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CD39/NTPDase-1 Activity and Expression in Normal Leukocytes

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Abstract

Introduction—CD39/NTPDase-1 is a cell surface enzyme expressed on leukocytes and endothelial cells that metabolizes ATP to ADP and AMP. CD39 is expressed on numerous different types of normal leukocytes, but details of its expression have not been previously determined.

Methods—We examined CD39 expression and activity in leukocytes isolated from healthy volunteers. Expression of CD39 on leukocytes was measured by FACS and activity of CD39 in lymphocytes and neutrophils was determined by an enzymatic radio-TLC assay.

Results—We established that CD39 is expressed on neutrophils, lymphocytes, and monocytes. The enzyme is found on >90% of monocytes, neutrophils, and B-lymphocytes, and 6% of T-lymphocytes and natural killer cells. Per cell density of expression varied, with the highest expression on monocytes and B-lymphocytes. ATPase and ADPase activities were highest on B-lymphocytes, lower on neutrophils, lowest on T-lymphocytes. The ratio of ADPase:ATPase activity was 1.8 for neutrophils and B-lymphocytes and 1.4 for T-lymphocytes. Hypertensive volunteers had lower levels of CD39 on their T-lymphocytes and NK cells. No correlation between age, gender, ethnic background, or cholesterol level and CD39 expression was observed.

Conclusions—We conclude that CD39 activity and expression are present to varying degrees on all leukocytes types examined. Differences between leukocyte types should be considered when examining CD39 in disease states.

Keywords

CD39; thrombosis; lymphocytes

CD39 (NTPDase-1, ecto-nucleoside triphosphate diphosphohydrolase) is an integral membrane protein that metabolizes extracellular ATP and ADP to AMP [1–3]. In 1991 Kansas et al. described the presence of CD39 on the surface of lymphocytes as well as endothelial cells [4]. The enzyme has also been reported on other leukocytes [5,6] as well as neoplastic cells in a number of hematologic malignancies [7–9]. Nevertheless, to date there has been no comprehensive description of the levels of CD39 activity and expression on normal circulating

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leukocytes. Alterations in the function of CD39 have been described in association with disease in humans and in animal models [10–12]. Thus, there is a need to define “normal” CD39 expression and function in order to develop further knowledge of the biochemistry of this enzyme.

The principal function of CD39 on the endothelial cell surface is to decrease platelet activation and recruitment by metabolizing ADP released from stimulated platelets [13]. From *in vitro* and *ex vivo* experimental evidence, we have proposed that CD39 on the endothelial cell surface represents the main control system for platelet reactivity and blood fluidity by acting as an antithrombotic agent [14]. CD39 in leukocytes has been demonstrated to have other effects as well, including modulation of cytokine expression and the inflammatory response, as well as cell-cell adhesion [4,6,15,16]. CD39 may also affect cell proliferation and apoptosis via its modulation of ATP levels in the pericellular fluid milieu [17]. Additionally, ATP and adenosine may have opposing effects in neutrophil activation., suggesting that CD39 may be a crucial regulator of neutrophil responses to stimuli [18].

Structure-function studies of CD39 suggest that the enzyme contains a single nucleotide binding domain that metabolizes both ATP and ADP. CD39 is usually more efficient in metabolizing ADP than ATP [19]. Five apyrase conserved regions are essential for preservation of CD39 enzyme activity. Importantly, enzymatic and biological activity of the enzyme are directly correlated [20].

Alterations in CD39 activity or expression in leukocytes have been described in humans in chronic lymphocytic leukemia (CLL) [8,21,22], cardiac disease [12], and HIV [10]. However, discussion of alterations in leukocyte CD39 activity is hampered by lack of a thorough description of what constitutes “normal” CD39 activity in leukocytes and each sub-type of leukocytes. As reported herein, we have addressed this question.

Methods

Donor population

Blood samples were obtained from normal healthy volunteers at the VA New York Harbor Healthcare System, Manhattan campus, and healthy controls as part of the thrombophilia in cryptogenic stroke (THICK) study currently underway at New York Presbyterian Medical Center/Weill Medical College of Cornell University. THICK is an ongoing case-control study that is investigating the role of prothrombotic disorders in the pathogenesis of cryptogenic stroke in younger adults (ages 18 to 64 years). Analyses were carried out for CD39 expression and function in addition to other procedures, including coagulation parameters. After informed consent was obtained, 45 ml of whole blood was collected from each donor via free flow into a plastic tube containing 4.6 ml 3.8% trisodium citrate. In addition, 3 ml of blood was collected in an EDTA anticoagulated tube for FACS analyses and measurement of complete hematologic parameters. Additionally, 3 ml samples of EDTA anticoagulated blood were taken from patients with acute myeloblastic leukemia (AML), chronic myelocytic leukemia (CML), and chronic lymphocytic leukemia (CLL) for FACS analysis of the neoplastic cells to compare CD39 expression in the neoplastic cells with expression in normal cells. The protocols were approved by the institutional review boards at the VA Medical Center and Weill Cornell Medical College.

Lymphocyte Isolation

Lymphocytes were isolated using a modified histopaque isolation protocol. Whole blood was centrifuged and platelet-rich plasma (PRP) removed. Phosphate-buffered saline (PBS) was added to the remaining blood to restore the original plasma volume. Blood was then diluted

1:1 with PBS and carefully layered on a histopaque gradient. Samples were centrifuged (30 min, 820g, 25°C) and the buffy coat isolated and washed with PBS (3X). Cells were then incubated in RPMI medium with 10% fetal calf serum (FCS) for 30 minutes (2X) in order to deplete monocytes, which adhered to the flask. Lymphocytes remaining in the fluid phase were washed with PBS, and re-counted. Short term FCS exposure (<24 hours) did not affect CD39 expression or function. Cell purity as assessed by FACS was >90% lymphocytes. Since B-lymphocytes tend to adhere to the flask during the monocyte depletion step, cells isolated by this method are generally >80% T-lymphocytes.

B-lymphocytes were isolated with the use of the RosetteSep (StemCell, Vancouver) B-lymphocyte isolation kit according to manufacturer's instructions. The RosetteSep B-lymphocyte isolation kit uses bispecific antibodies against CD2, CD3, CD16, CD36, CD56, CD66b, and glycophorin A to deplete non-B-lymphocytes from the histopaque layer. Cells isolated via this method were shown by FACS to be greater than 85% B-lymphocytes.

Neutrophils (PMN) were isolated via dextran gradient using the following protocol: Platelet-rich plasma (PRP) was isolated from whole blood by centrifugation and removed. Cells were diluted with normal saline to the original collection volume. Blood was then mixed with 3.5% DextranT-500 in 0.9% saline at a ratio of 35 mL dextran: 45 mL blood. Tubes containing the mixture were allowed to stand for 30–45 minutes, after which leukocyte-rich plasma (LRP) was removed and washed with saline (4°C). Remaining erythrocytes were lysed with hypertonic saline and the PMN were washed with saline. The cells were then analyzed for purity and studied if there were >90% PMN.

Assay for ADPase and ATPase Activity/TLC

To assay CD39 activity, cell suspensions were incubated with 50 μ M 14 C-labeled ADP or ATP in 50 μ L assay buffer for five minutes and the reaction terminated by addition of a "stop solution". The stop solution consisted of 160 mM EDTA (pH 7) and 17 mM ADP in 0.9% saline [13,23]. Suspended cells were then removed by centrifugation and the supernatant decanted for analysis of the reaction products. Thin-layer chromatography (TLC) was used to separate labeled nucleotides, nucleosides, and bases [12,13,24]. Their radioactivity was then measured to assess metabolism of 14 C-labeled ADP or ATP by CD39. Data are expressed as a percentage of ADP or ATP metabolized or as picomoles nucleotide metabolized per minute per 50,000 or 100,000 cells.

FACS Analysis

Cells were single-stained with CD3-APC, CD19-FITC, CD39-PE, CD4-PERCP, CD8-FITC, CD25-PE-Cy7, CD45RO-APC, and CD56-FITC (CD19-FITC and CD39-PE from Ancell, CD25-PE-Cy7 from Biolegend, all other antibodies from BD Biosciences). Cells were also double and triple stained for CD3/CD39/CD56, CD4/CD39, CD8/CD39, and CD19/CD39. CD3+ and CD19+ cells were further stained with CD25-PE-Cy7 to identify activated cells. CD4 and CD8 cells were stained with CD45RO-APC to identify activated/memory cells.

Staining was accomplished by the following procedure: 100 μ L of EDTA anticoagulated whole blood was placed in a 5 mL tube. Appropriate quantities of antibody were added to each tube. Cells plus antibody were incubated for 45–90 minutes at room temperature with gentle shaking. Erythrocytes were lysed with FACS lysing solution (BD Biosciences) and remaining leukocytes were then washed with PBS and resuspended in 0.5 mL PBS.

Acquisition and analysis of FACS data was performed on a FACSCanto apparatus (BD Biosciences) using FACSDiva software (BD Biosciences). Percentage positivity and geometric mean fluorescence (per cell density of expression) were determined. PMN,

lymphocytes, and monocytes were identified by their forward and side-scatter characteristics. T-lymphocytes were identified by expression of CD3, B-lymphocytes by expression of CD19, and natural killer (NK) cells by expression of CD56 in the absence of CD3. Killer/effector lymphocytes were identified via CD8 positivity and T-helper lymphocytes via CD4 positivity. CD25 was used to identify activated lymphocytes and CD45RO to identify activated and memory T-lymphocytes.

For measurement of activation, CD25 positivity was compared on cells which were double positive for CD3/CD39 and CD19/CD39 with those which were CD3+/CD39- or CD19+/CD39-. In addition, naïve versus activated/memory T-lymphocytes were compared.

Mitogen activation

T-lymphocytes were isolated via the Histopaque isolation technique as described above and incubated at 37°C for 1–15 days with medium (RPMI+10% fetal calf serum) alone, or medium plus lipopolysacchride (LPS) (100 ng/ml), or phytohemagglutinin (PHA) (1 µg/ml). Aliquots were removed at intervals for enzymatic activity or FACS analysis.

Statistics

When comparing groups, Student's two-sided t-test with unequal variance was used to generate p-values, except for comparisons of per cell CD39 expression in CD45RO+ versus CD45RO- T-lymphocytes and CD25+ versus CD25- lymphocytes. In those, a paired Student's two sided t-test was used.

Results

Volunteer Population

Samples were taken from 48 volunteers at the two institutions, 27 from the New York VA and 21 from Weill-Cornell. None of the volunteers had hematologic disease or overt vascular disease. Ten volunteers had hypertension (Table 1).

CD39 Activity in Resting Cells

CD39 activity was observed in T-lymphocytes, B-lymphocytes, and PMN. Activity was greatest in B-lymphocytes, lower in PMN, and lowest in T-lymphocytes (Figure 1a). The ratio of ADPase:ATPase activity was higher in B-lymphocytes and PMN than in T-lymphocytes, with an average ratio of 1.8 for both B-lymphocytes and PMN as compared with 1.4 for T-lymphocytes (Figure 1b).

CD39 Expression in Resting Cells

CD39 was expressed at varying levels on lymphocytes, monocytes, and PMN. Greater than 90% of PMN, monocytes, and B-lymphocytes expressed CD39 whereas only a minority of T-lymphocytes and NK cells expressed CD39 (Table 2). Expression was slightly greater in CD4+ T-lymphocytes than CD8+ cells. Geometric mean per cell CD39 expression was highest in monocytes and B-lymphocytes, lower in CD4+ T-lymphocytes, and PMN, and lowest in NK cells and CD8+ T-lymphocytes (Table 2). No difference in expression of CD39 was observed in male versus female volunteers, volunteers with hypercholesterolemia versus normal serum cholesterol, or smokers versus non-smokers. No correlations of CD39 expression with age were observed (data not shown). However, patients with hypertension were found to have lower CD39 expression levels in their T-lymphocytes ($p=0.01$) and NK cells ($p=0.02$) than patients without hypertension (Figure 2). This agrees with results reported by El Omar et al [12] in which subjects with hypertension had lower CD39 activity in T-lymphocytes than those without hypertension.

Moreover, CD39 expression was higher in activated (CD25+) T-cells than non-activated T-cells (Figure 3a), and activated or memory (CD45RO+) cells than naïve T-lymphocytes (Figure 3b). However, a small minority of naïve (CD45RO-) T-lymphocytes did express CD39 and most activated T-lymphocytes did not express CD39. This suggested that CD39 is not strictly an activation marker. In addition, CD39 per cell density was higher on CD4+CD45RO+ cells than CD4+CD45RO- cells ($p=0.02$) and CD3+CD25+ than CD3+CD25- cells ($p<0.0002$) (Figure 3c, 3d). A trend toward higher per cell density of CD39 was also seen on CD8+CD45RO+ versus CD8+CD45RO- cells and on CD19+CD25+ versus CD19+CD25- lymphocytes, but the trend did not reach statistical significance ($p=0.06$ and 0.08 , respectively).

CD39 was not identified on normal hematopoietic stem cells (CD34+/CD38- cells), leukemic cells in AML, or on Jurkat cells, a cell line derived from ALL. However, CD39 was seen in later bone marrow precursors, including CD34+/CD38+ cells and more differentiated leukemic cells, including cells from 20 CLL patients and one CML patient (data not shown). This suggests that CD39 appears relatively early in the differentiation of hematopoietic cells and appears in multiple lineages (i.e. both the lymphocytic and non-lymphocytic lineage) independently.

Effects of Mitogen Activation on CD39 Expression and Activity

CD39 expression and activation were measured after 1–15 days incubation with one of two mitogens: lipopolysaccharide (LPS) and phytohemagglutinin (PHA).

Enzyme activity was measured at intervals in T-lymphocytes from 5 individuals, isolated via the Histopaque procedure. Cells incubated with medium demonstrated stable to mildly decreased levels of CD39 activity over the incubation period. ADPase activity tended to decrease slightly more than ATPase activity, leading to a mildly reduced ADPase:ATPase ratio, although the significance of this effect is unclear at this time. In cells incubated with PHA, CD39 activity increased dramatically and consistently. However, the exact level of increase varied from person to person. Enzyme activity peaked at 7–10 days of incubation and fell gradually after this time (Figure 4). A similar pattern of activity was seen with LPS, but the extent of the increase in activity was much less (data not shown). In one donor, the cells did not respond to PHA and neither expansion of the lymphocyte population nor change in either CD25 or CD39 activity occurred, suggesting that the CD39 increase observed is directly related to stimulation of the lymphocytes.

We also quantitated the level of CD39 expression after incubation of T-lymphocytes with PHA or LPS. In all samples, CD39 expression in cells incubated with medium alone was stable or decreased over time in both CD4+ and CD8+ lymphocytes (Figure 5a and b). CD39 expression was slightly up-regulated in samples incubated with LPS (Figure 5a), with most of the increase being accounted for by an increase in CD8+/CD39+ cells. CD4+/CD39+ levels were stable or decreased in samples incubated with LPS. Interestingly, per cell expression of CD39 was stable in CD4+ cells and stable or slightly increased in CD8+ cells after incubation with LPS (data not shown).

When T-lymphocytes were incubated with PHA, a strong and consistent increase in CD39 expression was observed in both CD4+ cells and CD8+ cells, with a greater increase in CD8+ cells than CD4+ cells (Figure 5c). By day 3–6, the majority of CD8+ cells were CD39+. This contrasts with a baseline CD8/CD39 double positive level of about 5%. Moreover, the per cell expression of CD39 in CD8+ cells increased dramatically over time after incubation with PHA, with a 4-fold increase seen in one case, and over a 20-fold increase in another after 6–7 days of incubation (Figure 5d).

Discussion

Our studies demonstrate activity and expression of CD39 on leukocytes from normal subjects. B-lymphocytes and monocytes exhibit CD39 expression on greater than 90% of cells whereas T-lymphocytes and NK cells express CD39 on about 6% of cells. Per cell expression of CD39 was highest in B-lymphocytes and monocytes, lower in PMN and CD4+ T-lymphocytes, and lowest in CD8+ T-lymphocytes and NK cells. We also found that CD39 expression was up-regulated in T-lymphocytes upon activation and remained elevated in memory cells. This suggests that CD39, in addition to serving as the main control system for vascular fluidity, has an important role in immune modulation.

Historically, CD39 was initially cloned from B-lymphocytes and, therefore, its activity was thought to be greater in B-lymphocytes than T-lymphocytes. Interestingly, we found that a small percentage of T-lymphocytes were positive for CD39. However, 15 times as many B-lymphocytes as T-lymphocytes were found to express CD39. If we “correct” for the predicted expression of CD39, T-lymphocytes show an ADPase activity level of about 240 pmol/5 min/50K cells and an ATPase level of 165, as compared to 75 and 43, respectively, in B-lymphocytes. This might suggest that the T-lymphocyte activity of CD39 is much greater than that of B-lymphocyte CD39 per CD39 molecule or positive cell. However, interpretation of these results requires caution. First, neither the Histopaque technique nor the RosetteSep preparation produces an entirely pure population of cells. A small number of “contaminating” B-lymphocytes could alter the results of a T-lymphocyte preparation considerably. Second, some T-lymphocytes may become activated during the isolation process, although the results of the lymphocyte incubation experiments suggest that this is not a major factor since activation of T-lymphocytes was not observed in cells incubated in the absence of mitogen. We recently described several splice variants of CD39, some of which appear to decrease the activity of the full length form. Further characterization of these CD39 splice variants in different cell populations and in unstimulated versus stimulated lymphocytes may further clarify the differences observed [5].

Expression of CD39 did not vary with most measured patient characteristics. The exception to this is hypertension. Patients with hypertension tended to have lower levels of CD39 expression on their T-lymphocytes and NK cells. This difference was observed despite the fact that all volunteers with hypertension were well controlled on medications. We were not able to examine CD39 expression on endothelial cells from these subjects due to difficulties in obtaining samples. However, in the past we have examined CD39 activity on human umbilical vein endothelial cells (HUVEC). In these cells, the ADPase:ATPase ratio was similar to that of T-lymphocytes, with a ratio of 1.35 (unpublished data), suggesting that CD39 behavior in these cells is more similar to its behavior in T-lymphocytes than in other hematologic cells. We speculate that CD39 may be lower in the endothelial cells of patients with hypertension as well as in their T-lymphocytes. The lowered levels of CD39 in donors with hypertension may be a partial explanation for the observation that subjects with hypertension continue to have an elevated risk of vascular events, even after their hypertension is therapeutically corrected [25,26]. In addition, hypertension tends to be associated with a hypercoagulable state [27].

The finding that CD39 is up-regulated in activated and memory T-lymphocytes suggests that it may be involved in the inflammatory response. Its role is unclear at this time, although ATP and adenosine are known to play roles in immunity and inflammation, suggesting that CD39 may function as an immunomodulant via its ATPase activity. The function of ATP in the immune system is complex and may be concentration dependent, but in general ATP is involved in such pro-inflammatory functions as stimulation of lymphocyte proliferation [28] and cytokine release [15]. T-lymphocytes are capable of releasing ATP upon activation and the ATP is, in turn, capable of stimulating further T-lymphocyte proliferation. In addition,

generation of adenosine by CD73 from CD39's end product, AMP, may have anti-inflammatory effects [29]. This suggests that CD39 could act as a negative feedback mechanism, helping to reduce the immune response to keep it from becoming harmful to the system. If so, the relatively weak effect of LPS on CD39 induction may partially explain the extreme responses the human body develops upon exposure to LPS.

In summary, CD39 is expressed and active to a varying degree on a wide variety of leukocytes. These differences should be taken into account when examining possible alterations in CD39 activity or expression in disease states. Further information on CD39 splice variants and examination of the effects of CD39 on inflammation and immunity may lead to further insights into and therapy for a broad range of diseases including hematologic malignancies and arterial vascular diseases such as stroke. This study forms a baseline for further studies of CD39 in health and disease.

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Abbreviations

CLL	chronic lymphocytic leukemia
AML	acute myeloblastic leukemia
CML	chronic myelocytic leukemia
PRP	platelet-rich plasma
PBS	phosphate buffered saline
FCS	fetal calf serum
PMN	neutrophil
NK cell	natural killer cell
LRP	leukocyte-rich plasma
LPS	lipopolysacchride
PHA	phytohemagglutinin

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Figure 1.

Figure 1a. CD39 activity in normal leukocytes. CD39 activity as measured by radio-TLC in T-lymphocytes, B-lymphocytes, and PMN. The differences between the ADPase activity of T-lymphocytes versus B-lymphocytes or PMN and of B-lymphocytes versus PMN were highly significant ($p < 10^{-4}$). Differences in ATPase activity of T-lymphocytes versus B-lymphocytes or PMN, and B-lymphocytes versus PMN were significant as well, with a p-value of < 0.001 in each case. Error bars shown in this and all other figures represent standard deviations. Figure 1b: ADPase:ATPase ratios of normal T-lymphocytes, B-lymphocytes, and PMN. P-value for T-lymphocytes versus PMN was 0.03 and T-lymphocytes versus B-lymphocytes was 0.06.

Figure 2.

Comparison of CD39 expression in patients with and without hypertension. CD39 expression was essentially equal in volunteers with and without hypertension in PMN, monocytes, and B-lymphocytes. However, expression in T-lymphocytes and NK cells was significantly decreased in donors with hypertension ($p=0.01$ for T-lymphocytes, $p=0.02$ for NK cells). There was a trend towards decreased expression in donors with hypertension in CD8+ cells, but it was not significant.

Figure 3.

Figure 3a. CD39 status in activated (CD25+) and non-activated CD3+ T-lymphocytes. Error bars are one standard deviation. P-value for CD39 positivity in activated versus non-activated lymphocytes was less than 10^{-4} .

Figure 3b. CD39 positivity in activated/memory (CD45RO+) versus naïve T-lymphocytes. CD4+ and CD8+ lymphocytes were evaluated separately for this experiment. P-values were less than 10^{-3} for CD45RO+ versus CD45RO- lymphocytes in both the CD4+ and CD8+ populations. Notably, although almost all CD39+ cells are either activated (CD25+) or memory (CD45RO+) cells, most activated and memory T-lymphocytes are CD39 negative, indicating that CD39 is not simply a marker of activation.

Figure 3c. Per cell expression of CD39 in activated versus non-activated lymphocytes. P-value for CD3+CD25+ versus CD3+CD25- lymphocytes was 0.02 and for CD19+CD25+ versus CD19+CD25- lymphocytes was 0.08.

Figure 3d. Per cell expression of CD39 in activated/memory cells versus naïve cells. P-values were 0.0002 for CD4+ cells and 0.06 for CD8+ cells.

Figure 4.

CD39 activity after incubation of T-lymphocytes with PHA. Results represent mean results and standard deviations from four experiments. Activity peaked at 7–10 days. Control samples incubated with medium alone showed no change or a mild decrease in activity over time. Samples incubated with LPS showed a similar pattern to that of samples incubated with PHA, but to a much lesser extent.

Figure 5.

Figure 5a. CD39 expression after incubation. Results of a typical experiment showing percentage CD39 expression in all cells after incubation with medium alone, LPS, or PHA. Figure 5b: Average CD39 expression in T-lymphocytes after incubation with and without PHA. Degree of expression is shown in CD4+ cells incubated in medium only (blue bars), CD8+ cells incubated in medium only (brown bars), CD4+ cells incubated with PHA (yellow bars) and CD8+ cells incubated with PHA (light blue bars.)

Figure 5c: Example of CD39 expression in all T-lymphocytes, CD4+ and CD8+ cells after incubation with PHA for 0–10 days. CD39 up-regulation was seen in both CD4+ and CD8+

cells but the percentage increase and change in geometric mean per cell expression was greater in CD8⁺ cells.

Figure 5d: Geometric mean per cell expression of CD39 after incubation with PHA for 0–10 days in all T-lymphocytes, CD4⁺ lymphocytes, and CD8⁺ lymphocytes.

Table 1

Clinical characteristics of volunteers examined for this study.

Age	Mean 42 (N=48) (range 21–70 y/o)
Gender	Male 25
	Female 23
Ethnicity	White 28
	Black 6
	Hispanic 5
	Other 9
Smoker?	Yes 8
	No 38
	Unknown 2
Hypertension?	Yes 10
	No 38

Table 2

Average expression of CD39 in leukocytes of healthy volunteers. Percentages are presented as percentage of cell type expressing CD39. Per cell expression of CD39 is expressed as the geometric mean per cell expression by FACS for all cells of this type which are positive for CD39. CI=confidence interval

Cell type	Mean CD39 expression (%)	95% CI	Per cell CD39 expression	95% CI
PMN	98.4	97.8–99	1835	1607–2063
Monocytes	96.3	95.2–97.3	5357	4433–6281
Lymphocytes	17	15.5–18.5	2689	2466–2932
T-lymphocytes	6.1	4.7–7.6	1670	1448–1891
CD4+ T-lymphocytes	7.7	6.4–8.9	2098	1923–2274
CD8+ T-lymphocytes	5.4	3.6–7.3	989	850–1128
B-lymphocytes	94.5	93–96	3764	3355–4173
NK Cells	5.5	4.6–6.4	1079	925–1232