Morphologic, flow cytometric, functional, and molecular analyses of S100B positive lymphocytes, unique cytotoxic lymphocytes containing S100B protein.

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Abstract

Little is known about the $S100B^+$ lymphocytes, which are unique human peripheral blood lymphocytes (PBL) containing the S100B protein. It has recently been shown that S100B is released from various types of $S100B^+$ cells and exhibits varied cytokine-like activities. In this study, we precisely characterized the $S100B^+$ lymphocytes of healthy adults with respect to the proportion in the whole PBL, immunophenotypes, function, and their S100B mRNA expression and also evaluated their S100B-releasing activity upon stimulation. $S100B^+$ lymphocytes were detected in all individuals examined, and the proportion of $S100B^+$ lymphocytes in the whole PBL ranged from 0.42 to 16.15% (mean 4.21%). In addition, two subtypes of $S100B^+$ lymphocytes, a CTL subtype ($CD3^+$ $CD8^+$ $CD16^-$) and a NK subtype ($CD3^-$ $CD8^-$ $CD16^+$) were detected. The majority of the CTL subtype of $S100B^+$ lymphocytes expressed the αβ-T cell receptor. Surprisingly, S100B mRNA was detected not only in $S100B^+$ lymphocytes, but also in every $S100B^-$ lymphocytes, although the expression levels of S100B mRNA in $S100B^-$ lymphocytes

were much lower than those of $S100B^+$ lymphocytes. The CTL subtype of $S100B^+$ lymphocytes exhibited blastic morphological changes, proliferated and released S100B upon stimulation with PHA. The NK subtype of $S100B^+$ lymphocytes exhibited morphological NK activity when co-cultivated with NK-sensitive target, K-562 cells. Thus, the CTL subtype $S100B^+$ lymphocytes exhibit the biological characteristics of T cells, while the NK subtype $S100B^+$ lymphocytes exhibit the characteristics of NK cells. These results suggest that $S100B^+$ lymphocytes are a particular subtype of cytotoxic lymphocytes that play a unique role in anti-tumor immunity.

Key words: S100B, peripheral blood, cytotoxic T cells, NK cells,

Introduction

The S100 protein belongs to the EF-hand type calcium binding protein family, which includes calmodulin and the myosin light chain. S100 protein is involved in the Ca^{2+}-dependent regulation of a variety of intracellular activities, such as protein phosphorylation, enzyme activities, cell proliferation and differentiation, the dynamics of cytoskeleton constituents, the structural organization of membranes, intracellular Ca^{2+} homeostasis, inflammation, in the protection against oxidative cell damage, cell cycle progression and differentiation. The S100 protein family comprises 19 members, and is widely distributed among human tissues. Some S100 members are released or secreted into the extracellular space and thereby exert either trophic or toxic effects depending on their concentration, and thus can act as chemoattractants for leukocytes, modulate cell proliferation, or regulate macrophage activation (1-3).

S100B is localized mainly in astrocytes, and Schwann cells of the nervous system (4). In addition, the S100B protein is detected in chondrocytes, melanocytes, and

fat cells (5), dendritic cells (including interdigitating cells and Langerhans cells), and some of the peripheral blood T cells and activated macrophages in the human hematopoietic system (6, 7).

The intracellular functions of S100B include the regulation of calcium homeostasis and enzyme activity (8). In the nervous system, S100B is released by astrocytes and oligodendrocytes. Secreted S100B exerts paracrine effects on neurons and microglia, as well as autocrine effects on astrocytes (9). At the nanomolar level, extracellular S100B exerts a dual effect on neurons depending on its concentration, i.e., a pro-survival effect on neurons and stimulation of neurite outgrowth. In contrast, micromolar levels of extracellular S100B *in vitro* stimulate the expression of pro-inflammatory cytokines and induce apoptosis (10, 11). Furthermore, evidence has indicated that S100B interacts with the tumor suppressor p53 (12, 13). S100B overexpression can reduce p53 transcriptional activity by more than 50%. This effect is correlated with a decrease in p53 DNA binding activity and a reduction in the

accumulation of the MDM2 and p21 proteins (14).

S100B protein was first detected in the protein extract of whole T lymphocytes by Kanamori and colleagues (15). The $S100B^+$ lymphocyte subset was first identified by Takahashi and colleagues (16), and they reported that $S100B^+$ lymphocytes comprise approximately 1-4% (mean 3.4%) of peripheral blood mononuclear cells, and a part of $CD3^+$ $CD8^+$ cytotoxic T cells. They subsequently reported that $S100B^+$ lymphocytes are significantly decreased in the peripheral blood of patients with advanced cancer, comprising only 0.5% of the peripheral blood lymphocytes (PBL) (16-21). Cases of malignant proliferative disorders involving $S100B^+$ lymphocytes have been reported by several authors (22-26), and it has also been reported that the neoplastic counterparts of $S100B^+$ lymphocytes display NK activity, suggesting that $S100B^+$ lymphocytes are a particular type of cytotoxic T cells exhibiting NK activity. These reports suggest that $S100B^+$ lymphocytes play an important role in anti-tumor immunity. However, the physiological function of $S100B^+$ lymphocytes has been poorly understood, and remains

to be elucidated.

Recently, Steiner and colleagues analyzed the $S100B^+$ lymphocytes in human peripheral blood (27). They reported that the $S100B^+$ lymphocyte subset comprises two distinctive lymphocyte subtypes, a $S100B^+$ $CD3^+$ $CD8^+$ T lymphocyte subtype and a $CD3^-$ $CD8^-$ $CD56^+$ NK subtype, and that the T subtype of $S100B^+$ lymphocytes secrete S100B upon stimulation with an anti-CD3 antibody or phytohemagglutinin (PHA). Furthermore, high concentrations (μg/ml levels) of recombinant human S100B triggered the upregulation of CD11b and membrane shedding of CD62L in granulocytes and monocytes. These findings indicate that S100B might function as an interface to immunological processes, distinct from the known cytokine- and chemokine-mediated pathways. Moreover, Steiner and colleagues reported that $S100B^+$ NK cells are increased in schizophrenia patients, and they may be linked with stress axis activation (28). These findings suggest that $S100B^+$ lymphocytes play significant roles not only in anti-tumor immunity, but also in the regulation of inflammatory reactions and certain

physiological processes.

In this study, we attempted to precisely characterize the $S100B^+$ lymphocytes in healthy adult individuals with respect to the proportion, immunophenotypes, responses for T cells mitogen, the morphological response to NK-sensitive or –resistant target cells, and their S100B mRNA expression. We also attempted to confirm the release of S100B by $S100B^+$ lymphocytes after stimulation with mitogen.

Materials and Methods

Antibodies

Table 1 summarizes the specific primary and secondary antibodies used in this study.

Cell lines and cell culture

The cells were cultured in RPMI 1640 or DMEM medium containing 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml) and kanamycin (60 μg/ml). The human acute leukemia Jurkat cells (a T cell leukemia cell line), HUT78 (human Sezary syndrome cell line) and Daudi (human Burkitt's lymphoma cell line) cells were cultured in RPMI 1640 medium. A2058 (human melanoma cell line) and HeLa (human cervical adenocarcinoma cell line) cells were cultured in DMEM.

Preparation of peripheral blood lymphocytes

Peripheral blood mononuclear cells were obtained from the heparinized peripheral venous blood of 30 healthy adults [14 male, 16 female, ages ranging from 22 to 61 (mean 38.6, median 31) years old]. Mononuclear cells were collected by density gradient centrifugation using LSM® Lymphocyte Separation Medium (MP Biomedicals LLC, Irvine CA, USA), and were suspended in RPMI-1640 medium. The mononuclear cell suspensions were incubated in culture dishes that had been treated with autologous human serum for 15 min at 37°C. After removal of the adherent cells (monocytes), non-adherent cells were collected, washed, re-suspended in phosphate buffered saline (PBS), and examined as PBL.

Flow cytometric analysis and cell sorting

The PBL were re-suspended at a concentration of 1 x 10^6 /ml cells in PBS. First, the PBL were stained with various antibodies against lymphocyte cell surface molecules. When performing three-color staining, the PBL were incubated with 20 μl/ml

biotin-labeled mouse monoclonal antibodies against CD3 or CD8 or CD16 for 30 min on ice. The PBL were washed with PBS, and incubated with 20 μl streptavidin PerCP and PE-labeled mouse monoclonal antibodies against each of the cell surface molecules, as listed in Table 1, for 30 min on ice. When performing two-color staining, the PBL were incubated with 1 ml of PBS containing appropriately diluted PE-labeled mouse monoclonal antibodies for 30 min on ice. The PBL were then washed with PBS and treated with IntraPrep Permeabilization Reagent (Beckman coulter Corp., Miami, FL, USA) according to the manufacturer's instructions, for detection of the intracellular protein S100B. After washing, the PBL were re-suspended in 1 ml of PBL containing an appropriate amount of rabbit anti-S100B antibody for 30 min on ice. After washing, the PBL were re-suspended in 1 ml of PBS containing FITC-conjugated goat anti-rabbit IgG diluted according to the manufacture's instructions for 30 min on ice. After washing, the PBL were fixed with 4% paraformaldehyde in PBS.

The two- or three-color flow cytometric analyses were performed on a

FACSCalibur instrument (BD Biosciences, San Jose, CA, USA). Acquisition was done until 5000 lymphocytes were detected. The data analyses were performed using the Cell Quest™ Pro Software (BD Biosciences), which employs Forward Scatter (FSC)/Side Scatter (SSC) lymphocyte gating.

Furthermore, the PBL were sorted into the $S100B^-$ $CD3^+$ $CD8^+$ $CD16^-$ (CTL), $S100B^+$ CTL subtype, $S100B^-$ $CD3^-$ $CD16^+$ (NK cells), $S100B^+$ NK subtype, $CD4^+$ Th cells, and $CD19^+$ B cells, using the FACSAria instrument (BD Biosciences) and FACS Diva Software program (V5.0.3) (BD Biosciences).

RNA extraction and real-time RT-PCR

The S100B mRNA expression in cells, including cell lines, whole PBL, and sorted lymphocytes ($S100B^-$ CTL, $S100B^+$ CTL subtype, $S100B^-$ NK cells, $S100^+$ NK subtype, $CD4^+$ Th cells, $CD19^+$ B cells), was examined. Total RNA from cell lines and whole PBL were isolated with the RNeasy Mini Kit (QIAGEN, Hilden, Germany), and

total RNA from sorted lymphocytes were isolated with a RNeasy FFPE Kit (QIAGEN), according to the manufacturer's instructions. The expression levels of S100B transcription were measured relative to the expression of β-actin using an ABI Step One™ and Step One Plus™ Real Time PCR System (Applied Biosystems, Foster City, CA, USA). All reactions were performed using a One Step SYBR® Prime Script® PLUS RT-PCR Kit (Perfect Real Time) (Takara Bio Inc., Shiga, Japan). The expression levels are given as dCT, which were calculated by subtracting the cycle threshold value (the cycle number at which the message is first detected) for β-actin from the cycle threshold value for each transcript. Lower dCT values indicate higher RNA levels. As the cycle thresholds were in the exponential phase of amplification, a 1 dCT difference is equivalent to a 2-fold change in the mRNA levels (29). All reactions were carried out in triplicate. The primer sequences used are listed in Table 2. To confirm that the PCR products were of a specific sequence, they were separated by 2% agarose gel electrophoresis, and bands were visualized under ultraviolet radiation after staining with

ethidium bromide. The detected bands were excised from the agarose gels, and purified using the GENE CLEAN Kit (MP-Biomedicals) according to the manufacturer's instructions. Then, the purified DNA was sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Stimulation with T cell mitogen and quantification of S100B in the culture supernatants of lymphocytes

Fresh PBL (1 x 10^6 cells/ml) were suspended in RPMI 1640 medium and 10 μg/ml of PHA (Sigma Aldrich, St. Louis, MO, USA) or were left untreated (without mitogens) for 4 days at 37°C in 5% CO_2 and humidified air. Thereafter, the cells and the supernatants were harvested and analyzed at different time points. The cells were stained with S100B and various antibodies for lymphocyte cell surface molecules, as described above. Then, they were analyzed by the FACSCalibur instrument. The supernatants were evaluated with a Human S100B ELISA Kit (Millipore Corporation,

Billerica, MA, USA) according to the instructions provided by the manufacturer. The amount of S100B protein was corrected after setting the value of the RPMI 1640 medium as 0 pg/ml. Measurements were performed in duplicate. In addition, the cells cultured for 72 hours were smeared with Cytocentrifuge (Sakura Finetek, Tokyo, Japan).

Co-cultivation of PBL with K562 cells and BALL-1 cells

Fresh PBL (n = 1 x 10^6 cells) were co-cultivated with NK-sensitive K562 cells or NK-resistant BALL-1 cells (n = 1 x 10^5 cells) in 2.0 ml of the RPMI 1640 medium for 3 hours at 37^{o}C in 5% CO_2 and humidified air. These cells were then smeared with Cytocentrifuge.

Cell smear preparation

The cultured cells were smeared on Frontier slides (Matsunami Glass Ind., Ltd;

Osaka, Japan) with Cytocentrifuge, and immediately fixed with 4% paraformaldehyde for 30 min. The cell smears were washed with PBS and air-dried. The cell smears were examined by two-color immunofluorescence staining, and then were stained with May-Grünwald-Giemsa stain.

Two-color immunofluorescence staining

The cell smears were treated with 3% H_2O_2 and immersed in 10% normal goat serum. They were then incubated with rabbit anti-S100B at 4^{o}C overnight. After washing them with PBS, the cell smears were stained with FITC-conjugated goat anti-rabbit IgG at a dilution of 1:500, and a PE-conjugated mouse monoclonal antibody against CD3, CD8 or CD16 at a dilution of 1:250. The cell smears were observed with a LSM510 confocal laser scanning microscope (Carl Zeiss).

Statistical analysis

The statistical analyses were performed using the SPSW software program (version 18.0; SPSS Inc.).

Results

Immunophenotype of S100B$^+$ lymphocytes

S100B$^+$ lymphocytes expressed CD3, CD8, CD16, CD56, CD161, CD244 (2B4) and CD314 (NKG2D) (Figure 1A-F), but did not express CD4, CD25, CD19, or HLA-DR (Figure 1G-J). Although CD314 was expressed on all S100B$^+$ lymphocytes, CD3, CD8, CD16, CD56, CD161, and CD244 were expressed on only some of the S100B$^+$ lymphocytes. The three-color flow cytometric analysis indicated that two distinct subtypes of S100B$^+$ lymphocytes, the CTL subtype (CD3$^+$ CD8$^+$ CD16$^-$) and the NK subtype (CD3$^-$ CD8$^-$ CD16$^+$), were present in the peripheral blood. Almost all cells of the NK subtype of S100B$^+$ lymphocytes expressed CD56. The majority of the CTL subtype of S100B$^+$ lymphocytes expressed the αβ-T cell receptor (TCR) (Figure 2A). A small number of the CTL subtype of S100B$^+$ lymphocytes (around 5-10%) expressing the γδ-TCR were detected (Figure 2B). These cells strongly expressed CD3 and weakly expressed CD16. Such cells could not be detected as a distinct cell population in most

of individuals examined. The NK subtype of $S100B^+$ lymphocytes did not express the αβ-TCR or γδ-TCR. There were no $S100B^+$ lymphocytes that expressed Vα24-TCR, which is known to be expressed on NKT cells (Figure 2C).

The proportion of $S100B^+$ lymphocytes in the whole PBL of healthy individuals

The proportion of $S100B^+$ lymphocytes in the whole PBL varied considerably among the healthy individuals examined (Table 3). $S100B^+$ lymphocytes were detected in all individuals examined, but the proportion of $S100B^+$ lymphocytes in the whole PBL population ranged from 0.42 to 16.15% (mean 4.21%). There were no significant differences between males and females (t-test, $p = 0.578$) (Figure 3A). In contrast, the mean of the older group (> 32 years old) was 3.01%, while that of the younger group (< 32 years old) was 5.26%. Although this difference was statistically not significant (t-test, $p = 0.077$) (Figure 3B), it was suggested that there is a trend for the proportion to decrease with age.

Moreover, the average proportion of the CTL subtype of $S100B^+$ lymphocytes in the younger group was 3.59%, while that of the older group was only 1.18%, and this difference was statistically significant (t-test, $p = 0.012$). In addition, almost all $S100B^+$ lymphocytes in the younger group were of the CTL subtype. These findings indicate that there is a tendency for the proportion of the CTL subtype of $S100B^+$ lymphocytes to decrease with age, and suggested that the subpopulation of the NK subtype of $S100B^+$ lymphocytes increases with age although this difference was statistically not significant (t-test, $p = 0.406$) (Figure 3C). In contrast, there were no significant differences in the proportions of ordinary CTL and NK cells between the younger and older groups (Figure 3D).

S100B mRNA expression

Surprisingly, the expression of S100B mRNA was detected not only in $S100B^+$ lymphocytes, but also in $S100B^-$ lymphocytes, such as $CD4^+$ T cells and B cells,

although the expression levels of these $S100B^-$ populations were much lower than those of the $S100B^+$ lymphocyte populations (lower than one-hundredth) (Figure 4A). The transcription of S100B was confirmed by a sequential analysis. The mRNA expression of S100B was also detected in all cell lines examined, including both $S100B^+$ and $S100B^-$ cell lines, although the expression level of S100B mRNA in the $S100B^+$ melanoma cell line (A2058) was much higher than that of the $S100B^-$ cell lines.

In contrast, there were no significant differences in the p53 mRNA expression levels among the PBL subpopulations (Figure 4B), and there was no definite correlation between the S100B mRNA and p53 mRNA expression levels, although it has been demonstrated that S100B is functionally associated with p53.

Biological activities of $S100B^+$ lymphocytes

(1) Stimulation with the T cell mitogen, PHA

The expression of S100B protein was investigated after stimulation of PBL

with PHA. Non-stimulated PBL were used as controls. The number of PBL increased approximately two-fold in 96 hours upon stimulation with PHA. The majority of the PBL that were stimulated with PHA for 72 hours displayed marked blastic morphological changes. The CTL subtype of $S100B^+$ lymphocytes, as well as ordinary $S100B^-$ T cells, displayed these blastic morphological changes, while the NK subtype of $S100B^+$ lymphocytes and ordinary $S100B^-$ NK cells did not. The CTL subtype of $S100B^+$ lymphocytes increased in number upon PHA stimulation, whereas the population of NK subtype of $S100B^+$ lymphocytes decreased (Figure 5A). These findings indicate the distinctive T cell nature of the CTL subtype of $S100B^+$ lymphocytes. The $CD4^+$ lymphocytes and $CD19^+$ lymphocytes were not converted to be positive for S100B upon PHA stimulation.

Furthermore, we examined whether or not the CTL subtype of $S100B^+$ lymphocytes released S100B into the culture medium upon PHA stimulation. S100B was not detected in the culture supernatant of control PBL cultured without PHA. In

contrast, S100B was detected in the culture supernatant of PBL culture with PHA, and the concentration continuously increased during the culture (Figure 5B). These findings indicate that the CTL subtype of $S100B^+$ lymphocytes release S100B upon stimulation with PHA.

(2) Co-cultivation of PBL with NK-sensitive K-562 cells or NK-resistant BALL-1 cells

No method is currently available for purifying living NK subtype of $S100B^+$ lymphocytes to evaluate their NK activity directly. Therefore, we attempted to indirectly evaluate their NK activity. Briefly, we examined whether or not the NK subtype of $S100B^+$ lymphocytes attached specifically to NK-sensitive K-562 cells upon co-cultivation. We regarded only flattened cells adhering to K-562 cells as lymphocytes with NK activity, and regarded adherent lymphocytes without such morphological changes as accidentally adjoining cells. Two-color immunofluorescence staining indicated that both $S100B^-$ $CD16^+$ NK cells (ordinary NK cells; red) and $S100B^+$ $CD16^+$

cells (NK subtype S100B$^+$ lymphocytes; yellow) adhered to NK-sensitive K562 cells upon co-cultivation. These cells intimately attached to K-562 cells and became flattened in shape (Figures 6A and B). In contrast, none of the lymphocytes adhered to the NK-resistant BALL-1 cells (Figure 6C). These findings strongly suggest that the NK subtype of S100B$^+$ lymphocytes possess NK activity.

Discussion

In the present study, we examined the immunophenotype, functional characteristics and mRNA expression of the $S100B^+$ lymphocytes in human peripheral blood. We found that $S100B^+$ lymphocytes are present in the peripheral blood of all individuals examined as a distinct population of PBL (0.42-16.15%, mean 4.21%). There was no statistically significant difference in the average proportion of $S100B^+$ lymphocytes between males and females. As shown in this study, the average proportion of $S100B^+$ lymphocytes to the whole PBL population of the older individuals (> 32 years old) tended to be lower than that of the younger individuals (< 32 years old), although the difference was not statistically significant. These findings imply that $S100B^+$ lymphocytes play a crucial role in the immune system.

A flow cytometric analysis indicated that $S100B^+$ lymphocytes consist of two distinctive subtypes, the CTL subtype ($CD3^+$ $CD8^+$ $CD16^-$) of $S100B^+$ lymphocytes and the NK subtype ($CD3^-$ $CD8^-$ $CD16^+$) of $S100B^+$ lymphocytes. The present study

indicated that the proportion of NK subtype of $S100B^+$ lymphocytes to the whole population of $S100B^+$ lymphocytes of the older group tended to be higher than that of the younger group. These findings suggest that the proportion of the NK subtype of $S100B^+$ lymphocytes to the whole population of $S100B^+$ lymphocytes tended to increase, and conversely, that the subpopulation of the CTL subtype of $S100B^+$ lymphocytes tended to decrease with age. In contrast to the $S100B^+$ lymphocytes, such a tendency was not observed in the ordinary CTL and NK subtypes in this study. Previously, Xu and colleagues reported that there were significant differences in the proportion of ordinary CTL and NK subpopulations between an aged group (>70 years old) and a young group (18-25 years old) (30). Specifically, they demonstrated that there were fewer $CD3^+$ $CD8^+$ CTL and more $CD16^+$ NK cells in the peripheral blood of the aged subjects compared with the younger group. In the present study, however, we detected such differences in the $S100B^+$ lymphocyte subtypes, but not in the ordinary lymphocyte subtypes. This discrepancy may be due to the fact that the older group in

our study (32-60 years old) was much younger than the aged group of the study reported by Xu and colleagues (over 70 years old). It is possible that the $S100B^+$ lymphocyte subtypes are more sensitive to age than ordinary $S100B^-$ lymphocyte subtypes.

The $S100B^+$ lymphocyte population was discovered over 30 years ago. In the early reports, only the CTL subtype of $S100B^+$ lymphocytes ($CD3^+$ $CD8^+$) was described (16-21). Subsequently, several cases of neoplastic disorders involving $S100B^+$ lymphocytes have been reported (22-24, 26). All of these cases were characterized by an extensive leukemic increase of $CD3^+$ $S100B^+$ T cells and an aggressive clinical course. Interestingly, in some of these cases, the neoplastic counterparts of $CD3^+$ $S100B^+$ T cells were reported to simultaneously express NK cell antigens, including CD16, CD11b, CD11c and/or CD56, although such lymphocytes are not present in the peripheral blood (16, 25). A previous study by Steiner and colleagues and our present study indicate the presence of a NK subtype of $S100B^+$ lymphocytes in the peripheral blood of healthy individuals (27). These findings imply that the NK subtype of $S100B^+$ lymphocytes is

ontogenically closely related to the CTL subtype of $S100B^+$ lymphocytes.

In addition, both the CTL and NK subtypes of $S100B^+$ lymphocytes express CD161, 2B4 (CD244) and NKG2D (CD314). These molecules are known to be expressed on the surface of ordinary NK cells and $CD8^+$ T cells. CD161 is homologous to the Ca^{2+}-dependent lectin-like receptors. Although the function of CD161 has not been fully elucidated, it has been suggested that CD161 serves as a specific receptor for some NK cell targets (31). The 2B4 protein is known to be expressed on cells mediating non-MHC-restricted cytotoxicity. It is a member of the CD2 receptor subfamily, which have both activating and inhibitory functions in NK cells (32, 33). NKG2D was reported to be expressed on effector cells involved in both innate and adaptive immune responses. NKG2D binds to the MICA and MICB (MHC class-I chain-related protein A and B) expressed on epithelial tumor cells (34, 35). Therefore, the expression of these molecules on $S100B^+$ lymphocytes further suggests that $S100B^+$ lymphocytes have a cytotoxic function. CTL and NK cells are known to use a combination of several

mechanisms to lyse different target cells. For example, they mediate Ca^{2+}-dependent granule exocytosis and the release of cytotoxic proteins (perforin and granzyme) from intracytoplasmic granules; constitutively express or induce FasL expresson upon interaction with target cells, leading to the induction of Ca^{2+}-independent, Fas-mediated apoptosis; and produce membrane-bound or secreted cytokines (36). It seems possible that the S100B protein in $S100B^+$ lymphocytes may act as a calcium-binding protein involved in cytotoxicity.

The present analysis of the TCR revealed that the majority of the CTL subtype $S100B^+$ lymphocytes expressed αβ-TCR, while a small number of them (around 5-10%) expressed the γδ-TCR. The function of this minor population should be examined in further investigations. In contrast to $S100B^+$ lymphocytes, the NK subtype of $S100B^+$ lymphocytes did not express αβ-TCR or γδ-TCR, similar to ordinary CTL and NK cells.

The present finding that $S100B^+$ lymphocytes do not express Vα24-TCR

indicates that S100B$^+$ lymphocytes are different from NKT cells, because this molecule is reported to be expressed exclusively on NKT cells. NKT cells are a unique small subpopulation of lymphocytes (less than 0.1% of PBL) that represent a subset of mature T lymphocytes expressing the TCR as well as NK cell markers. NKT cells serve as a bridge between the innate and adaptive immune systems (37, 38).

Furthermore, the present study indicated that the CTL subtype of S100B$^+$ lymphocytes, but not the NK subtype of S100B$^+$ lymphocytes, displayed a proliferative response to PHA similar to that of ordinary T cells, and that the NK subtype of S100B$^+$ lymphocytes attached to NK-sensitive targets, such as K-562 cells, upon co-cultivation as did the ordinary NK cells. These findings indicate that there is no significant difference between the CTL subtype of S100B$^+$ lymphocytes and ordinary CTL or between the NK subtype of S100B$^+$ lymphocytes and ordinary NK cells, except for the expression of the S100B protein in S100B$^+$ lymphocytes. These results lend support to the finding by Steiner and colleagues that the functional characterization of S100B

positive and S100B negative T lymphocyte subsets showed that these cells are similar in their IFNγ and IL-2 production upon stimulation with PMA/ionomycin (27).

Curiously, S100B mRNA were expressed in not only $S100B^+$ lymphocytes, but also $S100B^-$ lymphocytes, including $CD4^+$ T cells and even B cells, in this study, although the expression levels of S100B mRNA in $S100B^-$ lymphocytes were much lower (less than one-hundredth) than those of $S100B^+$ lymphocytes. The presence of S100B mRNA in $S100B^-$ lymphocytes was confirmed by agarose gel electrophoresis and a sequence analysis. These results suggest that not only $S100B^+$ lymphocytes, but also $S100B^-$ lymphocytes, have the ability to produce the S100B protein following exposure to certain stimuli. However, the $CD4^+$ T cells and B cells did not express S100B upon stimulation with PHA in this study. Why $CD4^+$ T cells and B cells do not produce the S100B protein, despite the presence of S100B mRNA, and the biological significance of such low levels of S100B mRNA in $S100B^-$ lymphocytes should be determined in further investigations.

It has recently been reported that S100B interacts with the tumor suppressor p53 (8,13), which plays a pivotal role in the maintenance and regulation of normal cellular functions, and its inactivation can affect cell cycle checkpoints, apoptosis, gene amplification, centrosome duplication, and ploidy (39). Specifically, S100B inhibits p53 tetramer formation and p53 C-terminal phosphorylation mediated by protein kinase C, resulting in the inhibition of p53 DNA binding and transcriptional activity (14). Our present study, however, indicated that the expression levels of p53 mRNA are almost the same among the various PBL subpopulations in spite of the extensive differences in the expression levels of S100B mRNA among these subpopulations. These findings suggest that S100B is not directly involved in the inhibition of p53 gene transcription in $S100B^+$ lymphocytes.

We confirmed that PHA stimulation led to the extracellular release of S100B by $S100B^+$ lymphocytes. Moreover, we found that the number CTL subtype $S100B^+$ lymphocytes, as well as that of ordinary CTL, increased and exhibited blastic

morphological changes after PHA stimulation. In contrast, the proportions of whole NK cells and the NK subtype of $S100B^+$ lymphocytes in the whole PBL decreased 96 hours after PHA stimulation, and these lymphocytes did not display the blastic morphological changes. Steiner and colleagues have recently reported the extracellular secretion of S100B by $S100B^+$ lymphocytes upon stimulation with PHA or an anti-CD3 antibody (27). They also indicated that the number of $S100B^+$ T cells decreased significantly after PHA or anti-CD3 antibody stimulation, and suggested that $S100B^+$ T lymphocytes become negative for S100B after they release it. However, the present results do not support their view, because the numbers of both whole CTL and the CTL subtype of $S100B^+$ lymphocytes increased after PHA stimulation. It seems likely that CTL subtype of $S100B^+$ lymphocytes are continuously positive for S100B even after releasing S100B.

It has been reported that extracellular S100B exerts a dual effect on neurons depending on its concentration, i.e., a pro-survival effect on neurons and stimulation of

neurite outgrowth at nanomolar levels and a toxic effect at micromolar levels (neuronal death via apoptosis) (11). If the S100B released from $S100B^+$ T lymphocytes induces similar stimulatory biological effects on immune cells, $S100B^+$ lymphocytes might play an important role in the host defense. Accordingly, Steiner and colleagues reported that S100B induces granulocytes and monocytes to express the adhesion molecule CD11b and leads to the membrane shedding of CD62L, resulting in the functional stimulation of granulocytes and monocytes (27).

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor of the immunoglobulin superfamily and is involved in several diseases, such as complications of diabetes, cardiovascular diseases, Alzheimer's disease, and cancer. RAGE has been identified as a cell surface receptor for S100B, and triggers cellular signaling (40). Akirav and colleagues reported that RAGE is expressed in both $CD4^+$ and $CD8^+$ T cells at similar levels. Moreover, they reported that the proportion of T cells expressing RAGE is increased when peripheral blood cells are cultured with an

Epstein-Barr virus peptide and S100B (41). These findings suggest that the S100B released from $S100B^+$ lymphocytes induces the expression of RAGE in T cells, resulting in the activation of T cells.

In order to clarify the biological significance of S100B in NK subtype of $S100B^+$ lymphocytes, we examined whether or not the NK subtype of $S100B^+$ lymphocytes released S100B into the culture medium upon co-cultivation with NK-sensitive K-562 cells. But we failed to detect released S100B in the co-culture supernatant in this experiment (unpublished data). It remains unclear whether or not such an experiment is enough to activate NK-type of $S100B^+$ lymphocytes. The biological significance of S100B remains to be determined.

In conclusion, our study demonstrated the function and mRNA expression of the lymphocytes expressed S100B in the cytoplasm of human peripheral blood cells. We found that $S100B^+$ lymphocytes are a particular subset of lymphocytes that differ from those known so far, and that they appear to have an important role in the immune

system. Further studies should be performed to address what function, they perform based on different lifestyle- and living environment-related factors, and under pathological conditions, such as autoimmune disease and cancer.

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Tables

Table 1. List of the antibodies used in this study

Antibodies	Clone	Sources
Polyclonal Rabbit Anti-Human S100B	A5110	Dako Cytomation
PE-Conjugated Monoclonal Mouse Anti-Human CD3	UCHT1	BD Pharmingen
PE-Conjugated Monoclonal Mouse Anti-Human CD4	RPA-T4	BD Pharmingen
PE-Conjugated Monoclonal Mouse Anti-Human CD244	2-69	BD Pharmingen
PE-Conjugated Monoclonal Mouse Anti-Human HLA-DR	L243	BD Pharmingen
RPE-Conjugated Monoclonal Mouse Anti-Human CD8	DK25	Dako Cytomation
RPE-Conjugated Monoclonal Mouse Anti-Human CD16	DJ130c	Dako Cytomation
RPE-Conjugated Monoclonal Mouse Anti-Human CD25	ACT-1	Dako Cytomation
RPE-Conjugated Monoclonal Mouse Anti-Human CD19	HD37	Dako Cytomation
Phycoerythin (PE) anti-human CD56	CMSSB	eBioscience
Phycoerythin (PE) anti-human CD314/ NKG2D	1D11	eBioscience
Anti-Human CD19 APC	HIB19	eBioscience
Phycoerythin (PE) anti-human $\alpha\beta$TcR	IP26	eBioscience
Phycoerythin (PE) anti-human $\gamma\delta$TcR	B1.1	eBioscience
PE-Conjugated Monoclonal Mouse Anti-Human CD161	191B8	IMMUNOTECH
RPE-Conjugated Monoclonal Mouse Anti-Human TCR Vα24	C15	Serotec Ltd.
FITC Goat Anti-Rabbit IgG(H+L) Conjugated		ZYMED® Laboratories
Streptavidin PerCP		BD Pharmingen
Biotin Mouse Anti-Human CD3	HIT3a	BD Pharmingen
Biotin Mouse Anti-Human CD8	RPA-T8	BD Pharmingen
Biotin Mouse Anti-Human CD16	3G8	BD Pharmingen

Table 2. List of the primers used for real-time PCR.

Gene		Sequence	Product size	Gene Bank Acc.
S100B	F	CATCAACAATGAGCTTTCCCATTT	145	NM_006272.2
	R	GGCAGTAGTAACCATGGCAACAAAG		
p53	F	ACTAAGCGAGCACTGCCCAAC	130	NM_000546
	R	CCTCATTCAGCTCTCGGAACATC		
β-actin	F	TGGCACCCAGCACAATGAA	186	NM_001101
	R	CTAAGTCATAGTCCGCCTAGAAGCA		

Table 3. Proportion of $S100B^+$ lymphocytes in the PBLs of healthy individuals.

	distribution	mean	median
Age (year)	22 - 61	38.63	31
Male / Female	14 / 16		
$CD3^+$ T-cells (%)	48.38 - 83.23	64.41	64.28
$CD8^+$ T-cells (%)	11.76 - 42.73	27.24	27.61
NK-cells (%)	5.36 - 35.62	18.37	17.5
$S100B^+$ lymphocytes (%)	0.42 – 16.15	4.21	3.1
CTL-subtype $S100B^+$ lymphocytes (%)	0.16 - 11.22	2.6	1.97
NK-subtype $S100B^+$ lymphocytes (%)	0.01 - 5.76	1.48	0.97

Figure legends

Figure 1. Immunophenotyping of $S100B^+$ lymphocytes by flow cytometry. The PBL of a healthy adult were stained for various lymphocyte surface markers and cytoplasmic S100B protein. A three-color flow cytometric analysis of the PBL for S100B/CD3/CD8 (A), S100B/CD3/CD16 (B), S100B/CD56/CD16 (C), and a two-color analysis for S100B/CD161 (D), S100B/CD244 (E), S100B/CD314 (F), S100B/CD4 (G), S100B/CD25 (H), S100B/CD19 (I), and S100B/HLA-DR (J) were performed.

A and B: There are two subtype of $S100B^+$ lymphocytes, the $S100B^+$ $CD3^+$ $CD8^+$ $CD16^-$ subtype (CTL subtype; red dots) and the $S100B^+$ $CD3^-$ $CD8^-$ $CD16^+$ subtype (NK subtype; blue dots).

C: The NK subtype of S100B lymphocytes also express CD56.

Figure 2. T cell receptor (TCR) expression on $S100B^+$ lymphocytes detected by flow cytometry. PBL of a healthy adult were stained for various TCR and S100B. A three-color flow cytometric analysis of the PBL for S100B/αβ-TCR/CD3 (A), S100B/αβ-TCR/CD8 (B), S100B/αβ-TCR/CD16 (C), S100B/γδ-TCR/CD3 (D), S100B/γδ-TCR/CD8 (E), and S100B/γδ-TCR/CD16 (F) was performed. A two-color flow cytometric analysis was performed for S100B and Vα24-TCR (G).

A, B, C: S100B and αβ-TCR double positive lymphocytes are indicated by red blots, and S100B positive and αβ-TCR negative lymphocytes are indicated by blue blots.

D, E, F: S100B and γδ-TCR double positive lymphocytes are indicated by red blots, and S100B positive and γδ-TCR negative lymphocytes are indicated by blue blots.

Figure 3. Comparison of the proportion of $S100B^+$ lymphocytes to the whole PBL among healthy individuals (A-C). (A) A comparison of the younger group (< 32 years old) with the elder group (> 32 years old). (B) A comparison of males and females. (C) A comparison of the proportion of CTL subtype or NK subtype cells in the younger and older group. The proportion of the CTL subtype lymphocytes in the younger group was significantly higher than that of the older group ($p < 0.05$). (D) A comparison of the proportion of $S100B^-$ ordinary lymphocyte subpopulations ($S100B^- CD3^+$, $S100B^- CD8^+$, $S100B^- CD16^+$) to the whole PBL of the younger and older groups.

Figure 4. A comparison of the expression levels of S100B mRNA (A) and p53 mRNA (B) among the PBL subpopulations ($S100B^+$ CTL subtype, $S100B^-$ CTL, $S100B^+$; NK subtype, $S100B^-$ NK cells, $CD4^+$ T cells, B cells) and the different cell lines [Jurkat (T cell), HUT78 (T cell), Daudi (B cell), HeLa (epithelial cell), A2058 (melanoma)] by real-time RT-PCR. The expression levels of S100B transcripts were measured relative to the expression of β-actin. Note that $S100B^+$ cells exhibited high levels of S100B mRNA expression and that $S100B^-$ cells also exhibited low levels of S100B mRNA expression. There were no significant differences in the p53 mRNA levels among the PBL subpopulations.

Figure 5. Stimulation of cells with the T cell mitogen, PHA. Fresh PBL were cultured with PHA (10 μg/ml) or without PHA.

A: Proportional changes in the CTL or NK subtype of $S100B^+$ lymphocytes compared to the whole PBL cultured with or without PHA. ■: CTL subtype, ▲: NK subtype, ●: Whole $S100B^+$ lymphocytes. The solid line indicates PHA stimulation, and the dashed line indicates cells cultured without PHA. Note that the proportion of the CTL subtype of $S100B^+$ lymphocytes, but not the NK subtype, increased during stimulation with PHA.

B: The concentration of S100B protein in the culture medium of PHA-stimulated or control PBL measured by an ELISA. The S100B protein accumulated in the culture medium during PHA stimulation.

Figure 6. The results of the morphological analysis of the NK subtype of $S100B^+$ lymphocytes when they were co-cultivated with NK-sensitive K-562 cells (A, B) or NK-resistant BALL-1 cells (C) (original magnification x400).

A: May-Grünwald-Giemsa staining. Note that some of the lymphocytes adhered to K-562 cells.

B: The two-color immunofluorescence analysis of S100B (FITC) and CD16 (PE) expression. Note the flattened NK subtype of $S100B^+$ lymphocyte (yellow) adhering to the K-562 cells.

C: May-Grünwald-Giemsa staining. Note that none of the lymphocytes adhered to BALL-1 cells.

Figures

Figure 1

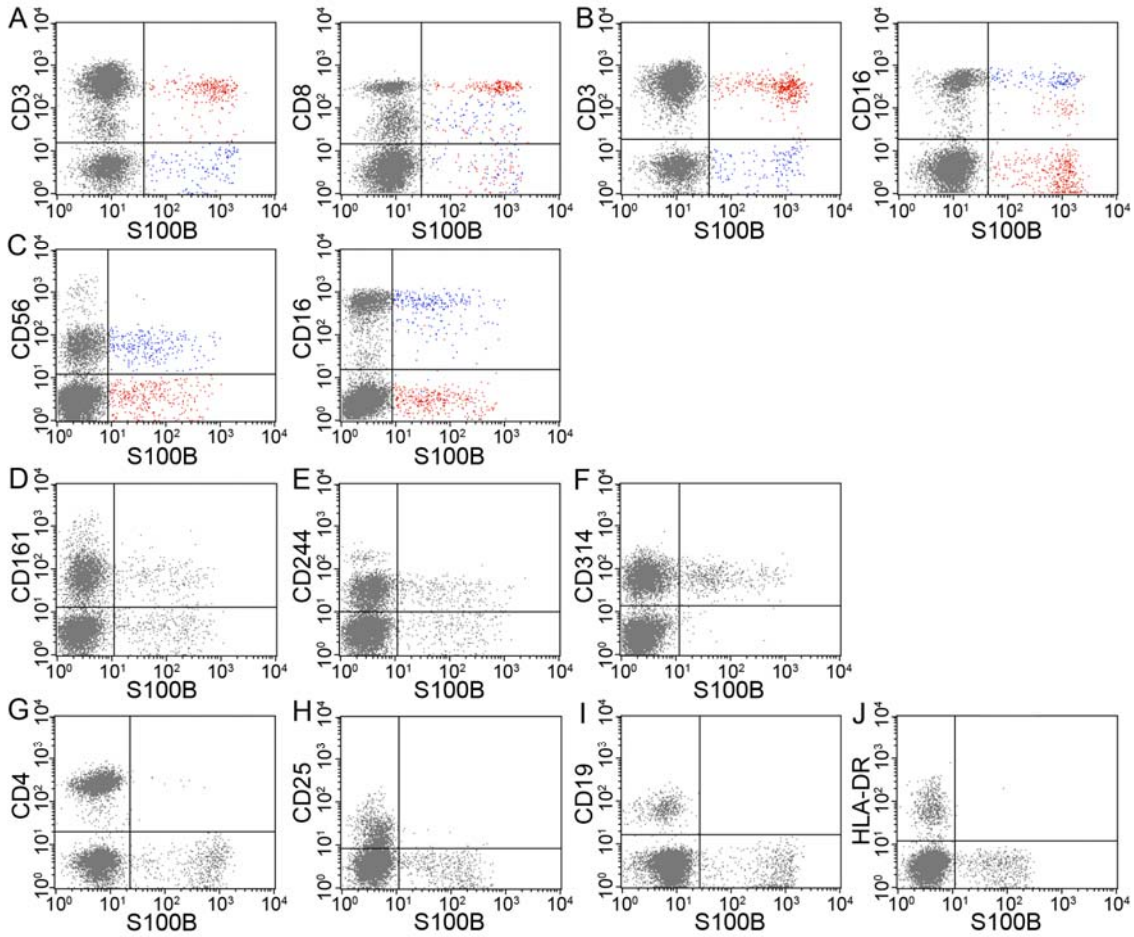

Figure 2

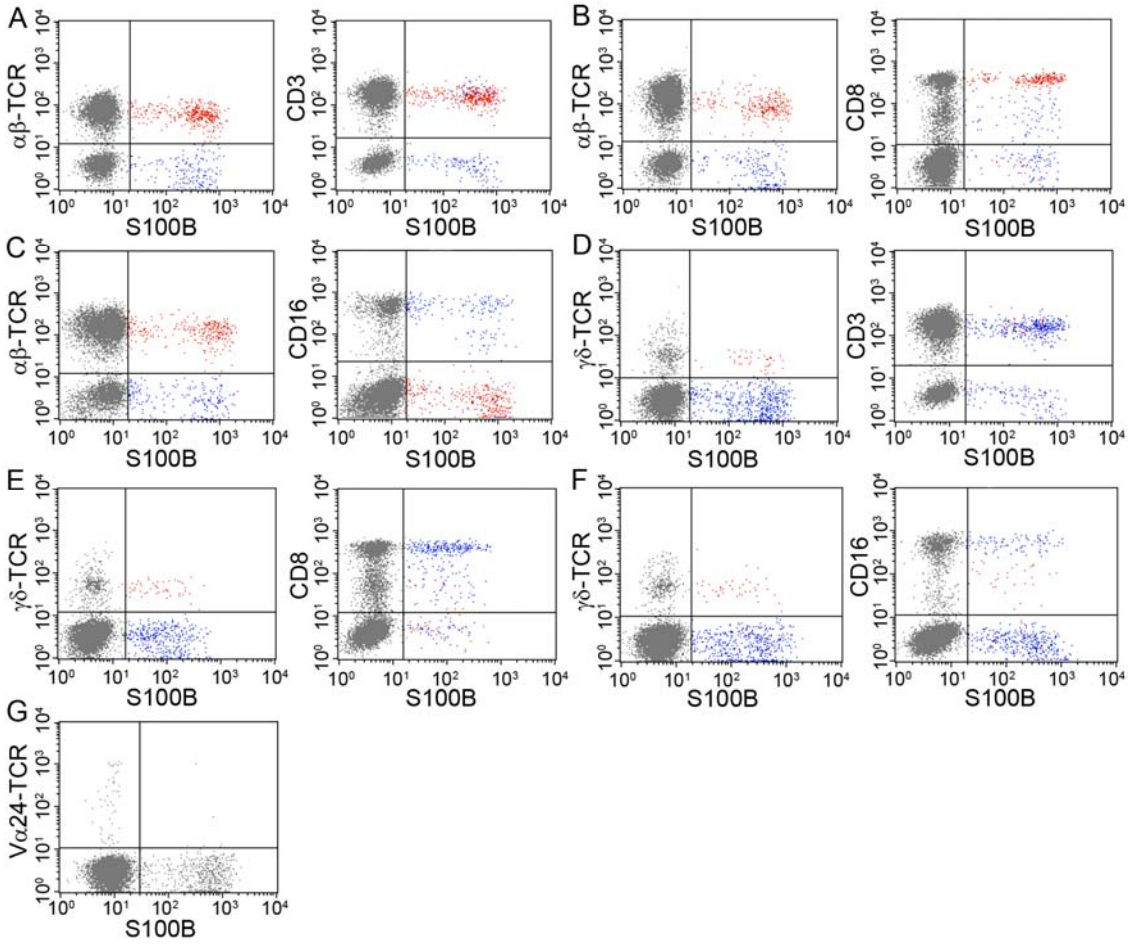
A
αβ-TCR
CD3
S100B
B
αβ-TCR
CD8
S100B
C
αβ-TCR
CD16
S100B
D
γδ-TCR
CD3
S100B
E
γδ-TCR
CD8
S100B
F
γδ-TCR
CD16
S100B
G
Vα24-TCR
S100B

Figure 3

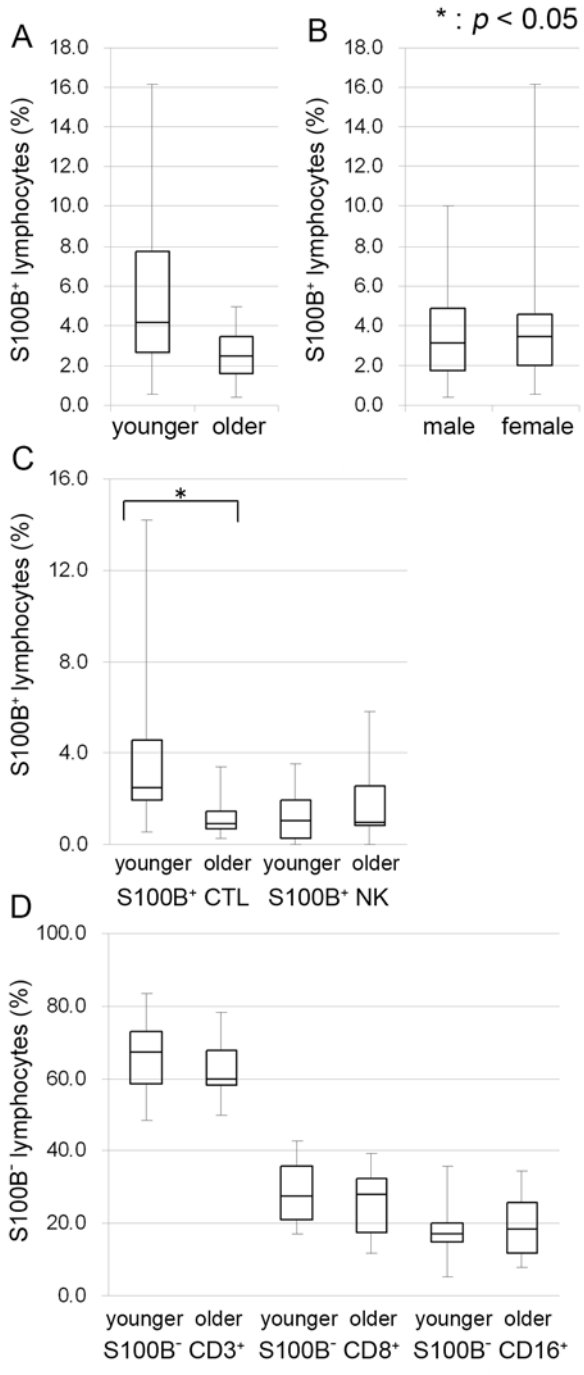
A
B
* : p < 0.05
S100B+ lymphocytes (%)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
younger
older
male
female
C
*
S100B+ lymphocytes (%)
16.0
12.0
8.0
4.0
0.0
younger
older
younger
older
S100B+ CTL
S100B+ NK
D
S100B- lymphocytes (%)
100.0
80.0
60.0
40.0
20.0
0.0
younger
older
younger
older
younger
older
S100B- CD3+
S100B- CD8+
S100B- CD16+

Figure 4

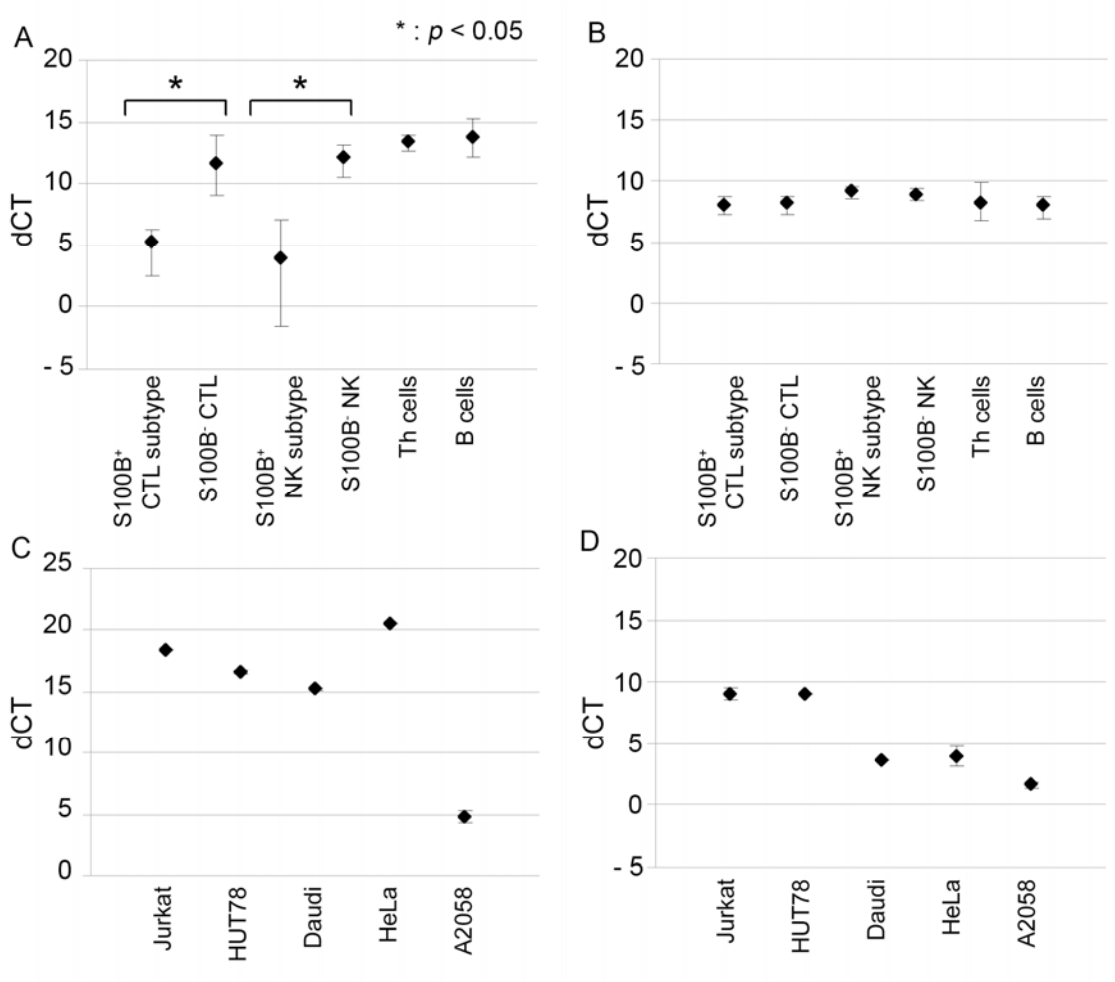
A
* : p < 0.05
dCT
S100B+ CTL subtype
S100B- CTL
S100B+ NK subtype
S100B- NK
Th cells
B cells
B
C
Jurkat
HUT78
Daudi
HeLa
A2058
D

Figure 5

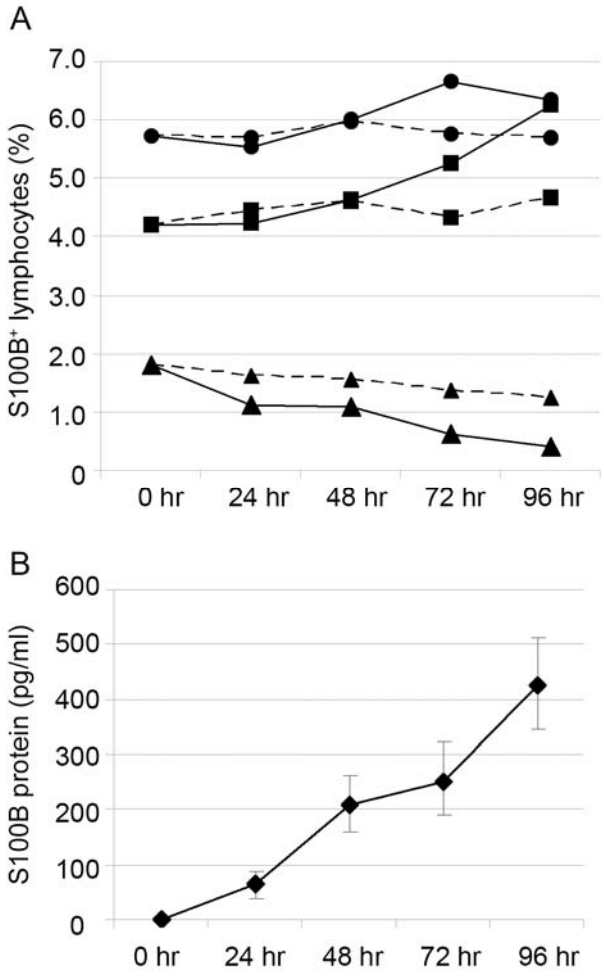
A
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
S100B+ lymphocytes (%)
0 hr
24 hr
48 hr
72 hr
96 hr
B
600
500
400
300
200
100
0
S100B protein (pg/ml)
0 hr
24 hr
48 hr
72 hr
96 hr

Figure 6