possessing multipotent activities in immune responses, are not well understood.

In this study, we focused on CD93 expression in broad and narrow human hematopoietic stem cell populations (CD34<sup>+</sup> and CD34<sup>+</sup>CD45<sup>dim+</sup> cells) and investigated the expression patterns of CD93 on these cell populations in human neonatal umbilical cord blood cells (UCBCs) using a CD93 monoclonal antibody (mAb) (mNI-11) established in our laboratories and flow cytometry to define the expression pattern on immature multipotent hematopoietic stem cells.

### Materials and Methods

#### Donors and preparation of peripheral blood mononuclear cells from normal adults and umbilical cord blood cells from neonates

We prepared human peripheral blood mononuclear cells (PBMCs) from four normal adults and human neonatal umbilical cord blood cells (UCBCs) from the umbilical veins of 17 neonates according to a previously described methodology.<sup>7)</sup> Informed consent was obtained from all the donors/parents.

#### Antibodies

A phycoerythrin (PE)-conjugated CD93 monoclonal antibody (mAb) (mNI-11) (mouse IgG1)<sup>8)</sup> was established in our laboratories. Fluorescein isothiocyanate (FITC)-conjugated CD34 mAb (My10) (mouse IgG1), allophycocyanin (APC)-conjugated CD34 mAb (My10) (mouse IgG1), and peridinin chlorophyll protein (PerCP)-conjugated CD45 mAb (2D1) (mouse IgG1) were purchased from BD Biosciences Co. (USA). Isotype-matched normal mouse IgG1 was purchased from MBL Co. (Nagoya, Japan).

#### Flow cytometry

The cells were washed in cold phosphate-buffered saline (PBS) containing 0.1% NaN<sub>3</sub> (subsequently referred to as the

washing buffer) and were then incubated in PBS containing 25% normal goat serum, 1 mg/mL of normal human IgG, and 0.1% NaN<sub>3</sub> for 10 min on ice to block the Fc receptor of IgG. The cells were then incubated with an optimal concentration of PE-conjugated CD93 (mAb) (mNI-11) and FITC-conjugated CD34 mAb (My10) for 40 min at room temperature. In some experiments, the cells were then incubated with an optimal concentration of APC-conjugated CD34 mAb (My10), PerCP-conjugated CD45 mAb (2D1), and PE-conjugated CD93 (mAb) (mNI-11) for 40 min at room temperature. Negative controls were provided by incubation with isotype-matched normal mouse IgG1 under the same conditions. Following a final wash with the washing buffer and resuspension in PBS containing 2% fetal calf serum (FCS) and 0.1% NaN<sub>3</sub>, the cellular debris and aggregates were excluded. The percentages of positively stained cells were analyzed using two-color and three-color flow cytometry using a FACScan (Becton Dickinson Co.). The analysis was repeated in triplicate per sample.

#### Statistical analysis

The results were expressed as the mean  $\pm$  standard deviation (SD). The statistical analysis was performed using the Student *t*-test. Differences with a *P* value of less than 0.05 were considered significant.

### Results and Discussion

First of all, we investigated the percentage of broad hematopoietic stem cells (CD34<sup>+</sup> cells) among normal adult PBMCs and neonatal UCBCs using an FITC-conjugated CD34 mAb (My10) and flow cytometry with side scattering; the gated CD34<sup>+</sup> cells were counted. The results are summarized in Fig. 1. As shown in Fig. 1, the percentages of gated CD34<sup>+</sup> cells among normal adult PBMCs and neonatal UCBCs were  $0.026 \pm 0.015\%$  and  $0.365 \pm 0.178\%$ , respectively. The percentage of CD34<sup>+</sup> cells among the neonatal UCBCs was significantly ( $P < 0.01$ ) larger than that among the normal adult PBMCs. Figure 2 shows typical histograms for the CD34<sup>+</sup> cells among normal adult PBMCs (panel A) and neonatal UCBCs (panel B). The results indicated that CD34<sup>+</sup> cells do not exist among normal adult PBMCs. Thus, only neonatal UCBCs were used for all subsequent

experiments in this study.

Next, we investigated the expression of CD93 on the gated CD34<sup>+</sup> cells from neonatal UCBCs using a PE-conjugated CD93 mAb (mNI-11), an FITC-conjugated CD34 mAb (My10), and two-color flow cytometry. The results are summarized in Table 1. As shown in Table 1, the percentage of CD93 expression on the CD34<sup>+</sup> cells (CD34<sup>+</sup>CD93<sup>+</sup> cells) was 89.1%, while the percentage of CD34<sup>+</sup>CD93<sup>-</sup> cells was 10.9%. Figure 3 shows typical histograms for the CD93 expression on CD34<sup>+</sup> cells.

Recently, a narrow hematopoietic stem cell population, in which the non-specific reactivity of CD34 mAb was excluded, was identified as CD34<sup>+</sup>CD45<sup>dim+</sup> cells.<sup>9,10</sup> We also investigated the percentage of CD34<sup>+</sup>CD45<sup>dim+</sup> cells among neonatal UCBCs using an APC-conjugated CD34 mAb (My10), a PerCP-conjugated CD45 mAb (2D1), and two-color flow cytometry; the gated CD34<sup>+</sup>CD45<sup>dim+</sup> cells were counted. The results are summarized in Table 2. As shown in Table 2, the percentage of gated CD34<sup>+</sup>CD45<sup>dim+</sup> cells among the neonatal UCBCs was 0.24 ± 0.069%. Next, we investigated the expression of CD93 on gated CD34<sup>+</sup>CD45<sup>dim+</sup> cells using a PE-conjugated CD93 mAb (mNI-11) and three-color flow cytometry. The results are summarized in Table 2. As shown in Table 2, the percentage of CD34<sup>+</sup>CD45<sup>dim+</sup>CD93<sup>+</sup> cells was 74.9%, while the percentage of CD34<sup>+</sup>CD45<sup>dim+</sup>CD93<sup>-</sup> cells was 25.1%. Figure 4 shows typical histograms for CD93 expression on CD34<sup>+</sup>CD45<sup>dim+</sup> cells.

Human CD93 has a molecular weight of about 90 – 100 kDa and is a heavily O-glycosylated type I transmembrane protein consisting of unique C-type lectin domains<sup>11</sup> that exhibits a strong homology (67 – 87% identity) with rat and mouse CD93 (C1qRp), also known as the AA4.1 antigen.<sup>11,12</sup> The selective expression of CD93 myeloid cell lineages (granulocytes and monocytes) and endothelial cells has been reported.<sup>5</sup> In addition, the regulation of CD93 expression has been investigated in a variety of cells, particularly granulocytes, and the inflammatory peptide FMLP has been shown to upregulate the expression of this molecule rapidly.<sup>13</sup> Furthermore, CD93 is strongly upregulated after the exposure of a human monocyte-like cell line (U937) to a PKC activator, PMA, and this upregulation is controlled by a PKC delta isoenzyme.<sup>6</sup>

On the other hand, mouse CD93, known as the AA4.1 antigen, was found to be expressed in primitive hematopoietic stem cells (CD34<sup>+</sup> cells),<sup>14</sup> suggesting that this molecule may be involved in some important biological functions of immune-related cells. These findings led to the speculation regarding the possibility that human CD93 may also be expressed on hematopoietic stem cells similar to mouse CD93 (AA4.1 antigen). From this point of view, we investigated the expression of CD93 on human hematopoietic stem cells in neonatal UCBCs. As shown in this study, CD93 defined by CD93 mAb (mNI-11) was clearly expressed in both broad and narrow hematopoietic stem cell populations (CD34<sup>+</sup> and CD34<sup>+</sup>CD45<sup>dim+</sup> cells, respectively) from neonatal UCBCs. Together, these findings suggest that human CD93 on hematopoietic stem cells may be associated with immune biological activities, such as cell proliferation and differentiation/maturation.

Human immature multipotent hematopoietic progenitor cells (hematopoietic stem cells) (CD34<sup>+</sup> and CD34<sup>+</sup>CD45<sup>dim+</sup> cells) reportedly have a much higher frequency among the UCBCs of neonates than among normal adult PBMCs.<sup>15</sup> In this regard, human neonatal UCBCs have been used as a source of hematopoietic stem cells for transplantation to enable hematopoietic reconstitution and reduce the occurrence of acute graft-versus-host-disease (GvHD) in recipients after myeloablative therapy during allogeneic transplantation.<sup>16,17</sup> Thus, the identification of these new hematopoietic stem cell populations, CD34<sup>+</sup>CD93<sup>+</sup> and CD34<sup>+</sup>CD45<sup>dim+</sup>CD93<sup>+</sup> cells, among neonatal UCBCs in this study may provide important information with potential application to the fields of basic and clinical immunology. Recently, we found that CD93 is also expressed on human naive T-lymphocytes (CD4<sup>+</sup>CD45RA<sup>+</sup> cells) among neonatal UCBCs using a CD93 mAb (mNI-11) probe and flow cytometry and western blot analyses.<sup>18</sup> These results indicate that multiple forms of CD93 exist on various cells, particularly on immature immune-related cells, and suggest that CD93 may be closely associated with the development and regulation of immature cells in neonatal immune responses, such as cell proliferation, differentiation, maturation.

In conclusion, we have found that CD93 is expressed on broad and narrow hematopoietic stem cell

populations (CD34<sup>+</sup> and CD34<sup>+</sup>CD45<sup>dim+</sup>) and that these cell populations (CD34<sup>+</sup>CD93<sup>+</sup> and CD34<sup>+</sup>CD45<sup>dim+</sup>CD93<sup>+</sup> cells) exist as novel hematopoietic stem cell populations in neonatal UCBCs. Further analyses are needed to demonstrate the detailed properties of these cell populations at the cellular and molecular (messenger RNA expression of CD93) levels in both basic and clinical fields.

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Table1. Percentages of CD93 expression on CD34<sup>+</sup> cells in neonatal UCBCs

Donor	CD34 <sup>+</sup> (%)	CD34 <sup>+</sup> CD93 <sup>+</sup> (%)	CD34 <sup>+</sup> CD93 <sup>-</sup> (%)
1	0.38	87.3	12.7
2	0.30	60.8	39.2
3	0.23	91.4	8.6
4	0.29	91.8	8.2
5	0.30	92.0	8.0
6	0.34	94.2	5.8
7	0.83	88.3	11.7
8	0.25	96.8	3.2
9	0.40	95.9	4.1
10	0.51	91.5	8.5
11	0.18	89.0	11.0

The neonatal UCBCs (n=11) were incubated with an FITC-conjugated CD34 mAb (My10) and a PE-conjugated CD93 mAb (mNI-11) for 40 min at room temperature. Negative controls were incubated with isotype-matched normal mouse IgG1. The percentages of positively stained cells were determined using two-color flow cytometry. The analysis was repeated in triplicate per sample

Table 2. Percentages of CD93 expression on CD34<sup>+</sup>CD45<sup>dim+</sup> cells in neonatal UCBCs

Donor	CD34 <sup>+</sup> CD45 <sup>dim+</sup> (%)	CD34 <sup>+</sup> CD45 <sup>dim+</sup> CD93 <sup>+</sup> (%)	CD34 <sup>+</sup> CD45 <sup>dim+</sup> CD93 <sup>-</sup> (%)
1	0.23	84.9	15.1
2	0.12	59.9	40.1
3	0.33	82.9	17.1
4	0.27	54.7	45.3
5	0.26	86.9	13.1
6	0.23	80.6	19.4

The neonatal UCBCs (n=6) were incubated with an APC-conjugated CD34 mAb (My10), a PerCP-conjugated CD45 mAb (2D1), and a PE-conjugated CD93 mAb (mNI-11) for 40 min at room temperature. Negative controls were incubated with isotype-matched normal mouse IgG1. The percentages of positively stained cells were determined using three-color flow cytometry. The analysis was repeated in triplicate per sample.

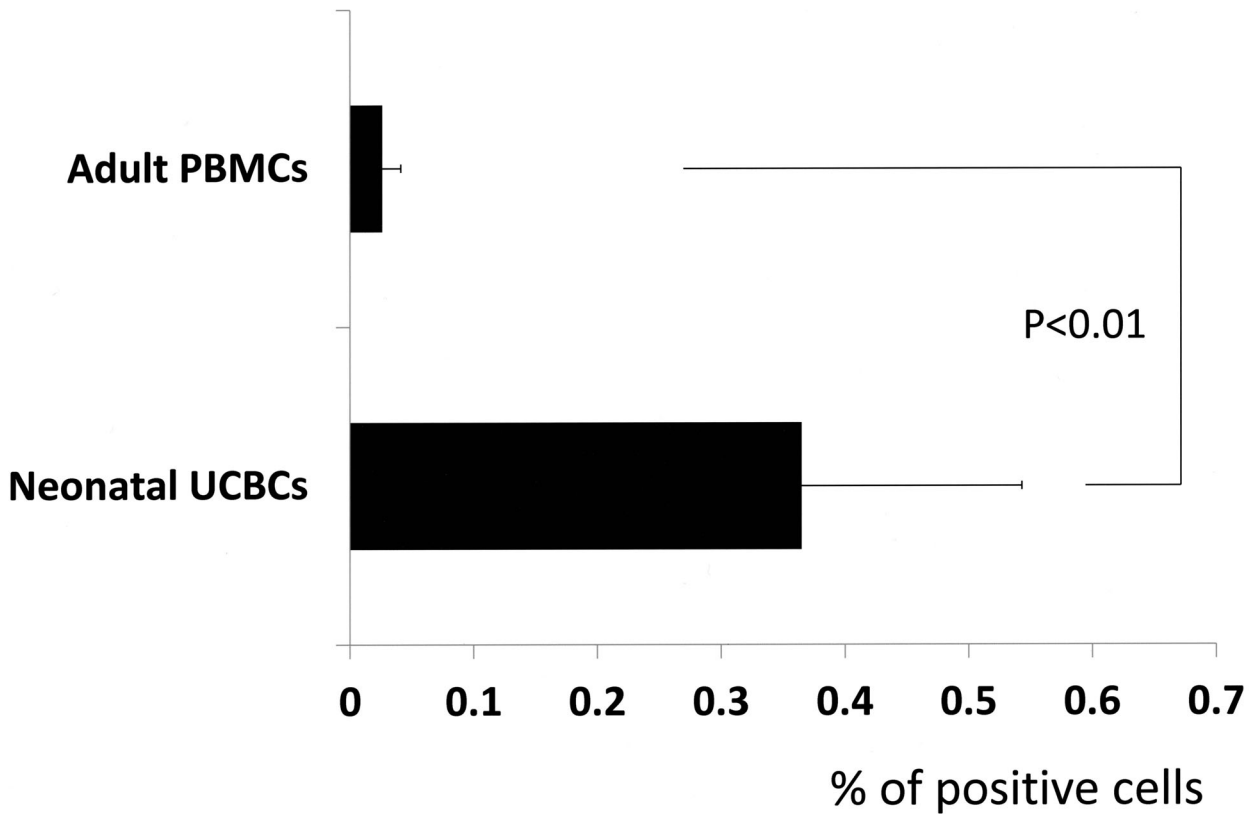


Figure 1. Percentages of CD34<sup>+</sup> cells among normal adult PBMCs and neonatal UCBCs. Normal adult PBMCs (n=4) and neonatal UCBCs (n=11) were incubated with an FITC-conjugated CD34 mAb (My10) for 40 min at room temperature. Negative controls were incubated with isotype-matched normal mouse IgG1. The percentages of positively stained CD34<sup>+</sup> cells were determined using CD34 mAb and flow cytometry with side scattering; the gated CD34<sup>+</sup> cells were counted. The analysis was repeated in triplicate per sample.  $P < 0.01$  (CD34<sup>+</sup> cells of neonatal UCBCs vs. CD34<sup>+</sup> cells of adult PBMCs).



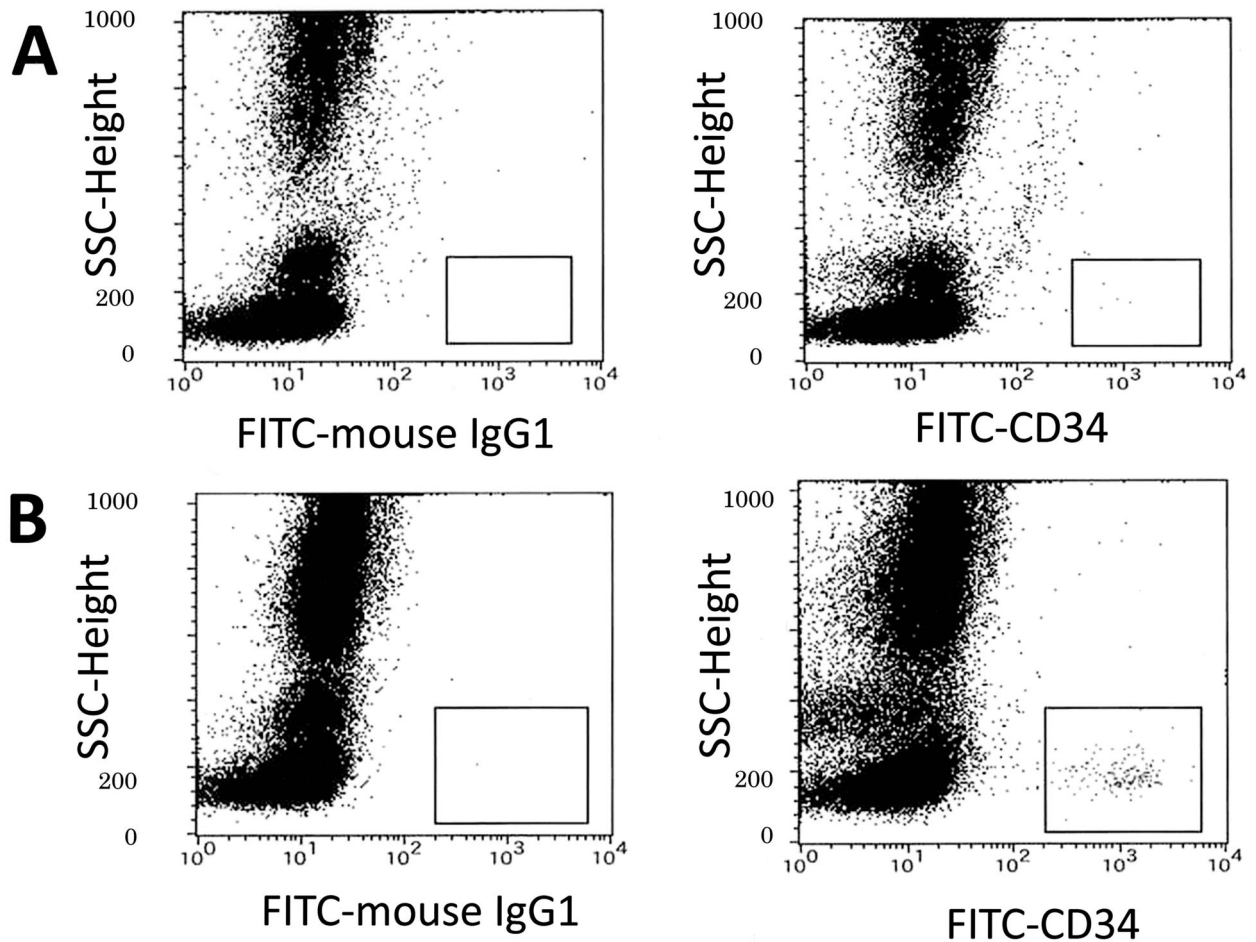


Figure 2. Typical flow cytometric histograms of CD34<sup>+</sup> cells in normal adult PBMCs and neonatal UCBCs. The normal adult PBMCs (panel A) and neonatal UCBCs (panel B) were incubated with an FITC-conjugated CD34 mAb (My10) for 40 min at room temperature. The percentage of CD34<sup>+</sup> cells among these cells was analyzed using CD34 mAb (My10) and flow cytometry with side scattering; the gated CD34<sup>+</sup> cells were counted. Negative controls were incubated with isotype-matched normal mouse IgG1. The analysis was repeated in triplicate per sample.

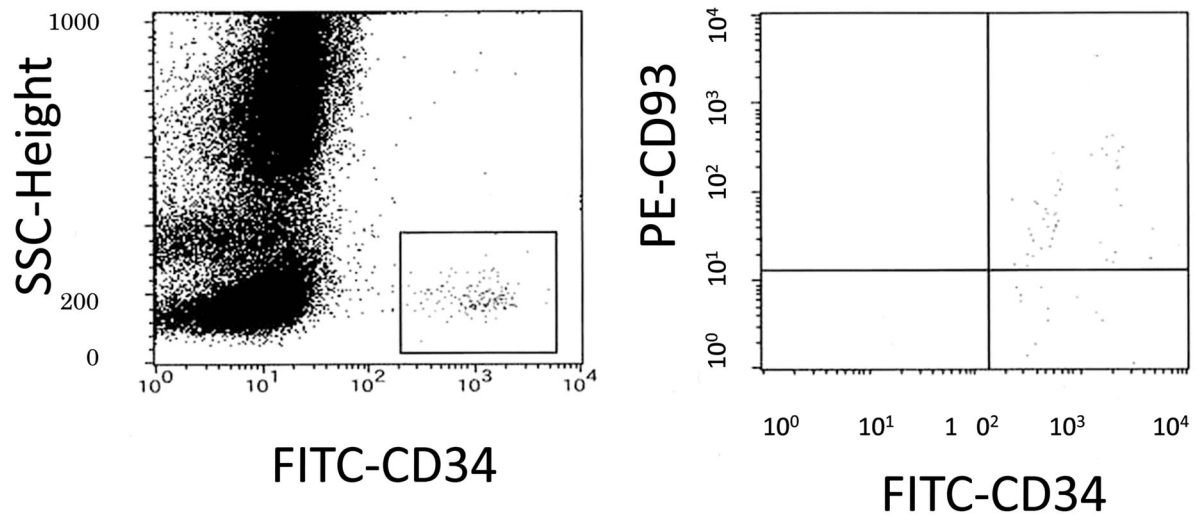


Figure 3. Typical flow cytometric histograms of CD93 expression on CD34<sup>+</sup> cells from neonatal UCBCs. The neonatal UCBCs cells were incubated with an FITC-conjugated CD34 mAb (My10) and a PE-conjugated CD93 mAb (mNI-11) for 40 min at room temperature. Negative controls were incubated with isotype-matched normal mouse IgG1. The percentages of positively stained cells were determined using two-color flow cytometry. The analysis was repeated in triplicate per sample.



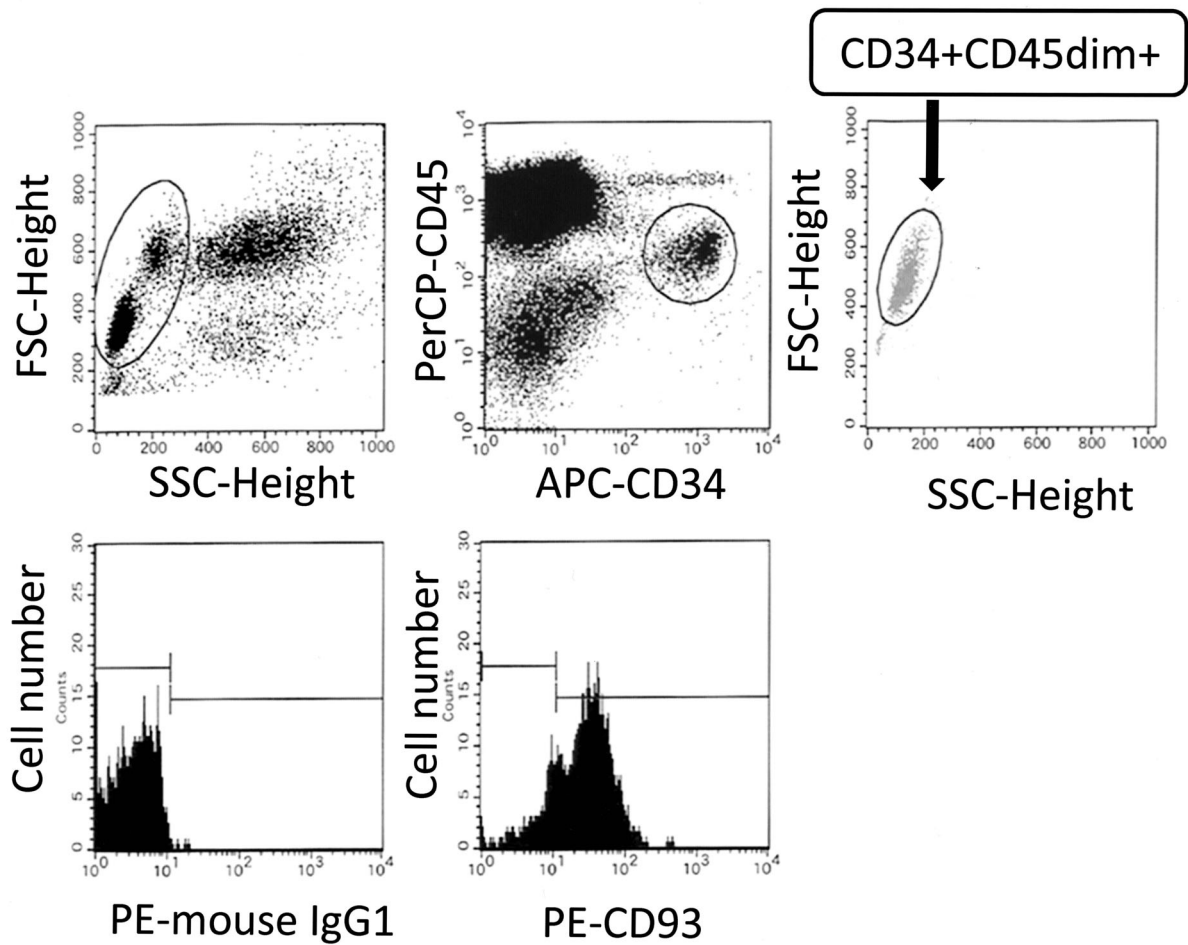


Figure 4. Typical flow cytometric histograms of CD93 expression on CD34<sup>+</sup>CD45<sup>dim+</sup> cells from neonatal UCBCs. The neonatal UCBCs were incubated with an APC-conjugated CD34 mAb (My10), a PerCP-conjugated CD45 mAb (2D1), and a PE-conjugated CD93 mAb (mNI-11) for 40 min at room temperature. Negative controls were incubated with isotype-matched normal mouse IgG1. The percentages of positively stained cells were determined using three-color flow cytometry. The analysis was repeated in triplicate per sample.

## 臍帯血由来造血幹細胞表面上のCD93の発現

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### 要旨

臍帯血由来造血幹細胞表面上のCD93の発現を我々が開発したCD93モノクローナル抗体(mNI-11)、既存のCD34抗体、CD45抗体を組み合わせたフローサイトメトリー法で解析した。その結果、臍帯血由来造血幹細胞（CD34<sup>+</sup>細胞およびCD34<sup>+</sup>CD45<sup>dim+</sup>細胞）表面上にはCD93が発現していることが分かった。以上の結果は、CD93が未熟な造血幹細胞の新たな細胞表面マーカーに成り得ると共に、造血幹細胞におけるCD93の免疫学的な機能解析に非常に有益な情報と考えられる。

キーワード：CD93 フローサイトメトリー解析 造血幹細胞 臍帯血細胞

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