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DBA Lectin Binds to Highly Proliferative Mouse Erythroleukemia Cells

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Abstract. *Background/Aim: Hematopoietic malignancies lead to disease states involving abnormal proliferation of blood cells. Ki-67 and carboxyfluorescein succinimidyl ester (CFSE) are assays used to examine the proliferation status of cells but affect cell viability. In this study, we used lectins to bind to surfaces of proliferating cells with different phenotypes while preserving cell viability. Materials and Methods: The mouse lymphocyte Friend leukemia F5-5.F1 cell line was stained using biotin-conjugated lectins from Canavalia ensiformis (ConA), Dolichos biflorus (DBA), Erythrina cristagalli (ECA), Lens culinaris (LCA), Phaseolus vulgaris (PHA-E4), Arachis hypogaea (PNA), Ulex europaeus (UEA) and Triticum vulgaris (WGA) and sorted by fluorescence-activated cell sorting. Morphology, gene expression and proliferation assays were performed on sorted cells. Results: DBA, LCA and PHA-E4 probing sorted cells based on surface phenotype. Gene expression analysis showed that myelocytomatosis oncogene (Myc), cyclin D1 (Ccnd1), and cyclinD2 (Ccnd2) were more highly expressed in the DBA^{High} fraction than DBA^{Int} and DBA^{Neg} fractions. Ki-67 expression and MTS assay correlated with the DBA-binding pattern, with DBA^{High} reflecting the highest proliferative tendency. Conclusion: Labeling with DBA allows selection of proliferating cells using flow cytometry.*

Hematopoiesis is the process by which hematopoietic stem cells differentiate into hematopoietic progenitors, which give rise to the various lineages of mature blood cells. Abnormalities in hematopoiesis, including chromosomal abnormalities and transcription factor alterations, can lead to leukemia, that is characterized as an increase of hematopoietic precursor and immature, non-functional blood cells in bone marrow (1). A large-scale global study noted that global deaths from leukemia have remained steady at approximately 281,300 per year in both 1990 and 2010 (2). There are several therapies available for the different forms of leukemia, many of which target proliferating cells (3). However despite advances in therapy, up to 60% of patients experience relapse (4, 5), suggesting a need for greater understanding of the molecular mechanisms of leukemia cell proliferation.

Some assays have been developed for evaluating the proliferative status of cells but the most common ones involve intracellular staining. Staining for nuclear Ki-67 protein (6) has been used as a method for identifying populations of reproducing cells. It has been used in immunohistochemistry, immunoblotting (7), immunoflourescence (8), and, more recently, in flow cytometric analysis (9). However the main limitation of this method is that even when used in flow cytometry, cells must be fixed and viable proliferative cells cannot be used for further downstream analysis. Another reagent, carboxyfluorescein diacetate succinimidyl ester (CFSE), has also been used as an intracellular staining method for the detection of cell proliferation (10), however, some studies have shown that CFSE causes cell death at some concentrations (11, 12). Previously, we used flow cytometry to investigate the expression of known markers on the surfaces of leukemia cell lines (13). Since many of the assays for proliferation status focus on intracellular staining, tools for identifying proliferating cells based on surface phenotype may address problems associated with intracellular toxicity/cell death.

Lectins are proteins present in plants and animals and are highly specific to glycoproteins on cell surfaces. There has been a long history of lectins being used for identification and separation of cell types and they are now recognized as

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pattern-recognition molecules (14, 15). Lectins have used in explorative studies and have been shown to bind to cancer cells during the progression of colorectal cancer (16) and to glioblastoma-derived cancer stem cells (17). Studies have been performed on leukemia cell lines to establish whether lectin binds to them (18) and several other studies have been published about the antiproliferative effect of lectins on some leukemia cell lines (19, 20). The use of lectins in conjugation with biotin to exploit the highly specific binding of lectins to glycoproteins in order to take advantage of the strong avidin–biotin complex thereby formed (21, 22) gives lectins utility as specific probes for investigating surface phenotypes of cells.

In this study we labeled mouse lymphocyte Friend leukemia F5-5.F1 cell line (23) with plant-based lectins in order to sort the cells into different populations based on their proliferative status. Morphology, gene expression and protein expression of the sorted cells were analyzed to elucidate whether our target lectins can be used as tools to separate cells based on proliferative status while preserving cell viability.

Materials and Methods

Cell culture. The mouse leukemia cell line F5-5.F1 (Riken, Tsukuba City, Ibraraki Prefecture, Japan) was cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) with 10 U/ml penicillin and 10 μg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) at 37˚C in 5% CO_2.

Biotin-conjugated lectin staining and flow cytometric analysis. F5-5.F1 cells were incubated with biotin-conjugated lectins (J-OIL MILLS, INC, Tokyo, Japan) on ice for 30 min. The eight lectins used in this study are listed in Table I. After incubation with lectins, the cells were washed twice with phosphate-buffered saline (PBS) (–) and incubated with purified anti-phycoerythrin-conjugated streptavidin (SA-PE) (BD Bioscience, San Jose, CA, USA) on ice for 30 min. The cells were washed twice with PBS (–) and then stained with 1:1,000 propidium iodide (PI) (BD Bioscience, San Jose, CA, USA) before being analyzed using a FACS Aria cell sorter (BD Bioscience). Once a major population of cells was determined by plotting side scatter against forward scatter, cells were sorted by gating out PI-positive cells to remove dead cells and were further gated based on SA-PE intensity. Cells that exhibited distinctive separation after staining were sorted for further analysis. The data were analyzed using FlowJo software (Tree Star, Inc., Sac Carlos, CA, USA). Each lectin stain was performed in triplicate. Data is presented as means±standard deviation (SD).

May-Grünwald Giemsa staining. After sorting the cells were attached onto glass slides (Matsunami glass, Osaka, Japan) by CytoSpin4 (Thermo Fisher scientific, Waltham, MA, USA) at 23 ×*g* for 7 min. Cells were dried at room temperature overnight and then stained with May-Grünwald reagent (Muto Pure Chemicals, Tokyo, Japan) for 5 minutes. The slides were then washed with tap water and incubated with PBS (pH 6.4), for 2 min. Cells were then incubated with 1:18 diluted Giemsa solution (Muto Pure Chemicals, Tokyo, Japan) at room temperature for 40 minutes after which they were washed with tap water and dried. Glass coverslips were attached to the slides by using MGK-S mounting solution (Matsunami glass, Osaka, Japan) then observed and recorded using an Olympus CKX41 microscope (Olympus, Tokyo, Japan) and ZEN 2 (blue edition) software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Table I. *Biotin-conjugated lectins used in this study.*

Lectin	Agglutinin
Con A	*Canavalia ensiformis* (Jack bean, Horse bean)
DBA	*Dolichos biflorus* (Horse gram)
ECA	*Erythrina cristagalli* (Cockspur coral tree)
LCA	*Lens culinaris*
PHA-E4	*Phaseolus vulgaris* (Kidney bean, French bean)
PNA	*Arachis hypogaea*
UEA	*Ulex europaeus*
WGA	*Triticum vulgaris* (wheat germ)

All lectins were sourced from J-OIL MILLS, INC, Tokyo, Japan and used at 1 μl/ 1×10^5 cells.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted using RNAqueous-Micro Kit (Life Technologies, Carlsbad, CA, USA). Total RNA was subjected to reverse transcription using a High-Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, CA, USA) according to established protocols. The mRNA levels of myelocytomatosis oncogene (*Myc*), Cyclin D1 (*Ccnd1*), and Cyclin D2 (*Ccnd2*) were analyzed by qRT-PCR using TaqMan® reagents with a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The mRNA level of each target gene was normalized to that of β-actin (*Actb*) as an internal control.

Immunocytochemistry. Cells were attached to slides using the method described above. Cells were fixed in 1% paraformaldehyde in PBS (–) at room temperature for 30 min. After washing with PBS (–), cells were blocked with 1% bovine serum albumin in PBS (–) and treated with 0.05% Triton-X 100 in PBS (–) at room temperature for 1 h. Cells were then stained with a monoclonal rat anti-mouse Ki-67 antibody (1:500; DakoCytomation, Glostrup, Denmark) at 4˚C overnight. The slides were incubated with AlexaFluor488-conjugated donkey anti-rat IgG (1:400; Invitrogen, Carlsbad, CA, USA) and TOTO-3 iodide (1:1500; Invitrogen) before attaching coverslips with fluorescence mounting medium (DakoCytomation, Glostrup, Denmark) and assessed using a Fluo View 1000 confocal microscope (Olympus, Tokyo, Japan). Slides were made in triplicate and 500-1,000 nuclei were counted on each slide as described in in a previous report (24).

MTS assay. Cells were sorted as described above and cultured in a 96-well plate in containing RPMI1640 medium without phenol red (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS with 10 U/ml penicillin and 10 μg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) at 37˚C in 5% CO_2 for 24 h (3,000 cells/well). The cells were then cultured in triplicate with 20 μl of CellTiter96® AQ_{ueous} One Solution Cell Proliferation

Assay Buffer (Promega, Madison, WI, USA) for 4 h. The absorbance was measured hourly with a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. The absorbance at 620 nm was recorded and subtracted from the initial reading to account for the background absorbance to determine the cell viability.

Results

DBA, LCA and PHA-E4 binding classifies F5-5.F1 cells into distinctive populations. After the incubation in the presence of biotin-conjugated lectins and SA-PE, the cells were analyzed by flow cytometry and gated according to the binding of SA-PE (Figure 1). Analysis showed that the lectins ConA, ECA, PNA, UEA and WGA did not form distinctive cell populations based on lectin binding. However, DBA, LCA and PHA-E4 separated cells into distinctive populations with ligand (L)-negative, intermediate and high populations of DBA-labeled cells (hereafter named $DBAL^{Neg}$, $DBAL^{Int}$, and $DBAL^{High}$); low- and high-binding populations of LCA-labeled (LCALLow and $LCAL^{High}$) and PHA-E4-labeled (PHA-$E4L^{Low}$ and PHA-$E4L^{High}$) cells (Figure 1A). Figure 1B shows a graphical representation of the flow cytometric analysis. The cells were separated into distinct populations with statistically significant differences between $DBAL^{Neg}$ and $DBAL^{High}$ ($p<0.001$), $DBAL^{Int}$ and $DBAL^{High}$ ($p<0.001$), $LCAL^{Low}$ and $LCAL^{High}$ ($p<0.001$), and PHA-$E4L^{Low}$ and PHA-$E4L^{High}$ ($p<0.001$) populations.

$DBAL^{Neg}$, $DBAL^{Int}$ and $DBAL^{High}$ cells exhibit morphological differences. The $DBAL^{Neg}$ cells had a high incidence of fragmented nuclei compared to $DBAL^{Int}$ and $DBAL^{High}$ cells. The incidence of fragmented nuclei was 45% in $DBAL^{Neg}$, 14% in $DBAL^{Int}$, and 4% in $DBAL^{High}$ populations (Figure 2A). No observable differences were found between the $LCAL^{Low}$ and $LCAL^{High}$ populations (Figure 2B). A similar pattern was observed for PHA-$E4L^{Low}$ and PHA-$E4L^{High}$ cells, where there were no observable differences in morphology (Figure 2C).

$DBAL^{High}$ cells highly express proliferation-regulated genes. To evaluate the proliferative status of cells, we assessed the expression of the cell proliferation-related genes *Myc*, *Ccnd1*, and *Ccnd2* by qRT-PCR. Figure 3A shows the differences in proliferation-related genes in cells sorted by DBA with DBALHigh cells as the reference sample. The expression of *Myc* in $DBAL^{Neg}$ cells was 4.5-fold lower than that of $DBAL^{High}$ cells ($p<0.001$) and that of $DBAL^{Int}$ cells was 1.8-fold lower than that of $DBAL^{High}$ cells ($p<0.01$). There was also a difference in the expression of *Myc* in $DBAL^{Neg}$ and $DBAL^{Int}$ cells ($p<0.01$). There was no observable difference in the expression of *Ccnd1*, and *Ccnd2* between $DBAL^{Int}$ and $DBAL^{High}$ cells, however, the level of *Ccnd1* was 9.1-fold lower ($p<0.01$) and that of *Ccnd2* was 1.7-fold lower ($p<0.01$) in $DBAL^{Neg}$ cells compared to the $DBAL^{High}$ cells. As shown in Figure 3B, the expression of Myc was 0.3-fold lower ($p<0.05$) in $LCAL^{Low}$ when compared to $LCAL^{High}$. No significant difference in the expression of *Ccnd1*, and *Ccnd2* were found in LCA-sorted cells. In PHA-E4 cells, the expression of *Myc* was 0.5-fold higher ($p<0.05$) in the low-binding when compared to the highly-binding population. No significant difference in the expression of *Ccnd1*, and *Ccnd2* in the PHA-$E4^{Low}$ and PHA-$E4L^{High}$ populations was found (Figure 3C).

$DBAL^{High}$ cells are highly proliferative. As DBA was able to separate cells into distinctive populations exhibiting different levels of expression of proliferation-related genes, the cell proliferative status was assessed by Ki-67 and MTS assay. Figure 4A shows representative images of DBA cells as sorted by binding to DBA lectin and stained with AlexaFluor488 (green) representing the Ki-67 protein and TOTO-3 (blue) representing nucleic acid. Samples stained with only the secondary antibodies are shown in the right column. The percentage of Ki-67-positive cells differed significantly between $DBAL^{Neg}$ and $DBAL^{High}$ cells ($p<0.01$) (Figure 4B). To confirm cell proliferative status, we performed an MTS assay using the CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Figure 4C). This showed greater viability of $DBAL^{Int}$ ($p<0.01$) and $DBAL^{High}$ ($p<0.01$) cells than $DBAL^{Neg}$ cells.

Discussion

All lectins used in this study were plant-based lectins (all but WGA were in fact legume-based). Although some lectins have been shown to attenuate proliferation of cancer cells (25), no specific lectin has been identified as a marker for proliferating cells.

When analyzing the flow cytometric profiles, we looked for populations that suggested differences in surface phenotype. A major advantage of using immortalized cell lines is that they have the ability to produce consistent populations of cells for the study of process such as cellular proliferation and differentiation (26). DBA, LCA and PHA-E4 separated cells into distinctive populations and although there was both positive and negative binding of the ECA and PNA and WGA lectins, no distinctive populations were found and thus these were eliminated rom further study.

The differences in fragmented nuclei between DBA^{Neg}, DBA^{Int}, and DBA^{High} displayed in Figure 2A correlate well with the gene-expression pattern shown in Figure 3A, where there was a relatively low expression of *Myc*, *Ccnd1*, and *Ccnd2* in $DBAL^{Neg}$ cells compared to $DBAL^{High}$ cells. This suggests that DBA is likely to bind more strongly to cells with high expression of proliferation-related genes and not to cells with low expression of proliferation-related genes

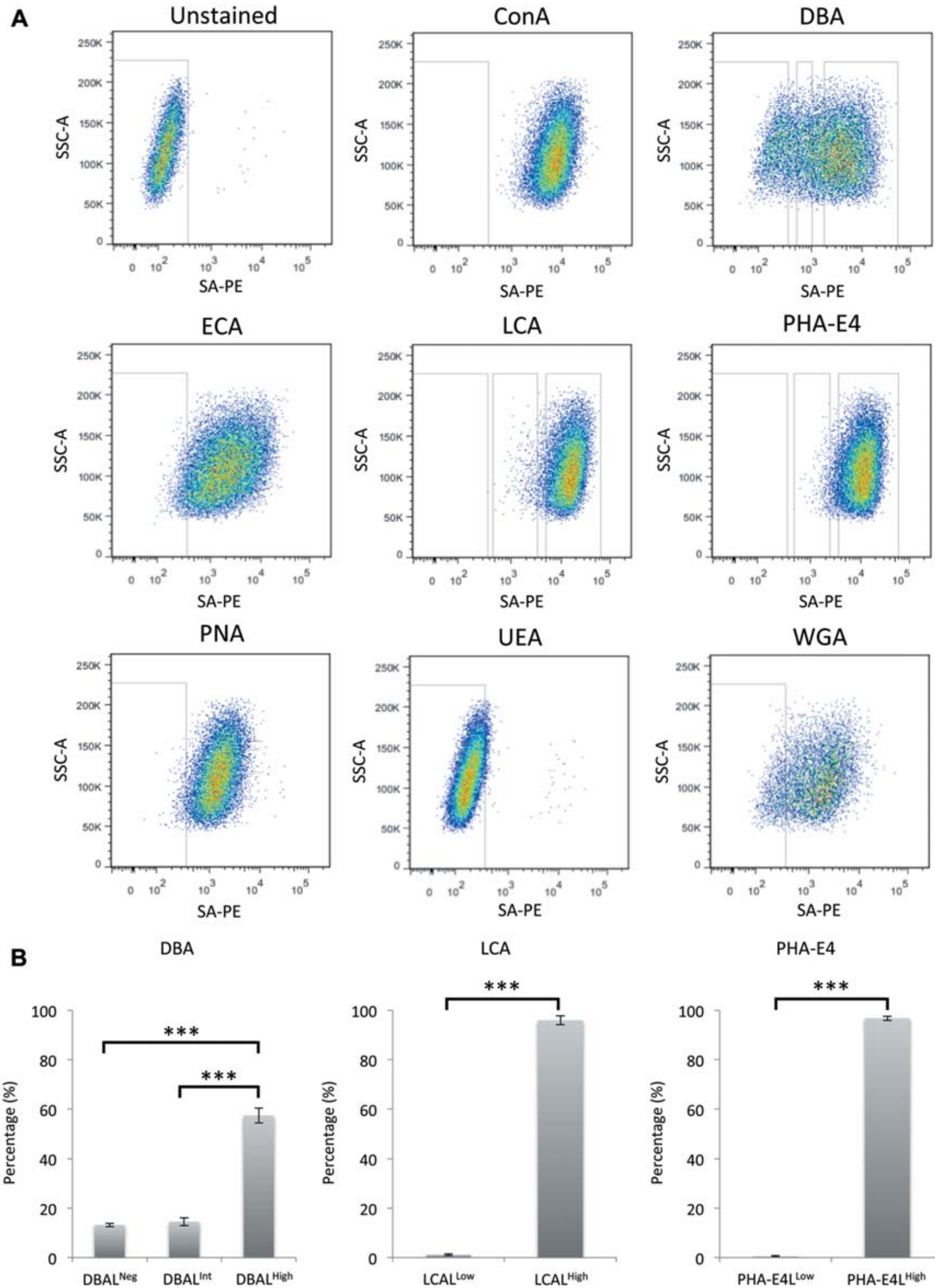

Figure 1. *Flow cytometric analysis of F5-5.F1 cells after staining with biotin-conjugated lectins. A: Flow cytometric profiles of F5-5.F1 cells after staining with biotin-conjugated lectins and anti-phycoerythrin-conjugated streptavidin (SA-PE). The flow cytometric profiles are representatives from three experiments with the panel at the bottom right showing cells stained by SA-PE but not labeled with any biotin-conjugated lectin. B: Graphical representations of the percentage of cells sorted by labeling with biotin-conjugated lectins. Cells labelled by Dolichos biflorus agglutinin (DBA) produced $DBAL^{Neg}$, $DBAL^{Int}$, and $DBAL^{High}$ populations, Lens culinaris agglutinin (LCA) produced $LCAL^{Low}$ and $LCAL^{High}$ populations, and Phaseolus vulgaris agglutinin (PHA-E4) produced PHA-$E4L^{Low}$ and PHA-$E4L^{High}$ populations (n=3). ***Significantly different at $p<0.001$.*

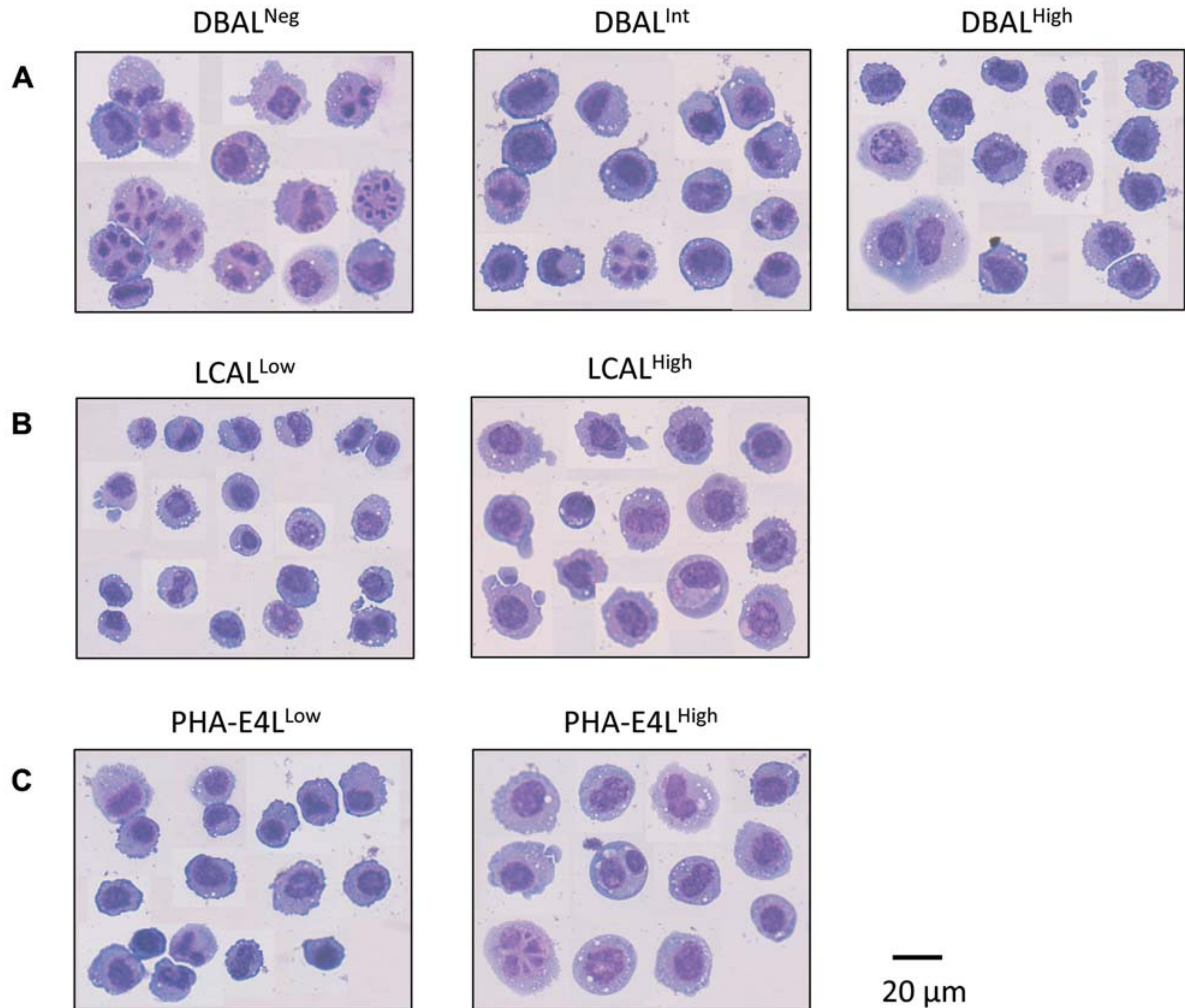

Figure 2. *Morphology of cells stained by May-Grünwald Giemsa. Sorted cells were attached to slides and stained using the May-Grünwald Giemsa staining technique. A: Representative images of cells labelled by Dolichos biflorus agglutinin (DBA). Representative fractions are $DBAL^{Neg}$, $DBAL^{Int}$, and $DBAL^{High}$. B: Representative images of cells labeled by Lens culinaris agglutinin (LCA). Representative fractions are $LCAL^{Low}$ and $LCAL^{High}$. C: Representative morphology of cells labelled by Phaseolus vulgaris agglutinin (PHA-E4). Representative fractions are PHA-$E4L^{Low}$ and PHA-$E4L^{High}$.*

and fragmented nuclei. From Figure 2B and C, it can be seen there was no obvious difference in the morphology of the cells and only a minor difference in the expression of *Myc*.

Ki-67 is a protein that is observed in the nuclei of dividing cells while in the G_1, S and G_2 phases and in mitosis, but not in the G_0 phase of quiescent cells, making it a useful marker for observing proliferating cells (27). Sorting of the cells by DBA implies that the greater the amount of DBA lectin that binds to the surface of F5-5.F1 cells, the more likely it is that Ki-67 is present in the nucleus (Figure 4A and B).

The MTS assay detects viable, metabolically active cells. The assay was performed independently from Ki-67 staining in order to confirm the proliferative status of cells by a second method. In metabolically active cells, the MTS reagent is metabolized into formazan, that can be detected by a microplate photometer. The gene expression in Figure 3A shows a correlation of the expression of *Myc* and *Ccnd1* with Ki-67 expression shown in Figure 4A and B, however, the expression of *Ccnd2* is higher in the intermediate population. The results of the MTS assay (Figure 4C) correlate with the expression of *Ccnd2* (Figure 3A), with a similar tendency.

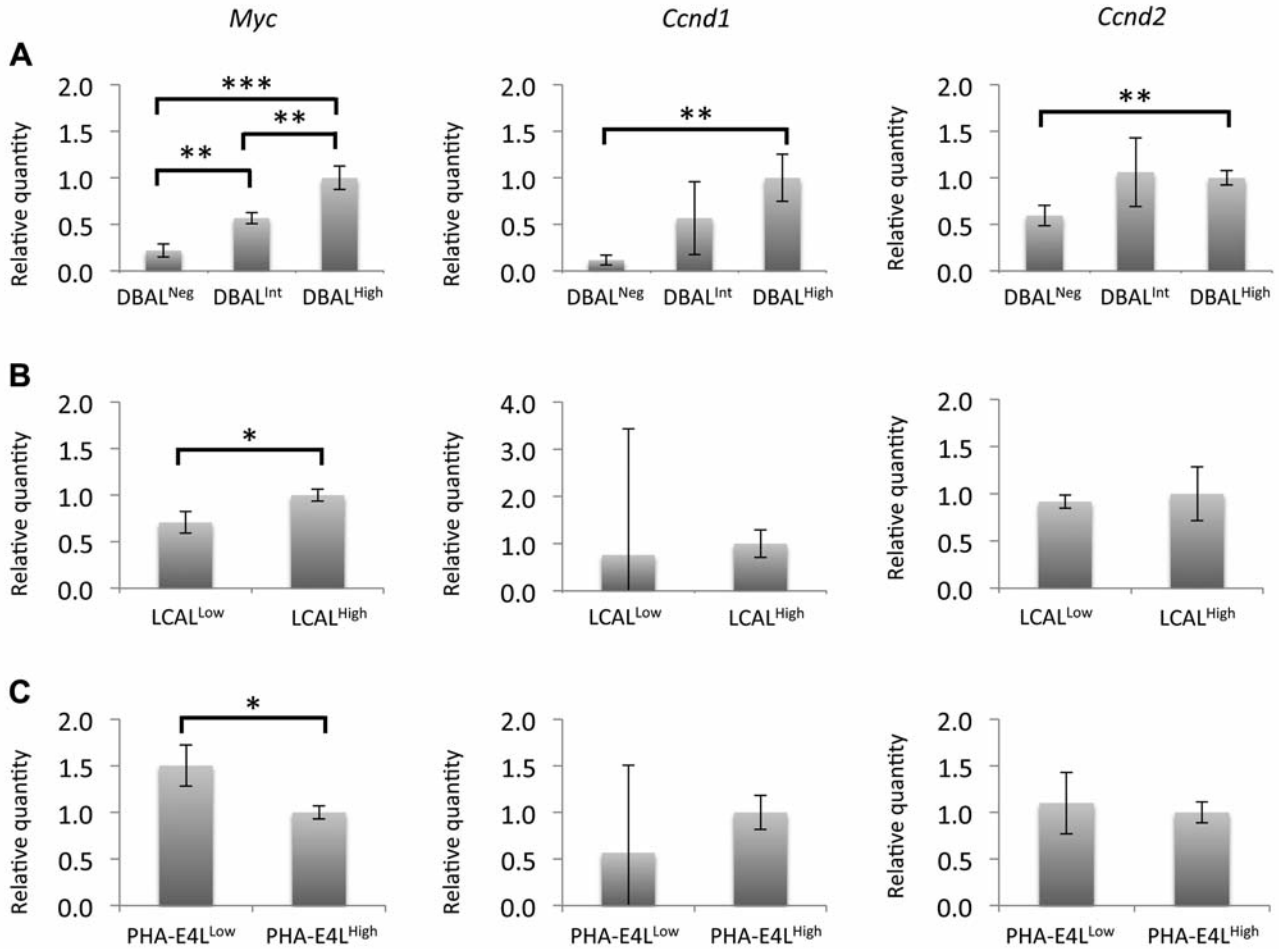

Figure 3. *Comparative gene expression of the relative quantity of proliferation genes in cells sorted by labeling with biotin-conjugated lectins. RNA was extracted from each cell population and the expression of the proliferation-related genes myelocytomatosis oncogene (Myc), cyclin D1 (Ccnd1), and cyclin D2 (Ccnd2) were investigated by quantitative real-time polymerase chain reaction. Cells sorted by labeling with Dolichos biflorus agglutinin (DBA) (A), Lens culinaris agglutinin (LCA) (B), and Phaseolus vulgaris agglutinin (PHA-E4) (C). Significantly different at *p<0.05, **p<0.01, and ***p<0.001. β-Actin was used as a reference gene and relative expressions were calculated compared to the expression level of DBAL^{High}, LCAL^{High} and PHA-E4L^{High} samples.*

Ccnd2 is required for the G_1/S cell cycle when the cell is undergoing normal metabolic activity followed by DNA replication (28). Since Ki-67 is present at G_1, S, and G_2 stages of the cell cycle, it is likely that DBAL^{Int} contains cells that are mainly in the G_1 and S phases, where *Ccnd2* is mostly expressed, but the DBAL^{High} population contains cells at G, S and G_2. Cells of the DBAL^{Neg} population had the lowest expression of proliferation-related genes and Ki-67 protein, and produced the lowest absorbance in the MTS assay. One potential explanation is that in the DBAL^{Neg} population there are fewer cells in the G_1, S and G_2 phases than in DBAL^{Int} and DBAL^{High} populations. In order to confirm the significance of these findings, further characterization of cell-cycle status in the DBA-sorted cells is required.

The information contained herein confirms that DBA lectin is able to characterize F5-5.F1 erythroleukemia cell lines based on proliferative status. This suggests that DBA can be used as a tool for understanding the mechanisms of leukemia cell proliferation and could potentially have similar applications in studies of other leukemia cell lines.

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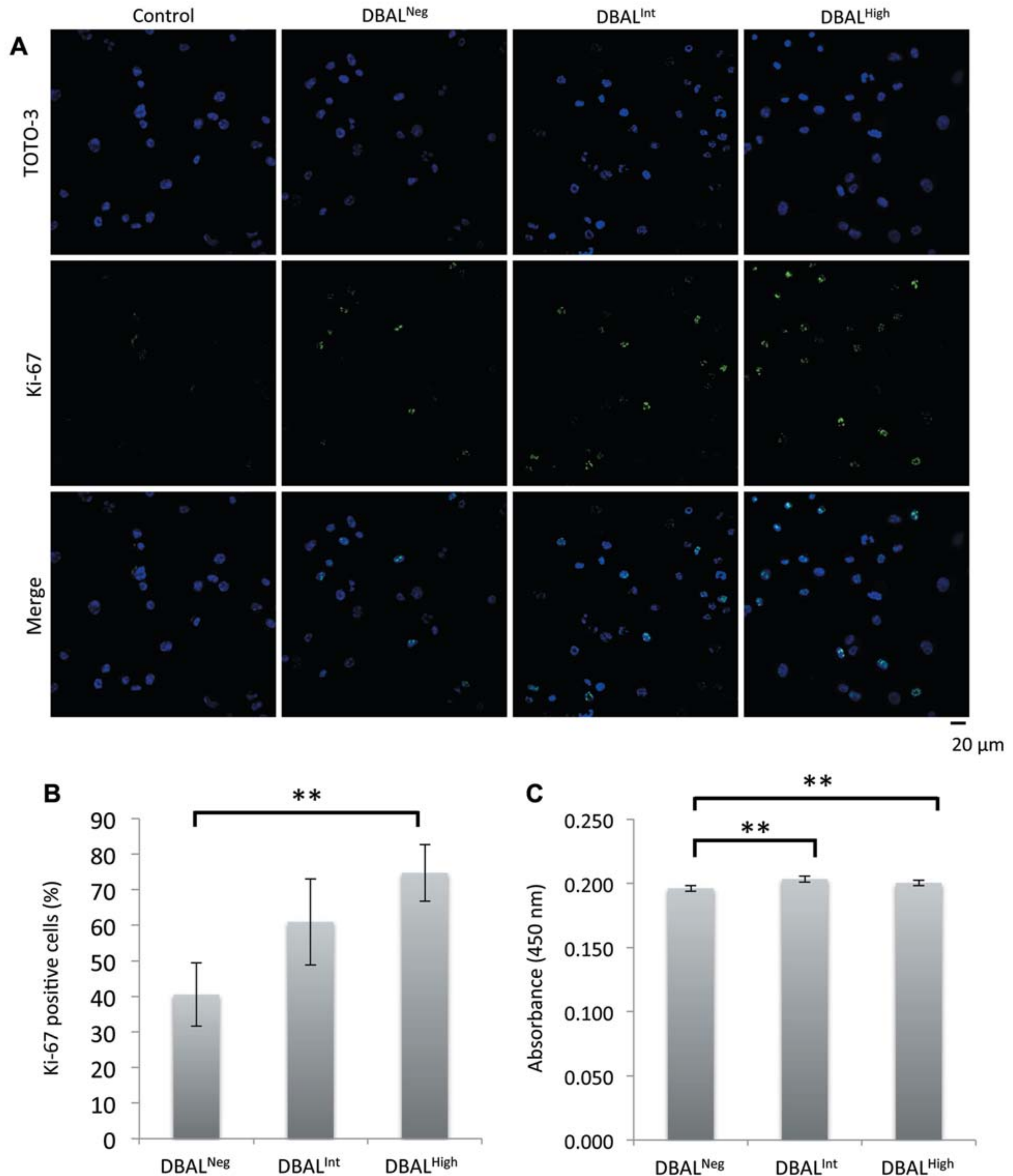

Figure 4. *Analysis of the proliferative status of F5-5.F1 cells sorted by labeling with biotin-conjugated Dolichos biflorus agglutinin (DBA). Two assays were performed in order to determine the proliferative status of cells sorted by labeling with DBA lectin. A: Representative images of cells F5-5.F1 cells sorted by labeling with biotin-conjugated lectin. The upper panel shows cells stained with AlexaFluor488-conjugated donkey anti-rat IgG, the middle panel shows nucleic acid stained by TOTO-3, and the lower panel merges the two images. The images on the far left represent slides that were not stained with the primary antibody (control). B: Graphical representation of the proliferative status through immunocytochemical staining (n=3). Each replicate of the experiment counted 500-1,000 nuclei to derive the data. C: Results from the CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay. Sorted cells were stained with CellTiter 96® AQ_{ueous} One Solution Reagent and the absorbance after 4 h of incubation is shown (n=3). **Significantly different at p<0.01.*

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