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Serial study of lymph node cell subsets using fine needle aspiration in pigtail macaques

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Abstract

Lymphoid tissues are of intense interest for studies of the pathogenesis of human immunodeficiency virus (HIV) in humans and simian immunodeficiency virus (SIV) in macaques but are relatively difficult to sample non-invasively. Fine needle aspiration (FNA) cytology, conventionally a diagnostic procedure for lymphadenopathy, can be used for longitudinal study of tissue cell subsets during HIV/SIV infection. In this study, we serially sampled lymph node (LN) FNA from pigtail macaques and studied cell subsets in the aspect of absolute count, frequency, and functionality by flow cytometry. The median recovered lymphocyte count from FNA samples was 2.01×10^5 (3.0×10^3 to 2.25×10^6, n = 38) and median CD4+ T cell subset recovered was 5.94×10^4 (277 to 6.17×10^5, n = 38). Although we observed a relatively large variation in the frequencies of cell subsets of FNA samples taken from different time points, the cell subset composition of FNA samples, in particular T cell and CD4+ T cell frequencies, was broadly comparable to whole excised LNs (n = 6) and distinct from peripheral blood. A subset of CD4+ T cells that is located almost exclusively in secondary lymphoid tissues, T follicular helper (T_{FH}) cells, were readily identifiable in LN FNAs and the T_{FH} cell frequencies were strongly correlated with B cell frequencies. *In vitro* functionality of FNA lymphocytes was demonstrated using polyclonal SEB stimulation, resulting in a median 6% of responding CD4+ T cells, comparable to circulating CD4+ T lymphocytes. We conclude that serial sampling of macaque LNs using FNA is a potentially useful method to study the immunopathogenesis of SIV infection and may be extended to HIV infection.

Abbreviations

HIV: human immunodeficiency virus, SIV: simian immunodeficiency virus, FNA: fine needle aspirate, LN: lymph node, T_{FH} cell: T follicular helper cell, GI tract: gastrointestinal tract, ART: anti-retroviral therapy, PBMC: peripheral blood mononuclear cell, min: minute, h: hour.

Keywords

Fine needle aspiration, flow cytometric immunophenotyping, lymph node, T follicular helper cells.

1. Introduction

Much has been learned from studying mouse lymphoid tissues through serial sacrifice studies (Harker et al., 2011; Poovassery and Moore, 2006), but serial studies of larger animal or human lymphoid tissues are more difficult. Elegant lymphatic cannulation studies in animals such as sheep have provided very useful information about trafficking of immune cells (West et al., 2001), but cannulation studies are rarely performed in higher mammals or non-human primates. Serial changes within lymphoid tissues such as gastrointestinal (GI) tract or LNs usually require excisional biopsy. The number of live excisional biopsies possible in any one animal is usually limited by the number of sites accessible and ethical constraints. There is a need for improved technologies to study serial lymphoid tissue samples in human and higher mammals such as non-human primates.

FNA cytology, which involves inserting a narrow gauge (25-22 G) needle into a mass and suction aspirating cell samples for microscopic examination, has been developed for the diagnosis and management of patients with malignancy and certain infections and has the advantage of being quick, low cost and less invasive compared to surgical excision or core biopsy (Roskell and Buley, 2004). Diagnostic accuracy, specificity and sensitivity are greatly increased when FNA cytology is used in combination with flow cytometric analysis, which has the ability to measure multiple parameters on individual cells and allows high throughput (Schmid et al., 2011).

One disease, in particular, that could benefit from longitudinal studies of lymphoid tissues using FNA is HIV infection. HIV infection is characterised by progressive,

systemic CD4+T cell depletion (Grossman et al., 2006). CD4+ T cell dynamics and dysregulation of homeostasis during all stages of HIV infection have been well described in peripheral blood. Cross-sectional studies on lymphoid tissues have revealed that the GI tract has a very profound CD4+ T cell loss during HIV infection, but LNs are believed to be the major sites where viral replication, immune activation, and greatly increased T cell proliferation and apoptosis occur (Brenchley et al., 2004; Grossman et al., 2006; Schacker et al., 2001; Zhang et al., 1999). However, serial examination of lymphoid tissues, especially during the extremely dynamic acute phase of HIV infection, are very limited, as current biopsy techniques to obtain tissue lymphocytes, such as peripheral LN excision and colonoscopy, are time-consuming and invasive, and have the potential for significant morbidity.

In the late 1990s, a few studies examined ultrasound-guided LN FNA sampling in chronic HIV infected patients in the aspects of lymph node cell phenotyping and viral burden (Bart et al., 1999; Burgisser et al., 1997; Fleury et al., 2000; Meylan et al., 1996), but this has not been followed up over the last 10 years. Similar studies have not yet been reported on subjects with acute HIV infection. In the setting of HIV infection, FNA is currently used for the diagnosis of HIV-relating infections and neoplasms by cytology (Lang et al., 2011). The utility of FNA for studying non-human primate LN samples is not well established. With the growing interest in LN cell subsets such as T_{FH} in HIV/SIV infection (Hong et al., 2012; Lindqvist et al., 2012; Petrovas et al., 2012; Xu et al., 2013), it is timely to investigate the utility of LN FNA in frequent serial sampling of non-human primates.

In this study, we used pigtail macaque (*Macaca nemestrina*) as a model to assess the safety of frequent sampling of FNA from peripheral (inguinal) lymph nodes, to measure the absolute cell number, to monitor the detailed composition of key populations of the lymphocytes longitudinally, and to look for antigen-specific CD4+ T cells.

2. Materials and Methods

2.1 Animals and ethics

Peripheral blood, inguinal LN FNA samples and/or whole inguinal LNs were taken from 24 healthy pigtail macaques (*Macaca nemestrina*), which are described in Table 1.

LN FNAs were taken at baseline from 24 animals. Six of them were longitudinally sampled a further two to three times over the following 70 days. Two passes were performed on an inguinal lymph node of each macaque, using a 25-gauge needle attached to a 1 ml syringe. A palpable inguinal lymph node was immobilised against the pubis bone with 2 fingers of one hand and with the tip of the needle within the lymph node the syringe plunger was pulled back and 2 passes through the lymph node made. If there was blood visible in the hub of the needle, the sample was discarded. The aspirates were dispensed into RPMI1640 medium (Gibco®) for flow cytometric analysis. At the same time, 9 ml of peripheral blood was drawn into sodium heparin-anticoagulated blood collection tube as a comparator.

For seven animals, a whole inguinal LN was excised for comparison to a FNA performed on the contralateral inguinal LN. A 1.5 cm incision was made over a palpable inguinal LN and the LN (usually 0.8 to 1.0 cm in diameter) excised was placed in RPMI1640 medium.

Lymphoid tissue experiments on pigtail macaques were approved by the Animal Ethics Committee of the Australian Animal Health Laboratory, Geelong, Victoria, CSIRO Livestock Industries, a department of the Australian Government. All animals were cared

for in accordance with the Australian code of practice for the care and use of animals for scientific purposes, issued by the National Health and Medical Research Council in conjunction with CSIRO Livestock Industries and the Australian Research Council. The Australian Animal Health Laboratory has Scientific Procedures Premises Licence (SPPL) number 113.

2.2 Flow cytometry

Monoclonal antibodies to human or non-human primate (NHP) proteins used in this study were CD3-APC, -PE-Cy7 and -Pacific Blue (PB) (clone SP34-2), CD4-PerCP-Cy5.5 and -Alexa Fluor (AF) 700(clone L200), CD8-APC and -AF700, and -APC-H7 (clone SK1), CD20-APC-Cy7 (clone L27), CD25-FITC (clone 2A3), CD27-PE-CF594 (clone M-T271), CD45-AF700 (clone DO58-1283), CD45RA-FITC (clone 5H9), CD134/OX40-PE (clone L106), CD279/PD-1-AF647 (clone EH12.2H7) from BD Biosciences (San Jose, CA, USA); and CD127-BV421 (clone A019D5) from BioLegend (San Diego, CA, USA).

For LN FNA collected in RPMI1640, cells were spun down in 15 ml BD Falcon tube at 335g for 5 min. Supernatant was removed by pipette aspiration without disturbing the cell pellet. Cells were resuspended in 100 µl of RF10 medium [RPMI1640 supplemented with 10% fetal calf serum (FCS)] and cell suspension volume was accurately measured by pipetting. Cell suspensions were divided into two portions: 50 µl was transferred to a 5 ml tube for phenotyping and 10-20 µl was transferred to a Trucount tube (BD Biosciences) to obtain absolute cell counts, according to manufacturer's instructions. Cells were stained with fluorochrome-conjugated antibodies according to manufacturers' instructions at room temperature for 15 min, washed once with PBA [Dulbecco's

phosphate-buffered saline (DPBS) containing 0.5% bovine serum albumin (BSA) and 0.1% sodium azide], and resuspended in 0.5% PFA (DPBS containing 0.5% paraformaldehyde). Panels for phenotyping and absolute cell count are described in Table 2.

Whole excised LNs were minced and pushed through a 70 µm sieve for single cell isolation. Aliquots of single cell suspension were stained with antibodies for phenotyping and absolute cell count as described for LN FNA samples. The rest of the cell suspension was pelleted and resuspended in freezing medium [90% FCS/10% dimethyl sulfoxide (DMSO)] for cryopreservation.

Aliquots of 170 µl peripheral blood were stained with antibodies for phenotyping as described for LN FNA samples. A red cell lysis step with FACSLyse (BD Biosciences) was performed according to manufacturer's instructions after antibody staining and before washing with Wash Buffer [DPBS containing 0.5% (w/v) BSA and 2 mM Ethylenediaminetetraacetic acid (EDTA)].

Stained macaque cells were acquired on LSR II or LSR Fortessa with FACSDiva 6.0 software (BD Biosciences) as previously described (Zaunders et al., 2006). For phenotyping tubes, a minimum of 50,000 total events were recorded except for samples with inadequate cells. For absolute cell count tubes a minimum of 20,000 beads were recorded. Flow data was analysed using FlowJo (v 8.8.6, Tree Star).

2.3 Functional assay

Functionally responsive CD4+ T cells were measured as previously described, identified as those expressing CD3, CD4, CD25 and CD134, following short-term culture with the polyclonal superantigen, staphylococcal enterotoxin B (SEB) (Zaunders et al., 2009). Briefly, 1 ml of sodium heparin-anticoagulated whole blood was diluted with 1 ml of RPMI1640. 400 µl of diluted blood was dispensed into each of two wells of a 24-well tissue culture plate (BD Biosciences) and incubated with no antigen or SEB (Sigma Aldrich; 1 µg/ml). Blood samples with or without stimulus were cultured for 44 h. At the end of the culture period, 100 µl of diluted blood was stained with CD3-PB, CD4-PerCP-Cy5.5, CD25-FITC and CD134-PE at room temperature for 15 min, lysed with Optilyse C (Beckman Coulter, Hialeah, FL) according to the manufacturer's directions, washed once with PBS, fixed with 0.5% paraformaldehyde in PBS, and analysed for flow cytometry analysis as previously described (Zaunders et al., 2009).

For FNAs, cells were resuspended in 300 µl of IF10PS culture medium [IMDM supplemented with 10% FCS and antibiotics (10 units/ml penicillin and 10 µg/ml streptomycin in final concentration)]. 100 µl of cell suspension was dispensed into two wells of 96-well U-bottom tissue culture plate (BD Biosciences). Cells were stimulated with no antigen or SEB for 44 h, stained with antibody cocktail and analysed by flow cytometry as above, except that the cells were not lysed with Optilyse C, and were washed with PBA after staining.

A cut-off for a positive CD25+CD134+ response was defined as mean plus three times the standard deviation (SD) of the background in the no antigen culture, as previously described (Zaunders et al., 2009); accordingly, the cut-off was 0.10% of CD4+ T cells for blood and 0.43% for FNA.

2.4 Statistics

Statistical significance was analysed by Wilcoxon signed rank test, Spearman correlation and linear regression using Prism 5.0 (GraphPad, La Jolla, CA). Unless otherwise indicated, data represent the median ± interquartile, with $p < 0.05$ considered statistically significant.

3. Results

3.1 FNA sampling of lymph nodes

FNA of peripheral LNs in macaques using a 25-gauge needle was performed safely without complications on 38 occasions (Table 1). The palpable lymph nodes in the inguinal region were readily immobilized with two fingers against the pubis bone. In one of four FNA attempts, a small amount of blood was visible in the needle hub. These aspirates were discarded and the LN was resampled. There was no observed bruising or infection resulting from the procedure.

3.2 Absolute cell count of LN FNA samples

The absolute lymphocyte count of each FNA sample or mononuclear cell suspension isolated from excised inguinal LN was determined using Trucount tubes. Absolute numbers of total lymphocyte (CD45+), B cells (CD3-CD20+), T cells (CD3+CD20-), CD4+ T cells (CD4+CD8-) and CD8+ T cells (CD4-CD8+) from each FNA sample and cell suspension isolated from excised ILN were calculated as shown in Figure 1.

Results from 38 separate FNA's are shown in Figure 2A. Lymphocyte counts were the primary index for yield from LN FNA or cell recovery from excised LN. For FNA samples, lymphocyte counts ranged from 3.0×10^3 to 2.25×10^6 and the median was 2.01×10^5 (n = 38). For excised LNs, lymphocyte counts ranged from 44×10^6 to 134×10^6 and the median was 77×10^6 (n = 7). The result shows that cell recovery from excised LN is relatively consistent (3-fold difference between maximum and minimum), whereas cell yield from LN FNA samples had a much wider range (750-fold difference between

maximum and minimum). A histogram plot of the lymphocyte counts from LN FNA samples demonstrated that the majority of samples had a yield between 10^4 and 10^6. Three samples had a yield of over 10^6 lymphocytes. There were only three of the 38 samples had a yield lower than 10^4 lymphocytes (Figure 2B). We found that as few as 1,000 CD45+ cells were sufficient for accurate phenotyping of major subsets of lymphocytes including B cells, T cells, CD4+ T cells and CD8+ T cells (Supplementary Figure S1A and S1B).

CD45+ lymphocytes in FNA samples are almost exclusively CD3-CD20+ B cells and CD3+CD20- T cells, which in total add up to more than 90% of CD45+ cells recovered. B cells and T cells recovered from 38 FNA samples were calculated and plotted in histogram (Figure 2C). For B cells, the median cell count was 7.52×10^4 (2.20×10^3 to 9.87×10^5, n = 38), with majority of samples ranging from 10^4 to $10^{5.5}$. For T cells, the median cell count was 1.17×10^5 (396 to 1.18×10^6, n = 38), with majority of samples ranging from $10^{4.5}$ to 10^6.

One cell population of particular interest to our group and others is CD4+ T cells within LNs. CD4+ T cells were readily identified in the LN FNA samples. The median CD4+ T cell count in FNA samples was 5.9×10^4 (n = 38), with a range between 277 and 6.17×10^5. The median cell count for CD8+ T cells was 4.14×10^4 (85 to 5.16×10^4, n = 38). The majority of samples had 10^4 to $10^{5.5}$ CD4+ T cells and CD8+ T cells (Figure 2D). These numbers are adequate for the measurement of HIV/SIV cell-associated mRNA levels and host gene expression and may also be enough for HIV/SIV DNA quantification.

3.3 Cell frequencies in lymph node and in peripheral blood

Blood contamination can exist in FNA, which is often associated with using a larger needle and increasing the times of sampling from the same LN. To evaluate the level of blood contamination in our LN FNA samples, we measured granulocyte (CD45+, high side scatter cells) frequencies of CD45+ cells and CD4/CD8 ratio in FNA and peripheral blood samples. The median granulocyte frequencies of CD45+ cells of LN FNAs were 0.81% (n = 38), much lower than that of peripheral blood (median 48.3%, n = 20), but similar to that of excisional LNs (median 0.72%, n = 7), consistent with the exclusion of granulocytes from LNs, except in certain forms of lymphadenitis. The median CD4/CD8 ratio in LN FNAs was 2.2 (n = 38), higher than that in the peripheral blood (median 1.5, n = 38), consistent with previous studies on human excisional LN and FNA (Meylan et al., 1996; Tedla et al., 1999). The large differences in granulocyte frequencies and the difference in CD4/CD8 ratio between blood and LN FNA samples suggest that the vast majority of the cells we sampled in the FNAs were LN cells.

3.4 Concordance of cell subset frequencies between FNA samples and excised ILNs

A question regarding LN FNA samples is how representative they are of the whole lymph node. In the current study, three to four LN FNA samples were taken from six macaques longitudinally over 70 days (Table 1). One inguinal LN was also surgically removed from each macaque in that period, with four macaques having both whole LN and FNA taken at the same time point (highlighted in Table 1). The average cell frequencies of longitudinal FNA samples were plotted against the cell frequencies of cell suspension of the whole LN from the same monkey in XY charts (FIG3A-D). First, non-parametric correlation analysis was performed for B cell and T cell frequencies of CD45+ lymphocytes and CD4+ T cell and CD8+ T cell frequencies of CD3+ T cells, with

standard deviation and sample size taken into account (Table 3 and Figure 3). Even though there were a limited number of whole LN studied, T cell frequencies, but not B cell frequencies, from LN FNA correlated with the frequencies from whole LN (Figure 3A and 3B). Within T cell sub-populations in LN FNAs, CD4+ T cell frequencies, but not CD8+ T cell frequencies, also correlated with the frequencies from whole LN (Figure 3C and 3D). In general, B cell and T cell frequencies of CD45+ cells were comparable from two specimen types. CD4+ T cell frequencies of CD3+ cells of whole LNs were slightly higher than those of FNAs, whereas CD8+ T cell frequencies of CD3+ cells were the converse, consistent with earlier studies on human LN FNAs (Bart et al., 1999). Thus, LN FNA samples appeared broadly representative of whole excised LNs, particularly for CD4+ T cell populations.

3.5 Detection of T follicular helper cells in FNA samples

T follicular helper (T_{FH}) cells are a specialized subset of memory CD4+ T cells that are almost exclusively found within the germinal centres of secondary lymphoid tissues, and are important for adaptive antibody responses and B cell memory (Crotty, 2011). T_{FH} cells in macaques can be defined as CD3+CD4+CD45RA-PD-1^{high}CD127- cells in secondary lymphoid tissues such as spleen and lymph node, as previous described (Xu et al., 2013). We found that T_{FH} cells could be identified as a distinct population in LN FNAs, as also observed in whole LNs (Figure 4A). Cell recovery of T_{FH} cell, as well as CD45RA- memory CD4+ T cell was calculated based on CD4+ T cell count determined by Trucount and cell subset frequency determined by phenotyping. 16 out of 21 samples had $10^{3.5}$ to 10^5 memory CD4+ T cells and 10^3 to $10^{4.5}$ T_{FH} cells (Figure 4B). Similar to B cells and T cells, the frequencies of CD45RA- memory cell of CD4+ T cell, and the

frequencies of T_{FH} cells of CD45RA- memory CD4+ T cells of multiple samples from each monkey were highly variable, but the mean values from three to four sequential FNA samples were close to the values derived from whole LN (Figure 4C and 4D). Thus, T_{FH} cells are readily identifiable in LN FNAs, providing an alternative means of studying T_{FH} cells compared to LN excision.

Interestingly, we found that for the same animal, when T_{FH} cell frequency of memory CD4+ T cells was higher in LN FNA than in whole LN, B cell frequency of CD45+ cells was also higher in FNA samples, and vice versa. This pattern was observed in 18 cases out of 21 samples (Figure 4D and 4E). As T_{FH} cells and B cells are enriched in the B cell area, it is suggested that each FNA in this study, which was the sum of two passes, may tend to slightly over-represent either B cell or T cell areas, presumably depending on the starting point within each LN where suction was begun.

3.6 *Superantigen-responsive CD4+ T cells in peripheral blood and FNAs*

The ability to perform functional assays on LN FNA samples would be advantageous in a research setting. We examined the functionality of cells taken from FNAs and compared it with that of peripheral blood cells by stimulating the cells for 44 h *in vitro* with superantigen SEB and measuring the response as the percentage of cells co-expressing CD25+CD134+ of total CD4+ T cells (Zaunders et al., 2009). FNA and peripheral blood from eight macaques were examined in this way (Figure 5). Cell number in each 100μl culture ranged from 6.3×10^4 to 1.67×10^5, equivalent to 6.3×10^5 to 1.67×10^6 cells per ml. Background response (the mean response of eight un-stimulated samples) was 0.03%±0.02% in blood culture and 0.16%±0.09% in FNA culture. For blood and FNA

cultures stimulated with SEB, high levels of CD25+CD134+ CD4+ T cells were detected and the response was 3.9%±1.3% in blood culture and 5.8%±1.6% in FNA culture (Figure 5).

4. Discussion

Previous studies of FNAs from HIV+ patients primarily focused on the diagnosis for lymphadenopathy (e.g. infection or malignancy) (Ellison et al., 2002; Grossl et al., 1997; Jayaram and Chew, 2000; Lowe et al., 2008; Martin-Bates et al., 1993; Michelow et al., 2010, 2012; Nayak et al., 2003; Reid et al., 2002; Schubert et al., 2011). A limited literature in the late 1990s on HIV pathogenesis studies used FNA to study lymphoid tissue in chronic HIV patients on HAART to determine CD4/CD8 ratios and HIV DNA and RNA copies (Bart et al., 1999; Burgisser et al., 1997; Fleury et al., 2000; Meylan et al., 1996). Here we have explored, for the first time, the safety and efficacy of serial LN FNA from macaques. We found that serial LN FNA could be safely performed, that an adequate number of lymphocytes were obtained by FNA in the vast majority of samples, and that specialized subsets of tissue lymphocytes such as T_{FH} cells and superantigen-responsive CD4+ T cells could be readily identified in LN FNA samples *ex vivo* and upon stimulation, respectively. Our results therefore provide evidence for the feasibility to use LN FNA samples to serially study these tissue lymphocyte subsets longitudinally in the pigtail macaque SIV model and can be extended to the detailed study of the effect of HIV infection on T_{FH}, germinal centers, and antigen-specific CD4+ T cells in human LNs. Such prospective studies are extremely important in primary HIV infection studies, and trials of antiretroviral therapies, immunotherapeutic or vaccine candidates. While frequent surgical excision of LNs is not feasible, our results showed that it is safe to take LN FNAs up to four times in a period of 70 days, without adverse event. This technique should be particularly useful to study the earliest events in HIV/SIV infection, but still allow later study of pathogenesis.

In this study, LN FNA was taken manually without ultrasound guidance since normal inguinal LNs were readily palpable in most macaques. Cell yield from two passes using 25-gauge needle was generally more than enough for flow cytometric immunophenotyping. Major lymphocyte subsets, including B cells, T cells and T cell subsets CD4+ and CD8+ T cells) could be reliably identified from as few as 1,000 lymphocytes (Supplementary Figure S1A and S1B). T_{FH} cells, a small but significant subset of CD4+ T cells in LNs, could also be reliably identified from samples of as few as 5,000 lymphocytes (Supplementary Figure S1C and S1D). Increasing the number of passes can increase cell yield (O'Malley et al., 2002). However, although up to 12 passes have been taken from human thyroid nodules (Pitman et al., 2008), considering patients' tolerance of the procedure, the risk of getting blood contamination, and the minimum cell number required for flow cytometric analysis, we believe five LN FNA passes should generate adequate LN cells for analysis. Ultrasound guidance should assist sampling of LNs in human subjects that are not readily palpable, such as those with more subcutaneous fat. Our data on the safety and utility of macaque LN FNAs has allowed us to obtain ethics approval for future human LN FNA studies in HIV-infected subjects.

With the development of sensitive PCR techniques, it is now possible to study gene expression at a single cell level (Sanchez-Freire et al., 2012). In this study, 32 out of 38 LN FNAs had a CD4+ T cell count of over 10,000, which is a promising number for the measurement of cell-associated HIV/SIV RNA and host gene transcripts. This will allow us to monitor productive infection, as well as host gene expression changes in lymphoid tissues, during all stages of HIV/SIV infection, including the most dynamic acute phase.

For HIV/SIV DNA, the copy number per cell is usually much lower than viral RNA; therefore, more starting material is required. A recent report published by our group showed that the median total HIV-1 DNA levels in PBMC were approximately 10,000 and 500 copies per 10^6 CD4+ T cells, from treatment naïve patients before and after ART, respectively. Based on the current quantification limit (10 copies per reaction) and qPCR setup (triplicate for HIV DNA and reference, respectively), the calculated median starting materials required for accurate quantification are 6×10^3 CD4+ T cells from treatment naïve patients and 1.2×10^5 CD4+ T cells from patients on ART, respectively. It has been reported that the HIV DNA copy number is three times higher in LN mononuclear cells than in PBMC (Pantaleo et al., 1995). Therefore, LN FNA samples may still be very useful for the measurement of HIV/SIV DNA reservoir in treatment naïve patients, or even in patients treated with ART, in particular with further development of sensitive PCR and very low quantification limits.

One potential problem with LN FNA that we identified is the large variation of cell subset frequencies. The CVs of B cell and T cell frequencies of LN FNAs from multiple visits of each monkey ranged from 10.69% to 36.99%. The CVs of CD4+ T cells and CD8+ T cells were 1.36% ~ 14.84% and 4.05% ~ 29.07%, respectively, slightly lower than those of the B cell and T cell frequencies. Several factors may contribute to the variation of cell frequencies in LN FNA samples. First, the aspirations were taken at different time points sampled from different lymph nodes. However, the captive macaques were healthy and were free from systemic or local infections at the time of FNA sampling. Therefore, variation in cell frequencies of FNA samples arose from subclinical infection was expected to be minor. Second, minor blood contamination could

alter cell frequencies in LN FNAs. However, very low granulocyte frequencies in our LN FNA samples indicated that blood contamination was minimal. Therefore, we believed there was a third factor that contributed to the large variation, in particular for T cell and B cell frequencies. As LN cells are compartmentalized, rather than homogenized, sampling with a fine needle may result in random bias at the B cell area, where B cells and T_{FH} cells are enriched, or at the T cell area, where T cells are enriched. This hypothesis is evidenced by the observation that T_{FH} cell frequency and B cell frequency of LN FNA changed in the same trend over that of whole LN, and is backed up by a strong positive correlation between T_{FH} cell frequencies and B cell frequencies in pooled data from all LN FNA samples (Supplementary Figure S2). Preferential sampling of different areas in the LN may also explains the slightly higher CD4/CD8 ratio observed in FNA samples compared with cell suspensions from whole LN (2.8 vs 2.5), as previously discussed by others (Bart et al., 1999). In future studies on infected macaques or humans, where enlarged LNs are present, and a higher number of passes could be achieved without substantially increasing the risk of blood contamination, increasing sampling to four to five passes may further increase the utility of LN FNA sampling.

A very strong correlation was observed for the frequency of T cells (as % of total CD45+ cells) in FNA compared to the frequency of T cells in the cell suspension from whole LN (spearman r = 0.9429, $p = 0.0167$). A significant correlation was also found for the frequency of CD4+ cells (as % of total T cells) between FNA and whole LN cell suspensions (spearman r = 0.9429, $p = 0.0333$). The correlations for the frequency of B cell (as% of total CD45+ cells) and the frequency CD8+ T cell (as % of total T cells), showed a trend to statistical significance (spearman r = 0.7714, $p = 0.1028$ for both),

despite the limited number of lymph node excisions available for this study. Therefore, cell subset composition of FNA samples, in particular for T cell and CD4+ T cell, are broadly comparable to that of the whole LN.

Recent publications have identified T_{FH} cells, while essential to germinal center reactions and high affinity antibody responses, also substantially contribute to the HIV/SIV reservoir in secondary lymphoid tissues (Brenchley et al., 2012; Klatt et al., 2011; Perreau et al., 2012; Petrovas et al., 2012; Xu et al., 2013). Furthermore, the proportion of T_{FH} cells as a percent of lymph node CD4+ T cells relatively increases during the early chronic stage (Brenchley et al., 2012; Hong et al., 2012; Lindqvist et al., 2012; Perreau et al., 2012; Petrovas et al., 2012; Xu et al., 2013), and such increase of T_{FH} cells may offset the loss of effector CD4+ T cells in the LN, resulting in a milder decrease of CD4+ T cells in this compartment than in the peripheral or the GI tract (Schacker et al., 2001). There is a need to study cells residing in the secondary lymphoid tissue longitudinally, both in acute and chronic phase of HIV/SIV infection. Our results show that T_{FH} cells, as well as other memory CD4+ T cell subsets, can be distinctly identified from as few as 5,000 lymphocytes in FNAs. Serial sampling of FNA will allow us to monitor the dynamics of LN CD4+ T cell subsets in detail.

We have also identified superantigen-responsive CD4+ T cells from LN FNAs, from as few as 6.3×10^4 lymphocytes in a 100μl culture. Although the background response of un-stimulated samples from FNAs was slightly higher than that from blood, the response to superantigen was also higher in FNAs than in blood, resulting in comparable background-subtracted response. Preliminary data on one SIV-infected macaque also

showed that antigen-specific CD4+ T cells responding to SIV Gag peptides can also be detected in FNA as well as in blood (not shown).

In summary, multiple sampling of LN FNAs is safe. Cell yield from two passes of aspiration allows accurate phenotyping of small cell subsets such as T_{FH} cells. More than half of our FNA samples also yielded enough cells to set up functional assays in addition to multi-panel phenotyping. Identification of other cell subsets from FNA, e.g. germinal center B cell and plasmablasts, will be feasible and of interest in future studies. Despite the relatively high variability of cell subsets over multiple time points in healthy macaques, larger changes in subset levels, such as dramatic CD4+ T cell depletion or increases in the proportion of T_{FH} cells, following the acute phase of SIV/HIV infection, should be readily detectable through serial FNA sampling. FNA specimens are an alternative to whole LN excision to study tissue cells and will be extremely useful for virological and immunological study.

Acknowledgements

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Figure legends

Figure 1. Gating strategy for absolute cell count of LN FNA and whole LN samples. Upper panel shows the gating strategy for identifying beads. **A.** Total events were plotted on FSC-A vs SSC-A dot plots and two distinct populations were shown. The population of lower FSC-A and higher SSC-A (gated) are enriched for beads and the population of higher FSC-A and lower SSC-A (ungated) are enriched for lymphocytes. **B.** The bead population was plotted on R670-A vs B525-A dot plot and a tight gate was drawn for the main $R670^{high}B525^{high}$ population to identify beads. Lower panel shows the gating strategy for discriminating different lymphocyte subsets. **C.** Total events were plotted on CD45-AF700 vs SSC-A dot plot and two distinct populations were shown. The population of higher SSC-A is enriched for beads and the population of lower SSC-A are enriched for lymphocytes, which can be further gated out based on high expression of CD45. **D.** Lymphocytes were plotted on CD3-PB vs CD20-APC-Cy7 dot plot for discrimination between B cells (CD3-CD20+) and T cells ($CD3+CD20-/^{dim}$). **E.** T cells were plotted on CD8-APC vs CD4-PerCP-Cy5.5 histogram for discrimination between CD4+ T cells (CD4+CD8-) and CD8+ T cells (CD4-CD8+).

Figure 2. A. Absolute count of lymphocyte, B cell, T cell, CD4+ T helper cells and CD8+ cytotoxic T cells from FNA samples (open circles, n = 38) and cell suspensions isolated from excised LNs (close circles n = 7). **B.** FNA lymphocyte count distribution. **C.** FNA B cell and T cell count distribution. **D.** FNA CD4+ and CD8+ T cell count distribution.

Figure 3. Cell frequency (of parent population) correlation between LN FNA samples and cell suspensions from whole LNs. The average value of cell frequencies of LN FNA samples taken from the same macaque at different time points were plotted against the cell frequencies of whole LNs from the same macaque at a single time point. Error bars represent the mean ± standard deviation of data from FNA samples and dotted curves show the 95% confidence intervals. Non-parametric correlation analysis was performed. Spearman r and *p* value were calculated based on the sample size n, which are shown in each graph.

Figure 4. A. Representative contour plots for identification of T follicular helper (T_{FH}) cells, which are memory CD4+ T cells of PD-1^{high}CD127- phenotype, from cell suspensions of whole LN (left) and LN FNA samples (right) of one macaque. **B.** FNA CD45RA- memory CD4+ T cell and T_{FH} cell count distribution. **C-E.** Cell subset frequencies (**C.** CD45RA- memory cell % of CD4+ T cell; **D.** T_{FH} cell % of memory CD4+ T cell; **E.** B cell% of CD45+ lymphocyte) from serial FNA samples (open legends) and from mononuclear cell suspension of whole LN sample (solid legends). Each column represents FNA and whole LN samples from the same macaque.

Figure 5. A. Representative plots for antigen-specific CD4+ T cells in blood and FNA cultures. CD4+ T cells were plotted against CD25 vs CD134 histogram. Antigen-specific cells are those co-expressing CD25 and CD134 in the top right quadrant. **B.** Summarized data of responding cell frequencies of CD4+ T cells in blood and FNA cultures, with or without stimulus.

Figure S1. A. Representative dot plots showing B cells and T cells frequencies of CD45+ lymphocytes (top panel), and CD4+ and CD8+ T cells frequencies of CD3+ T cells (bottom panel), from 1,000 lymphocytes (left panel) and from 10,000 lymphocytes (right panel). **B.** Summarized data of B cell, T cell, CD4+ T cell and CD8+ T cell frequency of parent population analysed from ~1,000 lymphocytes and from over 10,000 lymphocytes. **C.** Representative contour plots showing memory cells frequencies of CD4+ T cells (top panel) and T_{FH} cell frequencies of memory CD4+ T cells (bottom panel), from 5,000 lymphocytes (left panel) and from 10,000 lymphocytes (right panel). **D.** Summarized data of memory CD4+ T cell and T_{FH} cell frequency of parent population analysed from ~5,000 lymphocytes and from over 10,000 lymphocytes.

Figure S2. Non-parametric correlation between T_{FH} cell frequency of memory CD4+ T cells and B cell frequency of CD45+ cells from serial LN FNA samples.

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Table 1. Pigtail macaques included in this study

Monkey ID	Sampling time	Sample type		
		Peripheral blood	FNA	Whole LN
5878	Baseline	+	+	
	Day 14	+	+	
	Day 28	+		+
	Day 42	+	+	
18869	Baseline*	+	+	+
	Day 28	+	+	
	Day 42	+	+	
	Day 70	+	+	
35414	Baseline	+	+	
	Day 14	+	+	
	Day 28	+		+
	Day 42	+	+	
45610	Baseline	+	+	
	Day 14	+	+	
	Day 28*	+	+	+
	Day 42	+	+	
C3765	Baseline*	+	+	+
	Day 28	+	+	
	Day 42	+	+	
C3767	Baseline	+	+	
	Day 14	+	+	
	Day 28	+		+
	Day 42	+	+	
36142	Baseline*	+	+	+
19340	Baseline	+	+	
C3749	Baseline	+	+	
1024	Baseline	+	+	

1843	Baseline	+	+	
2467	Baseline	+	+	
16570	Baseline	+	+	
19430	Baseline	+	+	
26300	Baseline	+	+	
26301	Baseline	+	+	
26783	Baseline	+	+	
35377	Baseline	+	+	
36121	Baseline	+	+	
36271	Baseline	+	+	
B0399	Baseline	+	+	
C3752	Baseline	+	+	
C3763	Baseline	+	+	
C3770	Baseline		+	

* Time points that both LN excision and FNA sampling occurred were highlighted.

Table 2. Panels for phenotyping and absolute cell count.

Phenotyping	Absolute cell count
CD3-PE-Cy7	CD45-AF700
CD4-PerCP-Cy5.5	CD3-PB
CD8-AF700	CD4-PerCP-Cy5.5
CD45RA-FITC	CD8-APC
CD27-PE-CF594	CD20-APC-Cy7
PD-1-AF647	
CD127-BV421	

Table 3. Summary of lymphocyte subset frequencies

B cell and T cell frequencies of CD45+ cells in whole LNs and FNAs.

Macaque ID	B cell				T cell			
	Whole LN	FNA			Whole LN	FNA		
		Mean	CV%	N		Mean	CV%	N
1	29.3	28.43	32.71	3	62.7	65.07	20.41	3
2	29.9	36.53	26.28	4	57.9	55	19.76	4
3	40	47.07	14.68	3	50.2	47.2	11.65	3
4	50.4	62.28	10.69	4	42.3	25.48	35.01	4
5	37.4	33.47	36.99	3	55	59.13	23.74	3
6	36.6	35.47	18.16	3	58.8	59.23	18.2	3

CD4+ and CD8+ T cell frequencies of CD3+ T cells

Macaque ID	CD4+ T cell				CD8+ T cell			
	Whole LN	FNA			Whole LN	FNA		
		Mean	CV%	N		Mean	CV%	N
1	64.7	58.33	8.66	3	31.8	37.2	9.25	3
2	68.2	66.97	1.36	4	22	24.9	10.12	4
3	62.5	60.27	11.98	3	32.1	35.3	20.68	3
4	71.3	67.13	9.37	4	20.1	24.78	24.98	4
5	67.7	63.7	14.84	3	25.4	27.97	29.07	3
6	69.9	69	2.58	3	23.2	23.97	4.05	3

Memory CD4+ T cell frequencies of CD4+ T cell

Macaque ID	Whole LN	FNA		
		Mean	CV%	N
1	40.9	34.3	21.81	3
2	37.1	32.88	9.15	4
3	46.4	38.57	29.79	3
4	44.1	52.98	9.57	4
5	49.4	41.53	15.75	3
6	43.0	36.13	11.96	3

Tfh cell frequencies of memory CD4+ T cell

Macaque ID	Whole LN	FNA		
		Mean	CV%	N
1	11.2	7.93	20.3	3
2	12.3	14.56	40.32	4
3	27.2	31.7	51.39	3
4	13.2	18.2	26.87	4
5	15.2	18.2	50.71	3
6	9.92	10.41	7.59	3

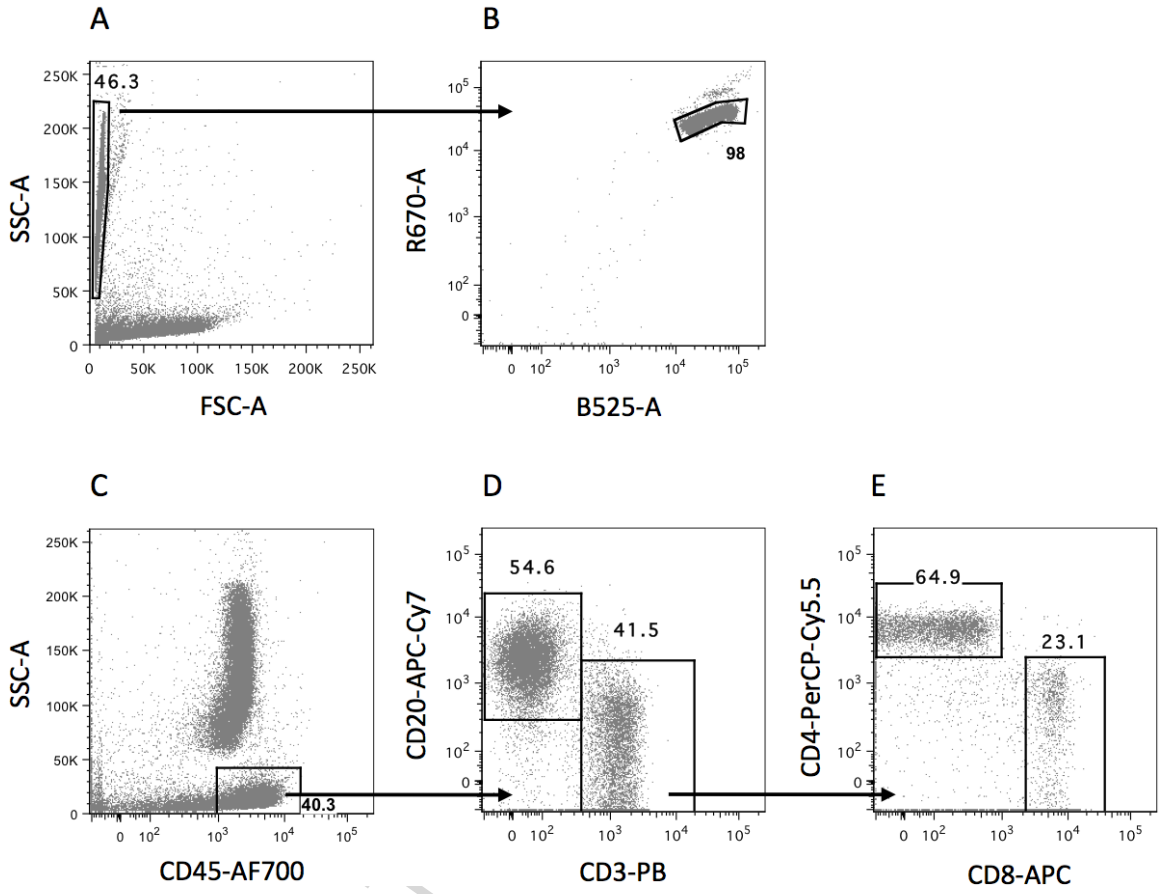

Figure 1

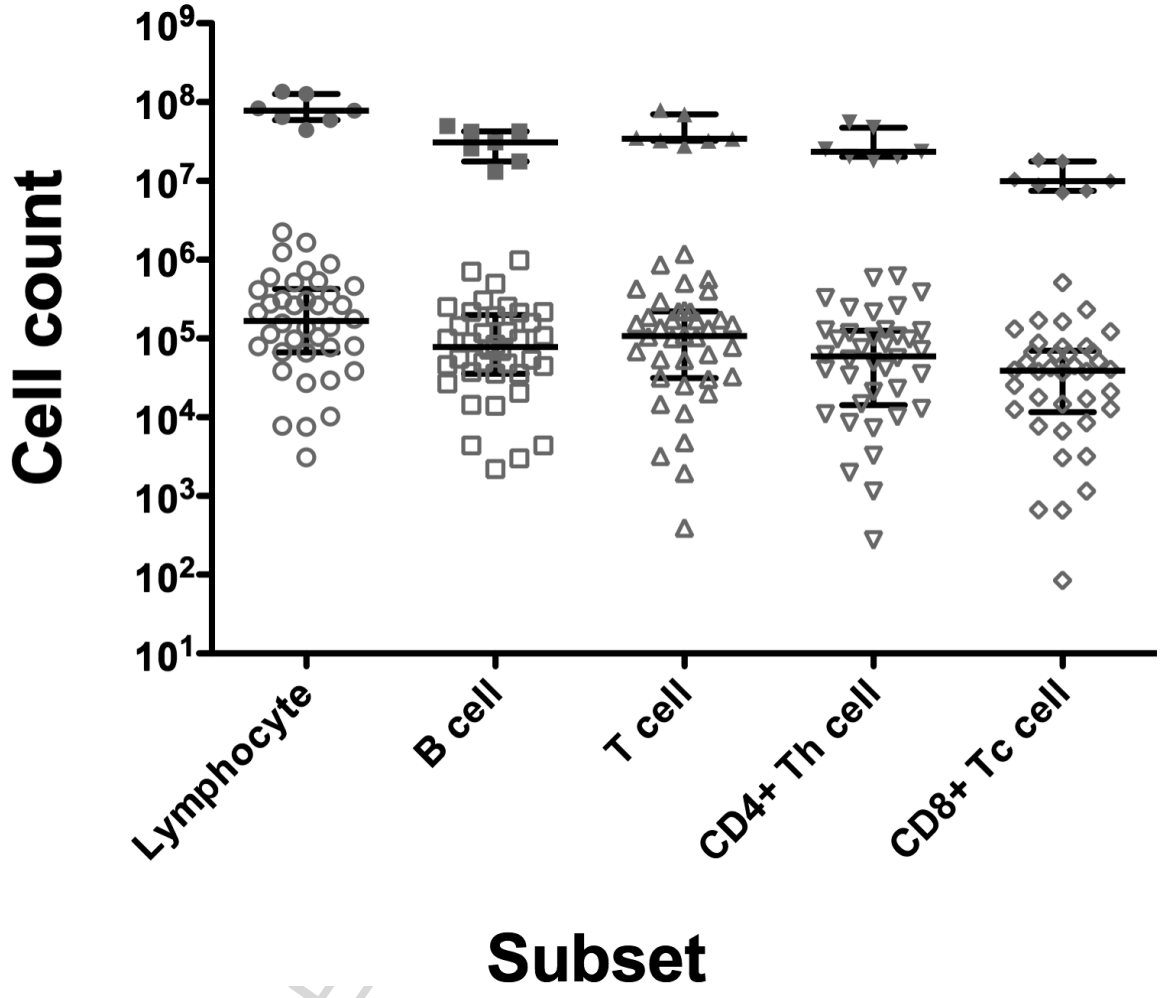

Figure 2A

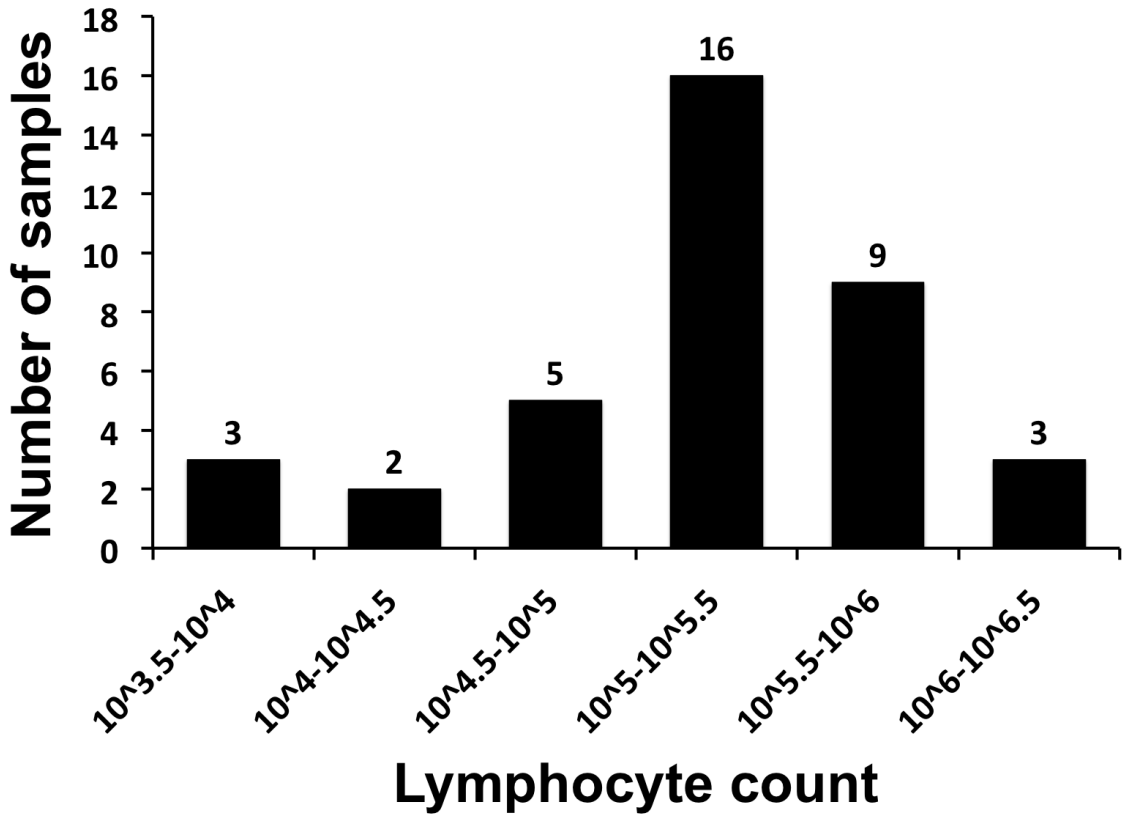

Figure 2B

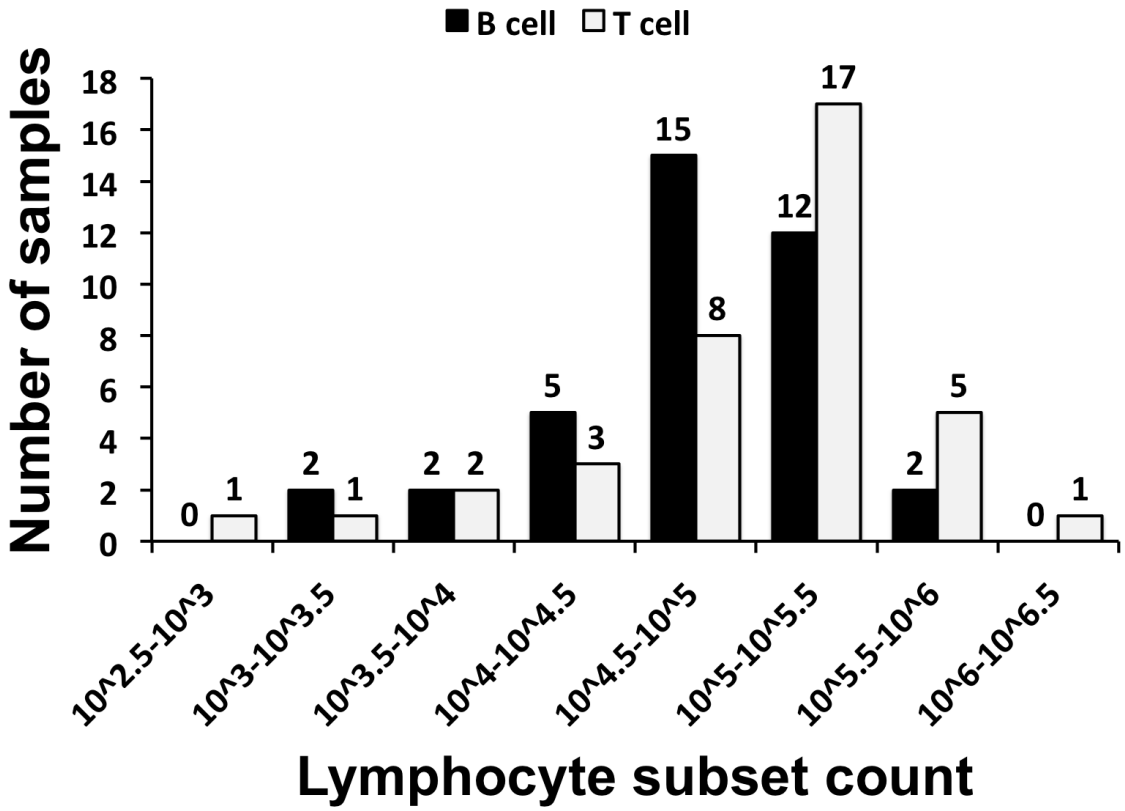

Figure 2C

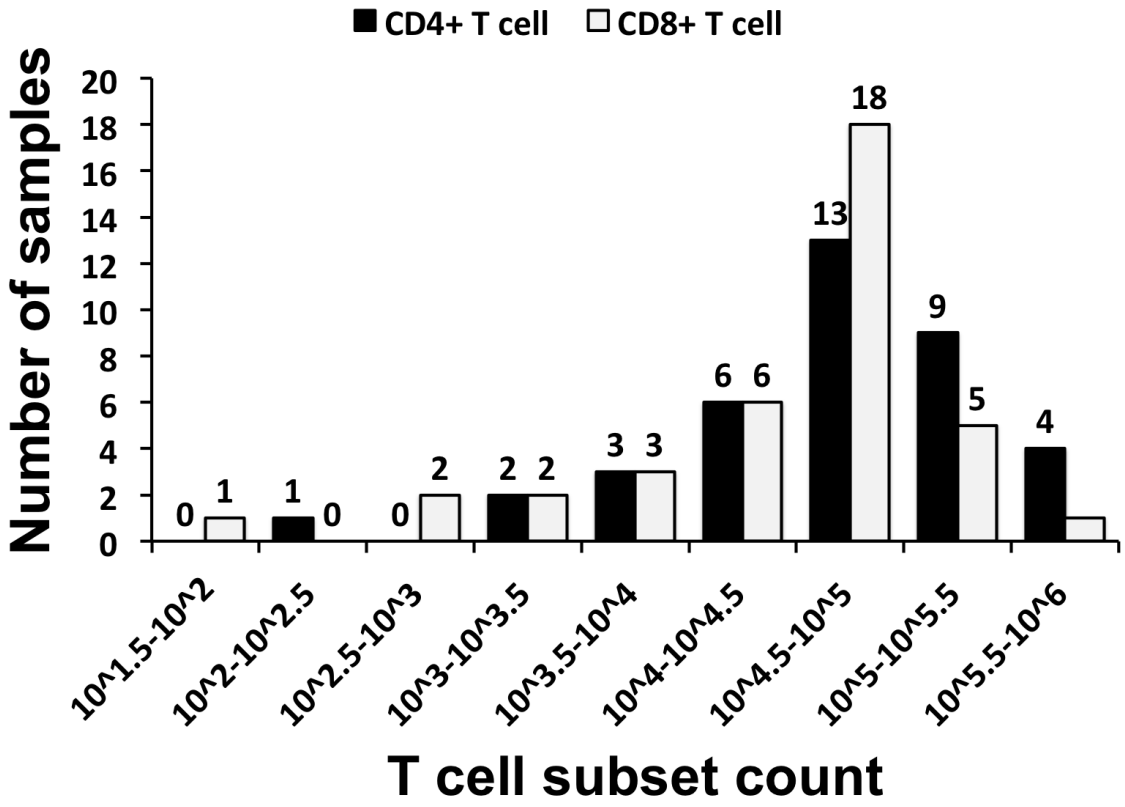

Figure 2D

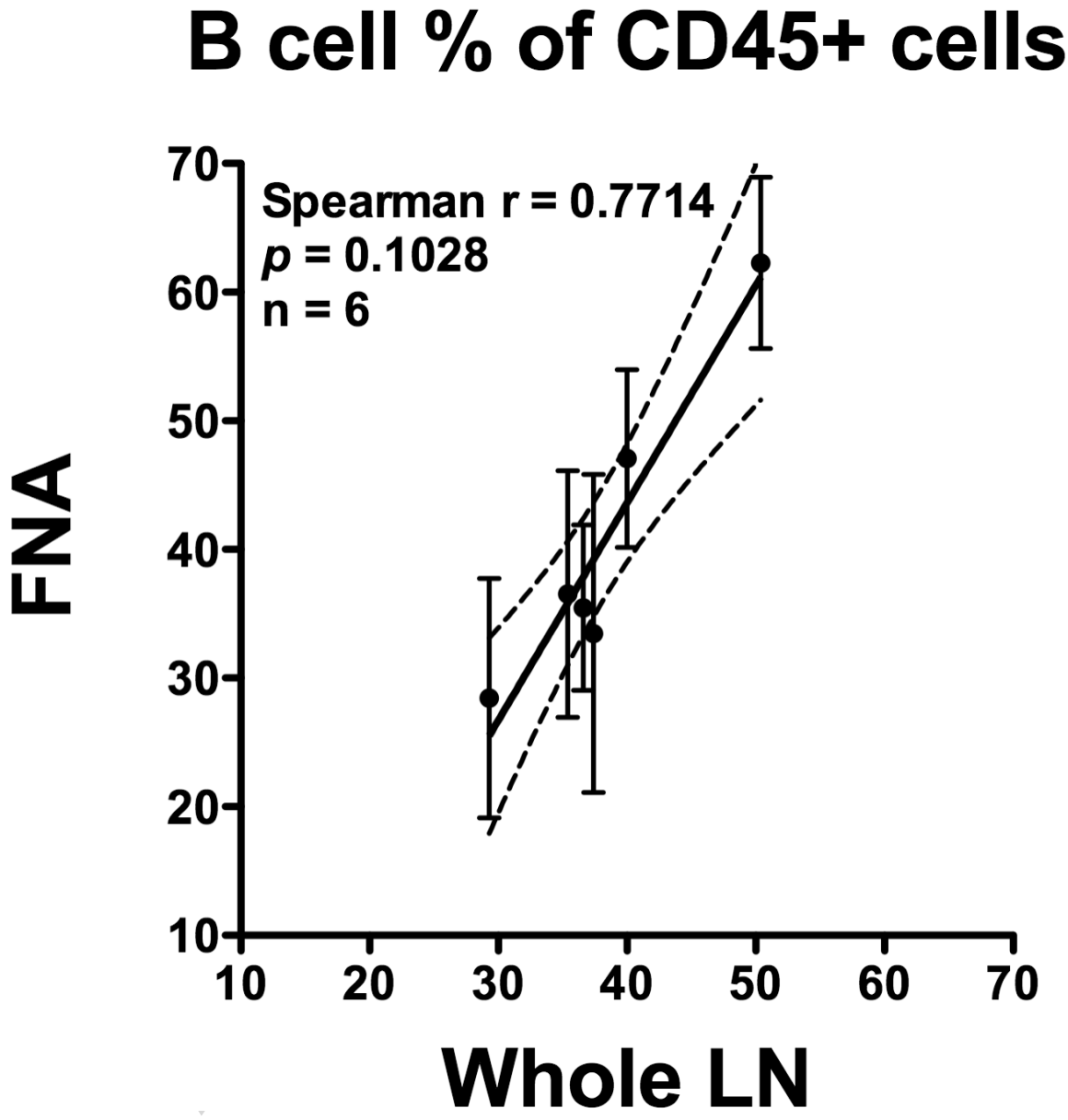

Figure 3A

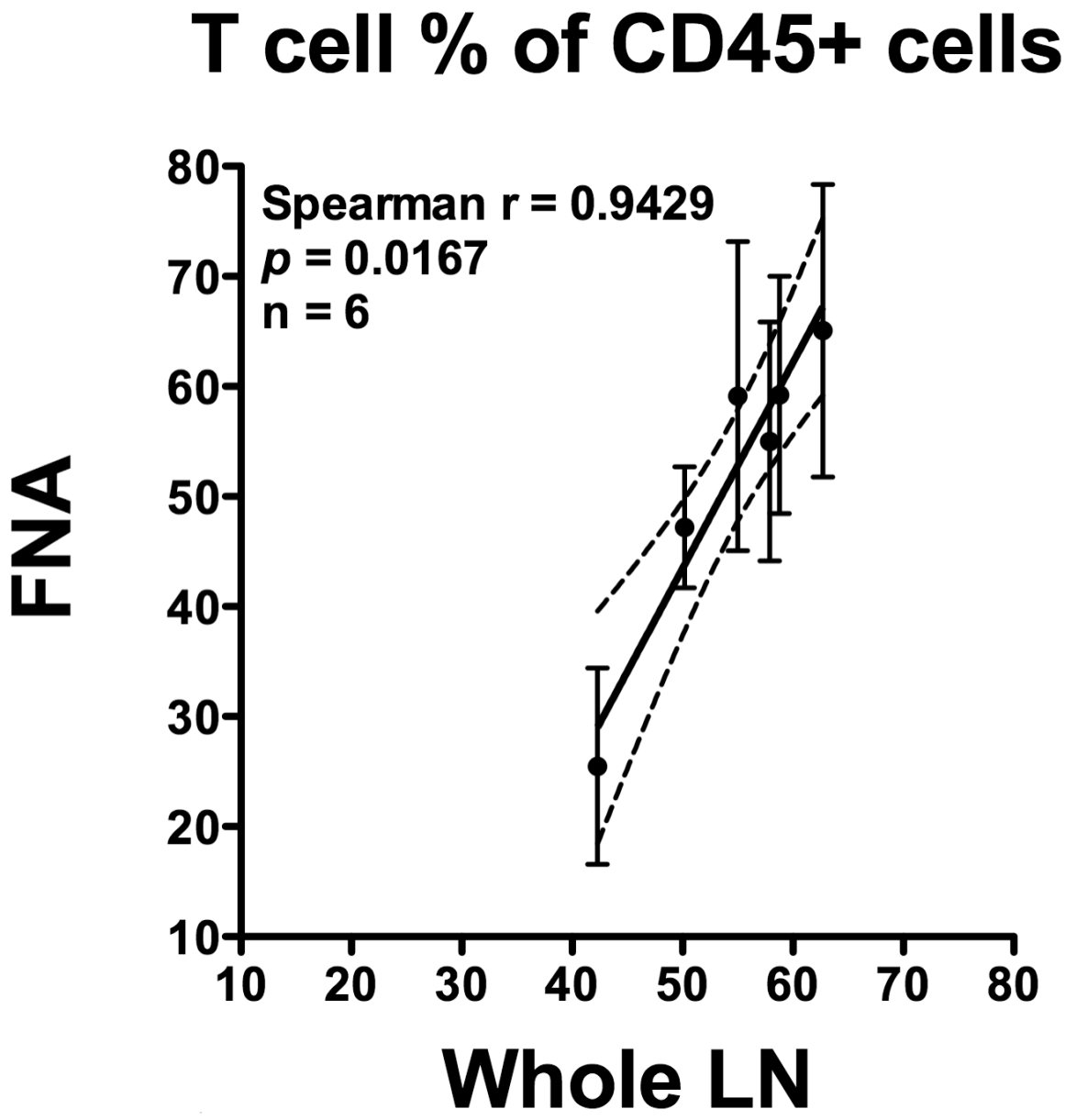

Figure 3B

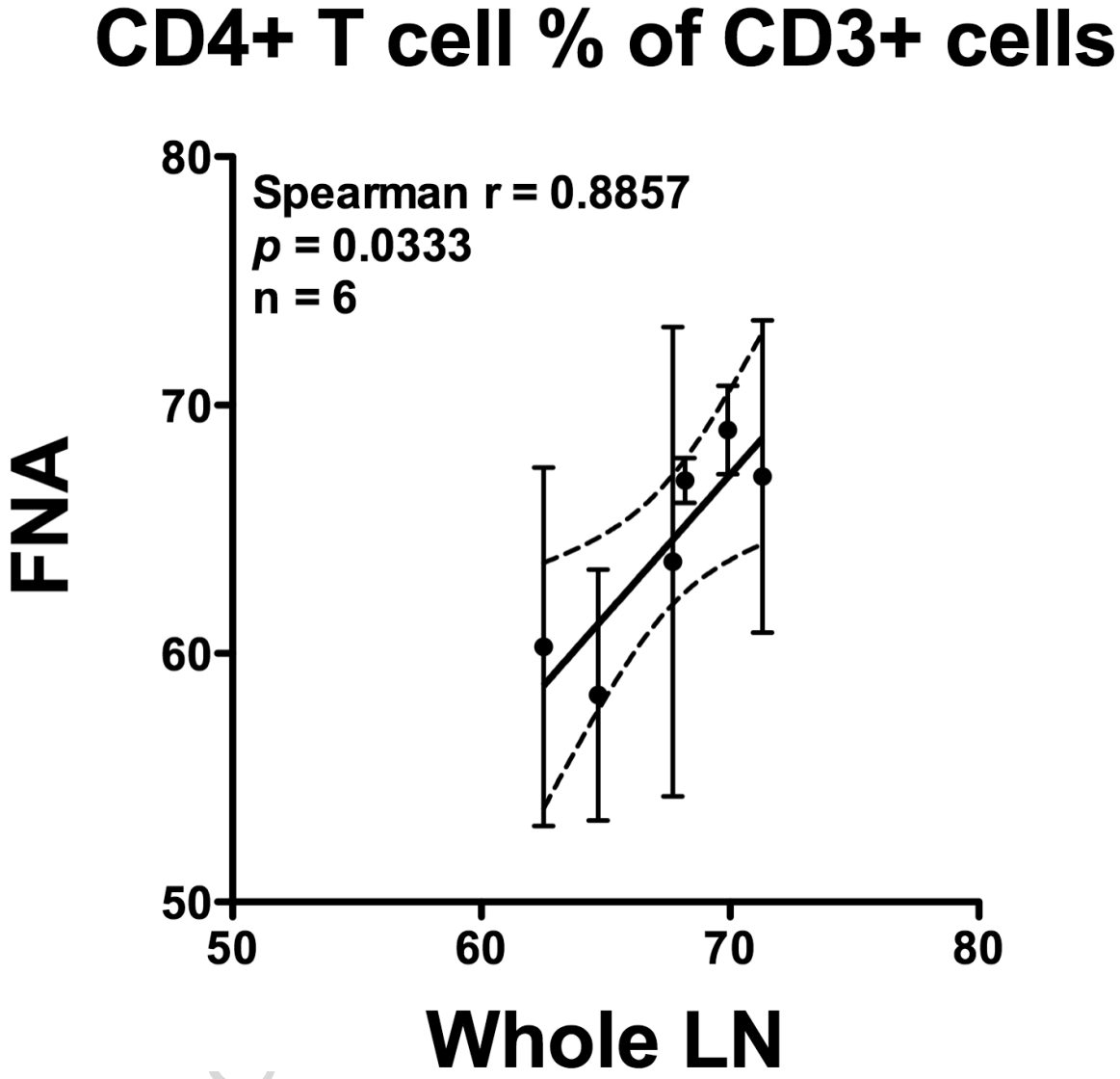

Figure 3C

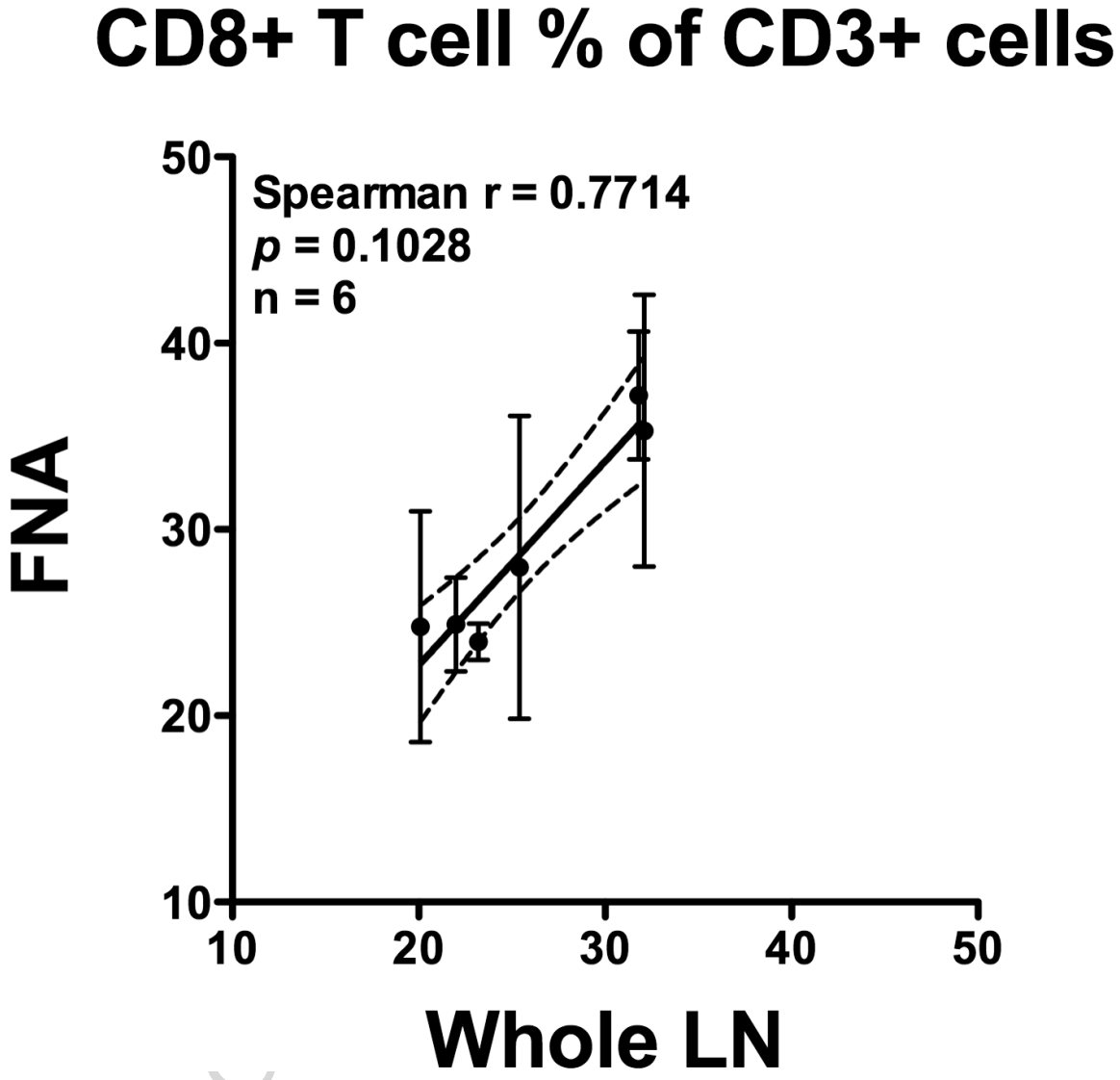

Figure 3D

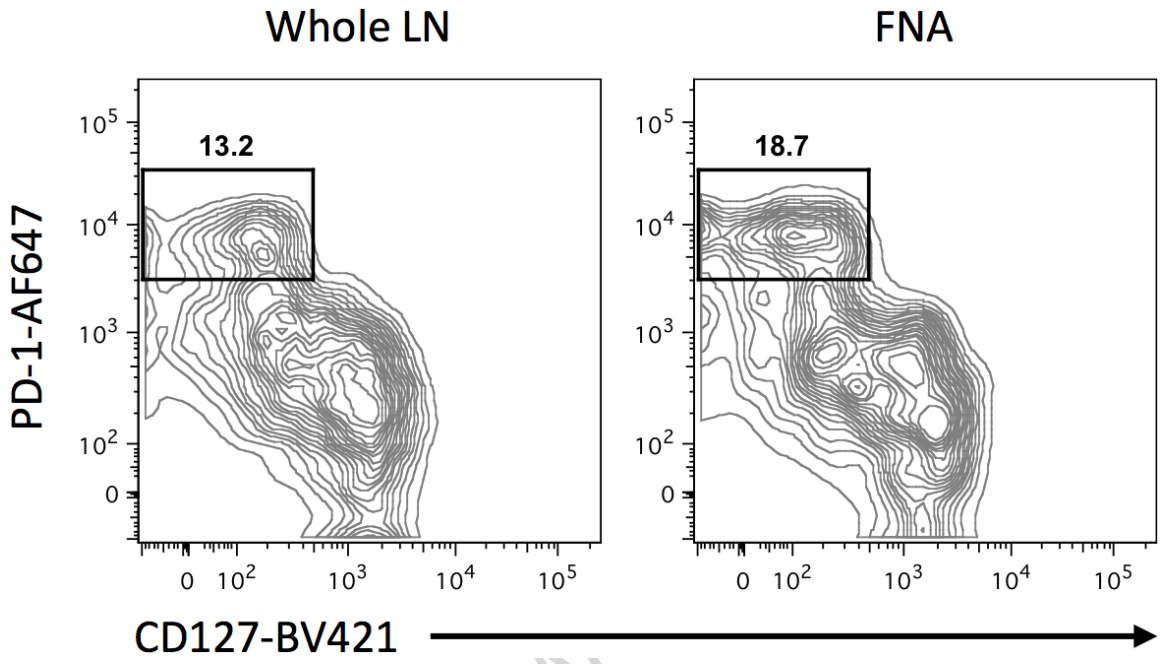

Figure 4A

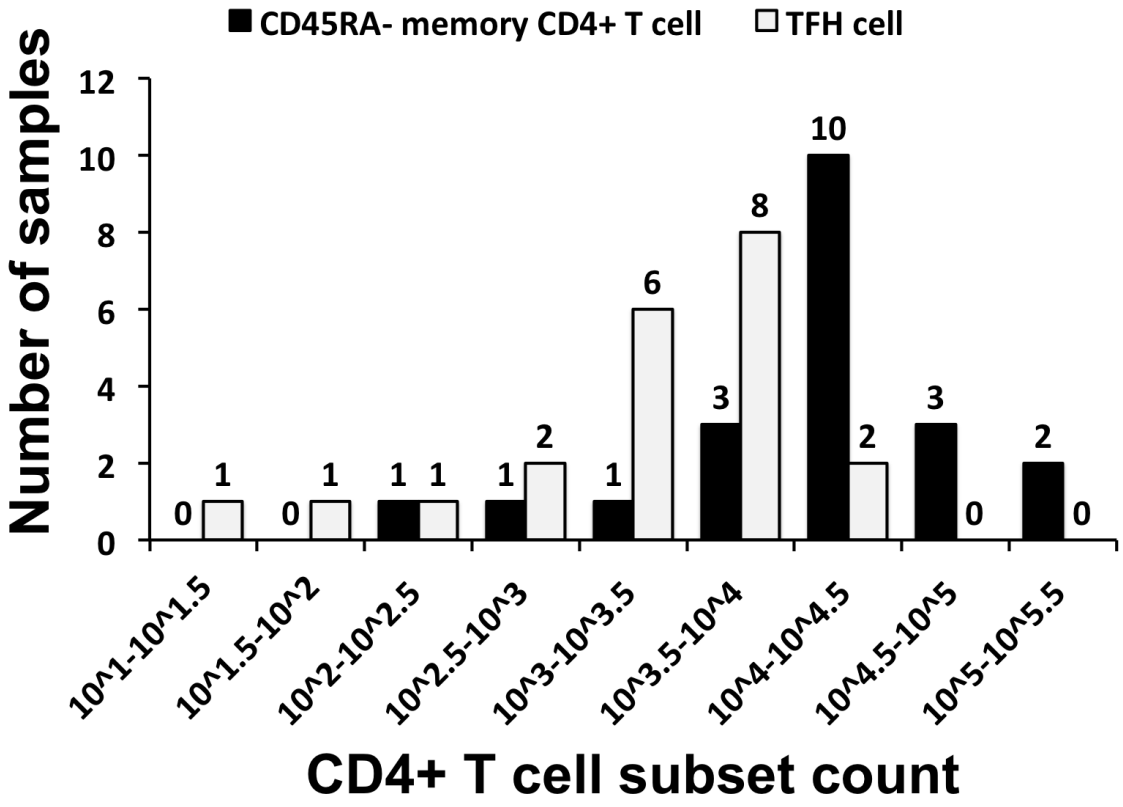

Figure 4B

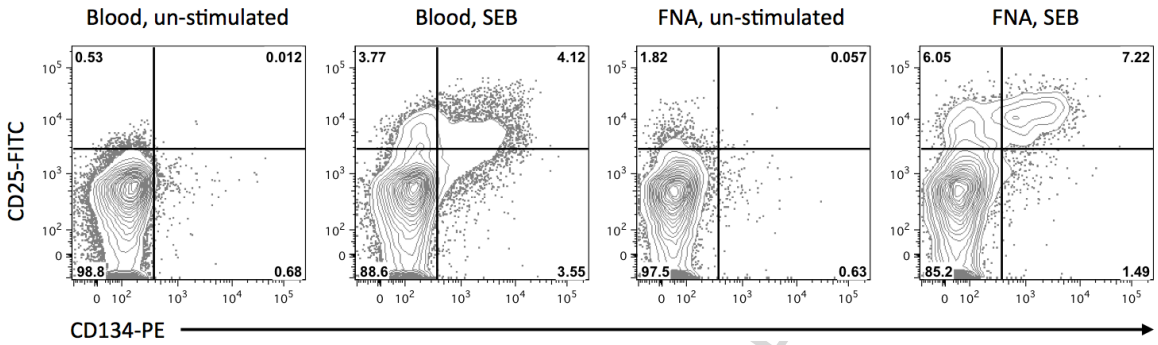

Figure 5A

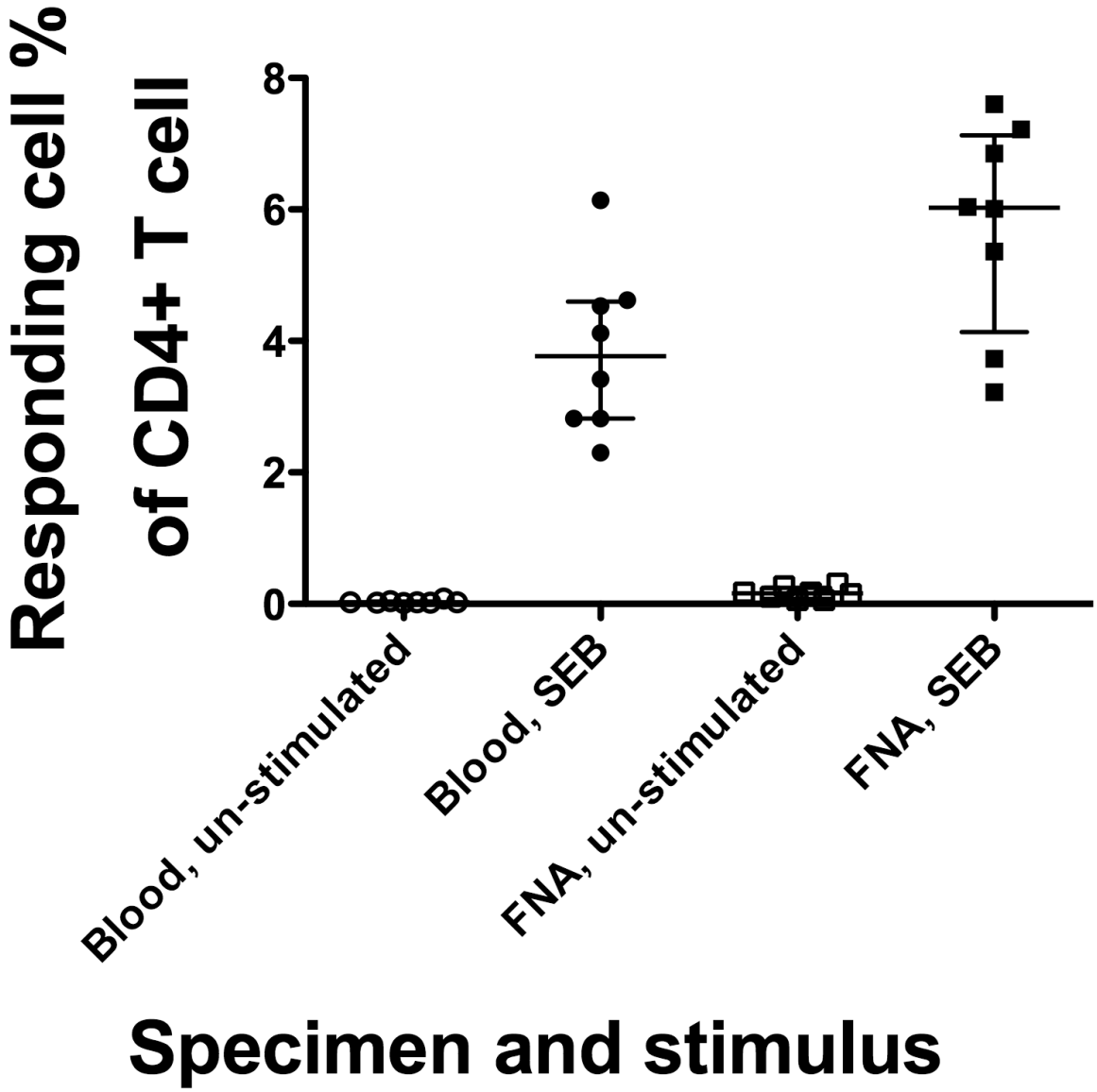

Figure 5B

Highlights

- LN FNAs were taken from pigtail macaques, on up to four separate occasions, from each individual over 70 days without adverse side effects.
- LN FNAs are representative of single cell suspensions of excisional whole LN.
- T_{FH} cells were readily identifiable in LN FNAs.
- Superantigen responsive CD4+ T cells were detected in LN FNAs upon stimulation.