The *Amaranthus leucocarpus* Lectin Enhances the Anti-CD3 Antibody-Mediated Activation of Human Peripheral Blood CD4⁺ T Cells

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Activation of CD4⁺ T cells plays a main role in adaptive immune response by regulating cellular and humoral immunity via processes associated with changes in cell surface oligosaccharide receptors. Lectins are glycoproteins that specifically recognize oligosaccharides and have been used to characterize changes in oligosaccharides present on T cell surface and their effects on activation. A lectin from *Amaranthus leucocarpus* seeds (ALL) is specific for glycoprotein structures containing galactose-*N*-acetylgalactosamine and is able to bind to human and murine CD4⁺ T cells, however, its effect on activation remains unclear. We examined the effect of ALL on the activation of peripheral blood human CD4⁺ T cells and analyzed cell proliferation, expression of the activation-associated molecule CD25, secretion of the activation-dependent cytokine interleukin (IL)-2 and intracellular calcium influx changes using flow cytometry. CD4⁺ T cells were stimulated with anti-CD3 antibodies that provided the first activation signal in the presence or absence of ALL. ALL alone did not induce CD4⁺ T cell activation but when also stimulated with anti-CD3 antibodies, ALL up-regulated CD25 expression, cell proliferation, IL-2 secretion and an intracellular calcium influx in a dose-dependent manner. In addition, ALL recognized CD4⁺ T cells expressing the CD69 and Ki67 molecules expressed only by activated T cells and induced production of the TH1-type cytokine interferon- γ . Our findings indicate that ALL binds to human activated CD4⁺ T cells and enhances the degree of activation of CD4⁺ T cells that are stimulated with anti-CD3 antibodies. ALL provides a new tool for analyzing T cell activation mechanisms.

Keywords: *Amaranthus leucocarpus*; lectins; T cell activation; surface glycosylation; activation signals
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T cell activation is a highly regulated process by which CD4⁺ T cell subpopulations proliferate and differentiate into activated effector T cells after binding to respective antigen targets resulting in antigen clearance and regulation of both cellular and humoral immune responses (Askonas 1988; Huppa and Davis 2003). The cellular immune response is regulated by the TH-1 CD4⁺ T cell subpopulation (T helper type-1), which produces the cytokines interleukin-2 (IL-2) and interferon gamma (IFN- γ). In contrast, humoral responses are regulated by the TH-2 subpopulation that produces the cytokines IL-4 and IL-10 (Zhu et al. 2010). Diminished or inefficient CD4⁺ T cell activation facilitates microbial invasion and tumor progression and overstimulation or activation of alternate signaling pathways can lead to

pathological conditions such as autoimmunity. T cell activation requires two signals: a primary signal, consisting of recognition by the T cell receptor complex (TCR-CD3) on the T cell surface of peptide antigen presented by class II major histocompatibility complex (MHC II) antigens expressed by antigen presenting cells (APC). This interaction requires stable adhesion between T cells and APCs and is mediated by adhesion molecules and co-stimulatory molecules such as CD28 (Acuto and Michel 2003; Huppa and Davis 2003; Kinashi 2005). In addition, activated T cells express early activation-associated molecules such as CD69 and the nuclear protein Ki67 as well as the IL-2 cytokine receptor CD25 (Palutke et al. 1987; Testi et al. 1989; Malek and Bayer 2004). T cell activation is also associated with

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cellular proliferation, secretion of cytokines such as IL-2 and an increased intracellular calcium influx (Malek and Bayer 2004; Feske 2007). Cross-linking of the TCR-CD3 complex by anti-CD3 or anti-TCR monoclonal antibodies can activate T cells *in vitro* but full activation requires a second activation signal mediated by costimulatory molecules such as the CD28 receptor expressed on the T cell surface (Geppert and Lipsky 1988; Acuto and Michel 2003). Therefore, adequate T cell activation involves critical cellular recognition events mediated by specific cell surface receptors, most of which are glycoproteins (Rudd et al. 2001; Marth and Grewal 2008). Advances in immunobiology have demonstrated that glycosylation of cell surface receptors regulates critical T cell processes including activation, and this regulation involves specific changes to cell surface oligosaccharide structures in a function-dependent manner (Rudd et al. 2001; Nguyen et al. 2001; Daniels et al. 2002; Comelli et al. 2006; Golks et al. 2007). Therefore, in order to understand T cell activation mechanisms it is essential to define how differential glycosylation affects this process.

Lectins are glycoproteins found in plants that recognize cell surface oligosaccharides and have been used as tools in immunology to characterize changes to oligosaccharides present on T cells and their effects on T cell activation (Galvan et al. 1998; Sharon and Lis 2004; Merant et al. 2005; Shanmugham et al. 2006; Keshewani and Sodhi 2007; Gavrovic-Jankulovic et al. 2008). Moreover, some lectins can activate T cells by cross-linking T cell surface receptors in a carbohydrate-dependent manner (Shanmugham et al. 2006; Keshewani and Sodhi 2007). Our laboratory isolated a lectin from *Amaranthus leucocarpus* seeds (ALL) that is specific for structures containing the galactose-*N*-acetylgalactosamine and *N*-acetylgalactosamine saccharides found in glycoproteins (Zenteno et al. 1992; Hernandez et al. 2004). ALL recognizes human and murine peripheral CD4⁺ T cells and ALL recognition increased three-fold on activated CD4⁺ T cells harvested from mouse lymph nodes (Lascrain et al. 1994; Ortiz et al. 2002). Moreover, ALL recognized murine CD4⁺ T cells expressing the activation-associated molecules CD69 and CD25 (Ortiz et al. 2002). Although these studies suggested that ALL selectively detected activation-related changes in CD4⁺ T cell surface carbohydrate moieties, the effect of ALL on the activation process remains unclear. In this study, we analyzed the effect of the ALL on the activation process of CD4⁺ T cells purified from the peripheral blood of healthy donors by analyzing cell proliferation, the expression of the activation associated cell surface marker CD25, the secretion of the activation-dependent cytokine IL-2 and changes in intracellular calcium influx using flow cytometry. In addition, we analyzed the secretion of IFN γ , IL-4 and IL-10 in pure CD4⁺ T cell cultures and the binding capacity of ALL to activated CD4⁺ T cells.

Materials and Methods

Reagents and antibodies

Bovine serum albumin (BSA) fraction V, GalNAc and chemical reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fluorescein isothiocyanate (FITC)-labeled antibodies to human CD4, IL-2, Ki67, phycoerythrin (PE)-labeled antibodies to CD4, CD25, IL-10, CD69 and IFN- γ and PE-Cy7-labeled antibodies to IL-4 were obtained from e-Biosciences (San Diego, CA, USA). Anti-CD3 and anti-CD28 antibodies, PE- or CyChr-streptavidin were purchased from PharMingen (New York, NY, USA). Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was obtained from Molecular Probes (Eugene Oregon, USA). The CD4 T cell isolation Kit II (MiniMACS system) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Lectin purification

A. leucocarpus seeds were collected from wild plants located in Tulyehualco (Mexico) and identified at the Centro de Investigaciones Biológicas de Baja California Sur, Mexico. ALL was purified by affinity chromatography on a column containing human type O red blood cell stroma as described by Zenteno and Ochoa (1988). ALL was then labeled with N-hydroxysuccinimide ester (biotin) (Pierce Chemical Co., Rockford, IL, USA) at a label/protein ratio of 2:1 (Savage et al. 1992).

PBMCs

Peripheral blood mononuclear cells (PBMCs) from healthy donors were separated by Ficoll-Hypaque density gradient and centrifuged at 1700 rpm for 30 min at 18°C. The cells were collected, washed twice and then suspended in phosphate buffered saline (PBS; 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.2). CD4⁺ T cells were purified from PBMCs by negative magnetic separation using the MiniMACS system (Miltenyi Biotec). The purity of CD4⁺ T cells was > 95% determined by flow cytometry using FITC- and PE-labeled anti-human CD4 antibodies. Cell viability (>90%) was determined by the trypan blue exclusion method. The study protocol was approved by the institutional Ethics Committee and written informed consent was obtained from all subjects.

Cell culture

PBMCs or purified CD4⁺ T cells (2×10^5) were cultured in 96-well flat bottomed cell culture plates (Costar, Cambridge, MA, USA) in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μ g/ml gentamicin and 10% heat-inactivated fetal calf serum (cRPMI) at 37°C in a 5% CO₂ humidified incubator.

Binding of ALL to activated CD4⁺ T cells

To analyze the binding capacity of ALL to activated CD4⁺ T cells, PBMCs were cultured with 10 μ g/ml of soluble anti-CD3 antibodies for 24, 48, 72 and 96 h. At each time point, cells were harvested, washed with PBS containing 1% BSA and 0.1% sodium azide (PBA) and incubated with biotin-labeled ALL for 20 min at 4°C followed by a CyChr-streptavidin and PE-labeled anti-CD4 antibodies for 15 min at 4°C. The binding of ALL to gated CD4⁺ T cells was analyzed by flow cytometry. Untreated cells were used as a non-activated cell control. The specificity of ALL binding was determined by competitive inhibition assays carried out using 200 mM of the inhibi-

tory sugar GalNAc (Zenteno and Ochoa 1988; Hernandez et al. 2001) at 37°C for 30 min after ALL staining.

Expression of activation-associated molecules CD69 and Ki67

PBMCs were cultured in presence of 10 µg/ml of soluble anti-CD3 antibodies for 48 h at 4°C. Cells were washed with PBA and stained with biotin-labeled ALL for 20 min at 4°C followed by staining with CyChr-labeled streptavidin and anti-CD4 and anti-CD69 antibodies for 15 min at 4°C. CD69 expression along with the ALL binding to CD4⁺ T cells was analyzed by flow cytometry. Untreated cells were used as non-stimulated cell controls.

To analyze the expression of the nuclear protein Ki67, PBMCs were cultured as described above and cells stained with biotin-labeled ALL and anti-CD4 antibodies as described. Cells were then washed with PBS and fixed with 4% (w/v) *p*-formaldehyde in PBS for 10 min at 4°C, washed twice with PBA and permeabilized with saponin buffer 1 (0.1% saponin, 0.01% pig IgG, 10 mM HEPES, 10% BSA in PBS) for 10 min at room temperature (Jung et al. 1993; Prussin and Metcalfe 1995). Cells were then incubated with FITC-labeled anti-Ki67 antibodies for 30 min at 4°C and the CD4⁺ T cell intracellular Ki67 expression (along with the binding of ALL to CD4⁺ T cells) was analyzed by flow cytometry.

CD4⁺ T cell proliferation

Purified CD4⁺ T cells (1×10^7) were stained with 1 µM CFDA-SE (Molecular Probes, Eugene, Oregon) for 15 min at 37°C in the dark and then incubated with cRPMI for an additional 10 min (Hasbold et al. 1999). Cells were washed and cultured in a 96-well plate in the presence of varying ALL concentrations, immobilized anti-CD3 antibody (2.5 µg/mL) or ALL in combination with immobilized anti-CD3 antibody for 72 h. Cells were then harvested and stained with PE-labeled anti-CD25 antibodies for 15 min at 4°C. Proliferation and CD25 expression levels were analyzed by flow cytometry. Some experiments were carried out in the presence of an anti-CD28 antibody (1.0 µg/mL). Untreated cells were used as a control. CFDA-SE is a cell permeable dye that binds to cytoplasmic proteins producing measurable fluorescence. When stained cells divide by mitosis the CFDA-SE concentration is halved between daughter cells and reductions in fluorescence detected as a measure of proliferation by flow cytometry analysis.

Detection of intracellular cytokine secretion

To analyze the production of cytokines, purified CD4⁺ T cells were cultured in the presence of ALL, anti-CD3 or both ALL and anti-CD3 for 24, 48 and 72 h. Five hours before harvesting, brefeldin-A was added (10 µg/ml) to respective wells. Cells were then washed with PBS and fixed in 4% (w/v) *p*-formaldehyde in PBS for 10 min at 4°C, washed twice with PBA and permeabilized with saponin buffer 1 (0.1% saponin, 0.01% pig IgG, 10 mM HEPES, 10% BSA in PBS) for 10 min at room temperature (Jung et al. 1993; Prussin and Metcalfe 1995). Cells were then incubated with antibodies specific for either IL-2, IL-4, IL-10 or IFN-γ for 30 min at 4°C and intracellular staining was analyzed by flow cytometry.

Flow cytometry

Cells were washed with PBS containing 1% BSA and 0.1% sodium azide (PBA) and incubated with the indicated antibody for 15 min at 4°C. After incubation, the cells were washed with PBA and analyzed by flow cytometry. A minimum of 1×10^4 cells were ana-

lyzed for all experiments. Analysis of lymphocytes was performed according to cell size and granularity. Debris and dead cells were excluded using side scatter and forward scatter plots. Measurements were performed on a FACScalibur flow cytometer (Becton & Dickinson, Franklin Lakes, NJ) and the data analyzed using FlowJo software (TreeStar Inc. Ashland Oregon). Flow cytometric analysis of PBMCs was carried out by gating first on the region corresponding to CD4⁺ cells, followed by analysis of Ia ALL binding and/or antibody staining.

Intracellular calcium influx

Purified CD4⁺ T cells (1×10^7) were incubated with 1 mM Fluo-4 (Molecular Probes) for 30–45 min at room temperature under shaking in the dark. After incubation, cells were washed and suspended in RPMI without fetal calf serum. Cells (1×10^6) were analyzed by flow cytometry for 30–40 s to define the basal calcium levels prior to stimulation with 10 µg/ml of soluble anti-CD3 and 5 µg/ml of ALL and measuring the calcium influx over a 5-min period (Gee et al. 2000). Ionomycin-stimulated cells were used as a positive control.

Statistical analysis

Statistical significance was determined using the SPSS14 software (Chicago, IL, USA) using the Student's *t*-test with a two-tailed distribution. Values were considered statistically significant at $p \leq 0.05$.

Results

Binding of ALL to activated CD4⁺ T cells

Previous studies indicated that ALL recognizes murine activated CD4⁺ T cells expressing activation-associated receptors (Ortiz et al. 2002). To analyze the binding of ALL to human activated CD4⁺ T cells, PBMCs were activated with soluble anti-CD3 antibodies for 24, 48, 72 and 96 h and the binding of ALL to CD4⁺ T cells was analyzed by flow cytometry (Fig. 1). Results indicated that ALL bound to $48.7 \pm 8.7\%$ of non-activated CD4⁺ T cells and to $81.2 \pm 3.3\%$ of activated CD4⁺ T cells by 72 h (Fig. 1A and B) indicating that ALL binding to CD4⁺ T cells increased upon activation. In order to investigate whether the binding of ALL to T cells was saccharide-dependent, we performed competitive binding assays using the ALL-specific carbohydrate *N*-acetyl-galactosamine (GalNAc) (Zenteno and Ochoa 1988; Hernandez et al. 2001), in which we used GalNAc to inhibit ALL binding to CD4⁺ T cells by competitively blocking the binding sites. Results showed that GalNAc inhibited ALL binding to non-activated and activated CD4⁺ T cells indicating a carbohydrate-dependent interaction (Fig. 1D).

To verify that ALL binds activated CD4⁺ T cells, we examined the expression of the known activation-associated molecules CD69 and Ki67 on CD4⁺ T cells. CD69 is a cell surface receptor expressed on T cells during the first stages of activation, and Ki67 is a nuclear protein associated with the cellular proliferation process that is upregulated after T cells receive the first activation signal. Because these molecules are detected during the first stages of activation they are considered early-activation markers. Results indicated

Fig. 1. ALL binding to activated CD4⁺ T cells. PBMCs were stimulated with soluble anti-CD3 antibodies for 24, 48, 72 and 96 h; cells were then harvested and stained with biotin-labeled ALL followed by CyChr-labeled streptavidin and anti-CD4 and anti-CD69 antibodies or anti-CD4 and anti-Ki67 antibodies. The binding of ALL to CD4⁺ T cells was analyzed by flow cytometry as well as the ALL binding to CD4⁺ T cells expressing the activation-associated molecules CD69 or Ki67. (A) Representative flow cytometric analysis with dot plot graphs of ALL binding to gated CD4⁺ T cells (inner squares) cultured at the indicated time points. The numbers represent the percent CD4⁺ T cells bound to ALL. (B) Percent CD4⁺ T cells bound to ALL following stimulation with anti-CD3 antibodies (black diamonds) and non-stimulated cells (grey squares). Data are expressed as the mean $\pm$ standard deviation of 4 observations ($n = 4$). (C) Representative flow cytometric analysis with dot plot graphs showing ALL binding to gated CD4⁺ T cells expressing CD69 (upper panel) or Ki-67 molecules (lower panel) at 48 h post stimulation inside the inner square ($n = 4$). (D) Representative flow cytometric analysis showing ALL binding to CD4⁺ T cells either un-stimulated or stimulated with anti-CD3 antibodies in the presence (thin line) or absence (thick line) of 200 mM GalNAc.

Fig. 2. ALL increases proliferation and CD25 expression of CD4⁺ T cells activated by anti-CD3 antibodies. Purified CD4⁺ T cells were stained with CFDA-SE and then cultured in the presence of ALL (at indicated concentrations), anti-CD3 antibodies, ALL plus anti-CD3 antibodies, anti-CD28 antibodies or anti-CD3 plus anti-CD28 antibodies for 72 h. Proliferation and expression of the activation-associated molecule CD25 were analyzed by flow cytometry. (A) Representative histogram showing the proliferation of CD4⁺ T cells (upper panel) and the CD25 expression (lower panel). Numbers represent percent proliferation and CD25 expression, respectively. (B) The effects of various stimuli on the ratio of proliferation (upper panel) and CD25 expression (lower panel) of CD4⁺ T cells. Bars represent the mean $\pm$ standard deviation of 5 observations ($n = 5$). * $p < 0.05$ using the Student's t test. Ratio = CD4⁺ T cells activated with anti-CD3 antibodies/CD4⁺ T cells activated by other stimuli.

that ALL bound to $88.7 \pm 3.6\%$ of $CD4^+$ T cells expressing the CD69 molecule (Fig. 1C upper panels) and to $59.65 \pm 2.7\%$ of $CD4^+$ T cells expressing the Ki67 molecule at 48 h (Fig. 1C lower panels). These data showed that ALL recognizes $CD4^+$ T cells expressing activation-associated markers.

ALL increased $CD4^+$ T cell proliferation and CD25 up-regulation following anti-CD3 antibody-mediated activation

To analyze the effect of ALL on the activation of $CD4^+$ T cells, we analyzed the proliferation and expression of the activation-associated molecule CD25 on purified $CD4^+$ T cells using flow cytometry and CFDA-SE staining. Purified $CD4^+$ T cells were stained with CFDA-SE and cultured in the presence of ALL (at indicated concentrations), anti-CD3 antibodies or ALL plus anti-CD3 antibodies. Untreated cells were used as a control. Additional activated T cell controls consisted of $CD4^+$ T cells stimulated with either

anti-CD28 antibodies or with anti-CD3 and anti-CD28 antibodies.

When $CD4^+$ T cells were treated with ALL alone ($2.5\text{--}20\text{ }\mu\text{g/mL}$) proliferation did not significantly increase compared to untreated cells (Fig. 2A and 2B, upper panels). However, the presence of ALL significantly increased the proliferation induced by anti-CD3 antibodies compared to the proliferation induced by anti-CD3 antibodies alone ($p = 0.028$) (Fig. 2B, upper panel). Moreover, the presence of ALL significantly increased CD25 expression on $CD4^+$ T cells stimulated with anti-CD3 antibodies compared to the CD25 expression induced by anti-CD3 antibodies alone ($p = 0.05$) (Fig. 2A and 2B, lower panel). These effects were dose-dependent (data not shown), with the optimal concentration of ALL at $5\text{ }\mu\text{g/mL}$. Interestingly, the proliferation induced by ALL together with anti-CD3 antibodies was similar to the proliferation induced by anti-CD3 and anti-CD28 antibodies (Fig. 2A and 2B, upper panel).

These data suggested that ALL alone did not induce

Fig. 3. ALL increases the IL-2 and IFN- γ production of $CD4^+$ T cells activated by anti-CD3 antibodies. Purified $CD4^+$ T cells were cultured in the presence of ALL, anti-CD3 antibodies, or ALL plus anti-CD3 antibodies for 24, 48 and 72 h and the intracellular production of IL-2, IFN- γ , IL-4 and IL-10 assessed by flow cytometry. Representative flow cytometric analyses of density plot graphs of cytokine production after culture for 24 h are shown. Numbers inside the inner square indicate the percentage of cells producing respective cytokines ($n = 4$).

Table 1. ALL enhances IL-2 and IFN- γ production in CD3-stimulated CD4⁺ T cells.

Cytokines	Medium	ALL	Anti-CD3+Anti-CD28	Anti-CD3	Anti-CD3+ALL
IL-2	4.9 $\pm$ 1.2	5.39 $\pm$ 1.3	6.34 $\pm$ 1.7	5.75 $\pm$ 0.7*	8.32 $\pm$ 1.6*
IFN- γ	6.13 $\pm$ 1.7	6.57 $\pm$ 0.6	10.01 $\pm$ 3.6	7.85 $\pm$ 1.3**	12.06 $\pm$ 2.7**
IL-10	4.4 $\pm$ 1.3	6 $\pm$ 2.3	4.77 $\pm$ 1.3	6.25 $\pm$ 0.4	7.19 $\pm$ 2.2
IL-4	4.98 $\pm$ 1.7	5.06 $\pm$ 1.4	4.87 $\pm$ 1.5	6.08 $\pm$ 1	6.16 $\pm$ 1.8

Cytokine production from purified CD4⁺ T cells cultured for 24 h. Data are represented as the mean percentage $\pm$ standard deviation of the positive cells for each cytokine ($n = 4$). * $p \leq 0.05$, ** $p < 0.05$ using Student's t test.

CD4⁺ T cell proliferation or CD25 expression but potentiated proliferation and CD25 expression induced by the activation signals triggered by anti-CD3 antibodies.

ALL increased the IL-2 and IFN- γ production of CD4⁺ T cells activated by anti-CD3 antibodies

T cell activation is characterized by IL-2 secretion which provides one of the necessary signals driving T cell proliferation. In addition, T cells can produce a pattern of cytokines that characterize TH-1 (IL-2 and IFN- γ) or TH-2 (IL-4 and IL-10) responses, respectively. To determine the cytokine profile elicited by ALL, purified CD4⁺ T cells were cultured in the presence of ALL, anti-CD3 antibodies or ALL and anti-CD3 antibodies for 24, 48 and 72 h and intracellular production of IL-2, IFN- γ , IL-4 and IL-10 assessed by flow cytometry. Fig. 3 shows a representative flow cytometric analysis of intracellular cytokine production at 24 h. This experiment demonstrated a significant increase in IL-2 and IFN- γ production when the cells were incubated with anti-CD3 antibodies and ALL ($p = 0.042$ and 0.044 , respectively) compared to CD4⁺ T cells treated with anti-CD3 antibodies only at the 24 h time point (Table 1 and Fig. 3). In contrast, we did not observe significant differences in IL-4 or IL-10 production in any of the cultures examined at any time point. The level of IL-2 and IFN- γ produced by CD4⁺ T cells stimulated with anti-CD3 antibodies plus ALL were similar to those produced by CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 antibodies (Table 1) suggesting that ALL has the capacity to provide a second activation signal.

ALL increased intracellular calcium influx in CD4⁺ T cells activated by anti-CD3 antibodies

During T cell activation antigen recognition via the TCR-CD3 complex on T cells (first activation signal) in the context of MHC II initiates a series of biochemical changes within the T cell. These intracellular changes include the release of calcium ions (Ca²⁺) from intracellular stores allowing an influx of extracellular Ca²⁺ (Feske 2007). When purified CD4⁺ T cells were stimulated with anti-CD3 antibodies in the presence or absence of ALL, flow cytometry revealed that the presence of ALL significantly increased the CD4⁺ T cell intracellular calcium influx if also incubated with anti-CD3 antibodies compared to the intracellular calcium influx levels observed in CD4⁺ T cells stimulated with

Fig. 4. ALL increases the intracellular calcium influx of CD4⁺ T cells activated by anti-CD3 antibodies. Purified CD4⁺ T cells were stimulated with anti-CD3 antibodies in the presence or absence of ALL or with ALL alone and the intracellular calcium influx was analyzed by flow cytometry ($n = 3$). The thick arrow indicates the start of stimulation and thin arrows indicate the stimulus used.

anti-CD3 antibodies alone (Fig. 4). ALL alone did not induce detectable changes in the intracellular calcium levels of CD4⁺ T cells.

Discussion

It has been shown that cell surface oligosaccharides undergo structural changes as a consequence of T cell activation (Piller et al. 1988), and recent evidence has indicated that these changes were involved in regulation of T cell function (Rudd et al. 2001; Nguyen et al. 2001; Daniels et al. 2002; Comelli et al. 2006; Golks et al. 2007). Because activation of the CD4⁺ T cell subpopulations plays a critical role in mediating adaptive immune responses, a better understanding regarding how differential glycosylation affected CD4⁺ T cell activation is needed, and lectins have been proven to be powerful tools in analyzing the role of cell surface oligosaccharides in T cell activation.

The present study demonstrated that a lectin extracted

from *A. leucocarpus* seeds (ALL) bound to activated human CD4⁺ T cells resulting in elevated proliferative responses when combined with anti-CD3 antibodies. ALL-mediated effects on CD4⁺ T cell activation were due to specific interactions with cell-surface carbohydrates but unlike other lectins (*e.g.*, Con A) that induce T cell activation alone (Ashraf and Khan 2003), ALL without a primary signal did not induce CD4⁺ T cell activation. Instead, ALL enhanced the intensity of signal one delivered by anti-CD3 antibodies in our stimulation model. Although ALL bound to non-stimulated CD4⁺ T cells (Fig. 1A), the lack of signals resulting from ALL ligation alone can not be explained by a lack of ALL receptors. This observation suggested that ALL alone was incapable of properly activating CD4⁺ T cells but acted synergistically with signal one if delivered via CD3.

Only jacalin, a lectin from *Artocarpus integrifolia* has been shown to enhance activation signals in human T cells (Tamma et al. 2006a, 2006b). This lectin has an affinity for structures containing the disaccharide Gal-GalNAc similar to ALL, however, it has been reported that jacalin alone induced proliferation of human CD4⁺ T cells through interactions with CD45 and only induced IL-4 synthesis (Tamma et al. 2006a; Baba et al. 2007). In contrast, ALL alone did not induce proliferation of CD4⁺ T cells and enhanced IFN- γ cytokine secretion during activation triggered via the ligation of anti-CD3 antibodies, indicating that ALL contributed to the development of TH-1 responses. Reports of lectins with similar specificities toward simple sugars displaying different biological effects are not unusual (Benoist et al. 2009). Many studies have shown that lectins can recognize subtle glycosylation modifications and these observations may explain how lectins bind to distinct molecules, thereby eliciting different functions (Nakamura-Tsuruta et al. 2008). In this regard, Hernandez et al. (2004) reported that the affinity of ALL for glycans was determined by the spatial conformation of saccharides present in the protein backbone, suggesting that ALL expressed allosteric restrictions that may have allowed it to recognize different ligand domains.

To our knowledge this is the first report of a lectin specific for glycans containing GalNAc or Gal-GalNAc structures that synergistically increased CD4⁺ T cell activation triggered by anti-CD3-mediated signals that was unable to induce CD4⁺ T cell activation alone. This finding could provide of a new tool for the analysis of T cell activation mechanisms involving signal one delivered via CD3 ligation and the recognition of cell surface oligosaccharides. In addition, it could provide an alternative tool for the detection of activated T cells.

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