

Comparison of Two Flow Cytometric Methods for Detection of Human Invariant Natural Killer T Cells (iNKT)

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ABSTRACT

Background: Invariant natural killer cells (iNKT) are an important immunoregulatory T cell subset. Currently several flow cytometry-based approaches exist for the identification of iNKT cells, which rely on using the 6B11 monoclonal antibody or a combination of anti-V α 24 and anti-V β 11 antibodies. **Objective:** The aim of this study was to compare the ability of two flow cytometry-based methods for detecting the frequency of circulating iNKT cells. **Methods:** The frequency of iNKT cells was detected in the peripheral blood of 37 healthy adult donors by flow cytometry using the 6B11 antibody or a combination of anti-V α 24 and anti-V β 11 antibodies. **Results:** The frequency of iNKT cells detected by 6B11 antibody or by combination of anti-V α 24 and anti-V β 11 antibodies was significantly different (0.54% vs. 0.31%, respectively, $p < 0.001$) but the values were highly correlated (Spearman $r = 0.742$, $p < 0.0001$). **Conclusion:** The results of this study indicate that different combinations of mAbs detect different frequencies of peripheral blood iNKT cells and a consensus in the field needs to be established to allow better assessment of iNKT-related studies and suggest using different methods for accurate identification of iNKT cells.

Keywords: Flow Cytometry, Invariant NKT Cells, Monoclonal Antibody

INTRODUCTION

Invariant natural killer T cells (iNKT) are a small subset of T lymphocytes that recognize glycolipid antigens in the context of the non-classical MHC class I molecule CD1d. In humans iNKT cells express a highly limited TCR repertoire, composed of an invariant TCR α chain (V α 24-J α 18) and a biased set of TCR β chains (predominantly V β 11) (1,2). iNKT cells rapidly secrete a broad range of cytokines, including Th1 and Th2 cytokines upon activation (3). They are implicated in many immune responses including

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infections (4), autoimmunity (5), anti-tumor immunity (6) and allergic airway inflammation (7). Because of the significant contribution of iNKT cells to immunity, they have become a potential target for immunodiagnostic and immunotherapy (8) and a few therapeutic approaches using iNKT cells have entered clinical trials (9,10).

Defect in the number of circulating iNKT cells is implicated in some immune-mediated disorders such as multiple sclerosis (11), type I diabetes (12), rheumatoid arthritis (13), inflammatory bowel diseases (14), some types of cancers (15) and some other diseases (16).

Most of the studies evaluate the circulating number of iNKT cells for assessing their role in health and disease and to monitor the efficacy of iNKT-based treatments. At the moment several flow cytometry-based approaches exist for detection of iNKT cells including use of CD1d-tetramer, 6B11 monoclonal antibody, which detects invariant CDR3 domain of the canonical V α 24J α 18 TCR, or a combination of antibodies against V α 24 and V β 11 chains. Because of the low frequency of iNKT cells in peripheral blood, the accurate identification of iNKT cells is the most important parameter in their analysis, however, the reagents have been used interchangeably in this field without a clear understanding of how different approaches compare to one another (3,17).

In this study, we compared the frequency of iNKT cells in a cohort of healthy adults using 6B11 antibody and a combination of anti-V α 24 and anti-V β 11 antibodies to have a better estimation about the distinguishing power of different antibodies and the validity of different approaches for detection of iNKT cells in order to decrease the amount of controversies that exist in this field.

MATERIALS AND METHODS

Study Population. This study was conducted in the Immunology Research Centre of Mashhad University of Medical Sciences, Mashhad, Iran. Thirty-nine healthy adult volunteers (22 males, 15 females, mean age 28 years) without any history or symptoms of acute or chronic immune-mediated diseases at the time of blood sampling enrolled in this study. The study was approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran. All patients provided informed consent to participate in the study.

Flow Cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood using Lymphosep (Biosera, UK). Cell viability and number were determined by trypan blue exclusion. Phenotypic analysis of conventional T lymphocytes and iNKT cells in PBMCs was performed by three-color flow cytometry using Facscalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). To reduce non-specific binding, the cells were incubated with 10% human AB serum in RPMI 1640 for 30 minutes. Subsequently, the cells were washed twice with 1% BSA-PBS and then incubated with respective monoclonal antibodies in the dark at 4°C for 20 minutes. After two washes with 1% BSA-PBS, the cells were re-suspended in 250 μ l PBS with 1% BSA and were subjected to flow cytometry analysis. Specific monoclonal antibodies used for this study were: Anti-CD3 peridinin chlorophyll protein (PerCP), 6B11 phycoerythrin (PE) anti-invariant NKT cell TCR, anti-V β 11 (clone C21) fluorescein isothiocyanate (FITC) and anti-V α 24 (clone C15) phycoerythrin (PE) (Immunotech, Marseille, France).

For all staining experiments, appropriate isotype controls were included. Furthermore, because of the low frequency of iNKT cells in PBMC, at least 2×10^5 cells were ana-

lyzed. Except where indicated, all monoclonal antibodies (mAbs) were purchased from Becton Dickinson (San Jose, CA, USA).

Flow Cytometry Data Analysis. Flow cytometry data were analyzed by using FlowJo software. For more validity and to prevent any bias because of non-specific binding of dead or granular cells, which can cause overestimation of iNKT cells as has been shown in some studies (18,19), we used a stringent serial gating strategy. First, all dead cells and non-lymphocytes were excluded using lymphocytic gate (based on the forward versus sideward scatter) and then the CD3 positive cells were gated based on the sideward scatter and anti-CD3, and were used for the analysis of the frequency of iNKT, V α 24 and V β 11 cells (Figure 1). Because iNKT cells were identified in two ways, we briefly referred to them as 6B11-iNKT when 6B11 monoclonal antibody was used and V α 24-V β 11 iNKT when a combination of both anti-V α 24 and anti-V β 11 monoclonal antibodies were used.

Statistical Analysis. Data were analyzed by SPSS software package version 13 (Chicago, USA). When appropriate, two-tail Mann-Whitney test or Student *t*-test was used, depending on data normality. For evaluation of the association between different surface markers or cell subsets, Spearman's non-parametric correlation test was used. $p < 0.05$ was considered significant.

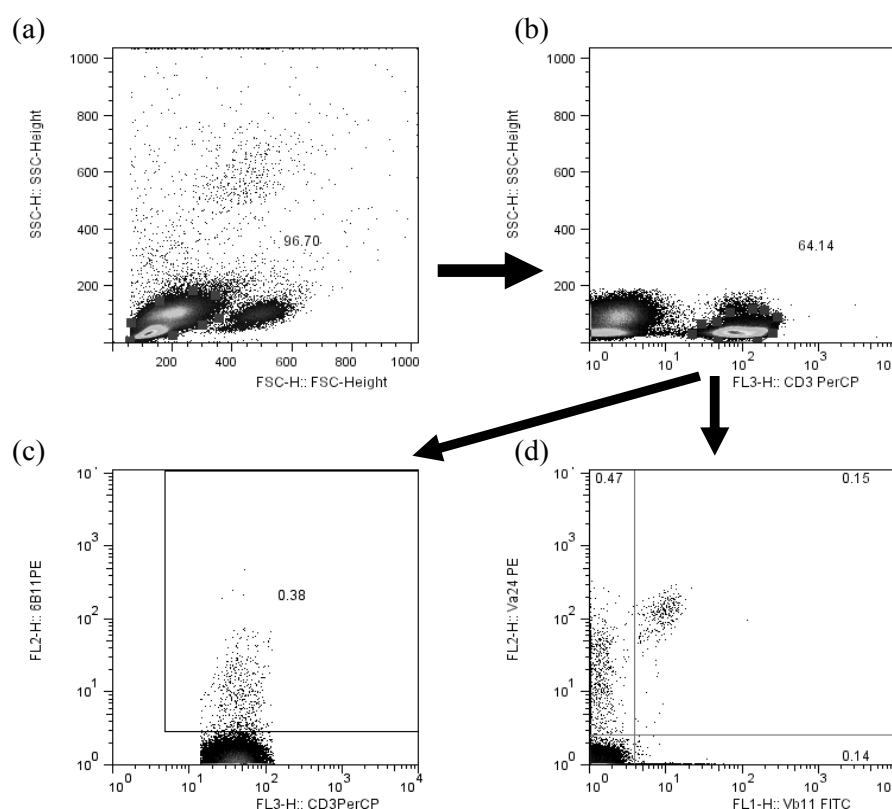


Figure 1. Gating strategy for analyzing iNKT cells. (a) Lymphocytes were gated on FSC-SSC plot. (b) CD3⁺ cells were then gated based on the sideward scatter and anti-CD3. The NKT cells were identified (c) using a combination of anti-CD3 and 6B11 antibodies or (d) by co-expression of V α 24 and V β 11.

RESULTS

The mean frequency of 6B11-iNKT cells was 0.54% (0.14-1.78%), while those positive for V α 24V β 11 were 0.31% (0-1.6%). This difference was statistically significant ($p < 0.001$) and indicates that, in total, 6B11 detected approximately two times more iNKT cells than co-staining with V α 24V β 11 antibodies (Figure 2).

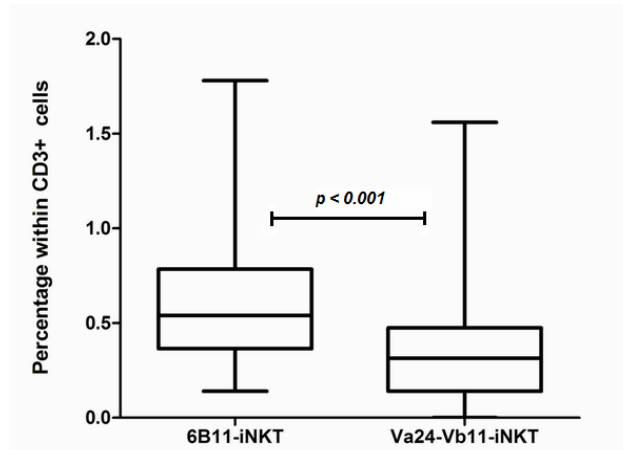


Figure 2. Frequency of 6B11-iNKT and V α 24-V β 11-iNKT T cells in the peripheral blood.

There was a strong positive correlation between the frequency of iNKT cells detected by both methods (Spearman $r = 0.742$, $p < 0.0001$, Figure 3). Although men had higher percentage of iNKT cells in both methods than women, there was no significant difference in the percentage of either 6B11-iNKT cells or V α 24-V β 11-iNKT cells between male and female donors.

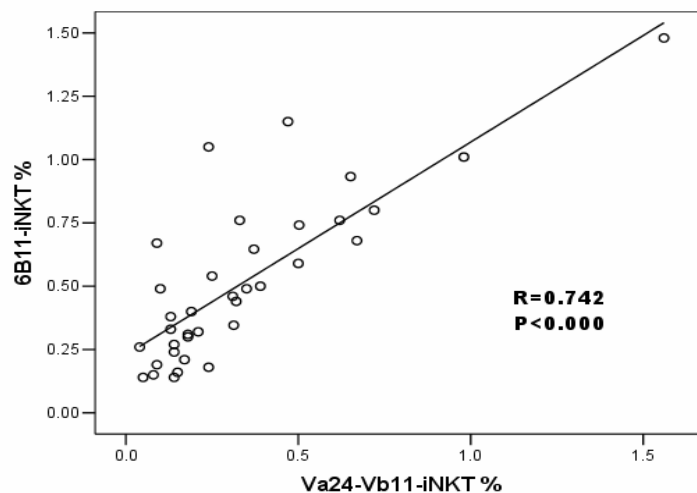


Figure 3. The correlation between the percentages of circulating iNKT cells detected by 6B11 antibody and a combination of anti-V α 24 and anti-V β 11 antibodies.

DISCUSSION

iNKT cells have important immunoregulatory functions and many studies have evaluated the role of iNKT cells in human diseases by measuring their number in peripheral blood or local tissues. Because different methodology and reagents were used in previous studies to identify iNKT cells, it is difficult to consolidate their findings. In this study, we analyzed the frequency of circulating iNKT cells by different monoclonal antibodies to evaluate the usefulness of these antibodies for identification iNKT cells. Currently, there are three common flow cytometry-based approaches for detecting of iNKT cells in peripheral blood including CD1d loaded glycolipid tetramer, 6B11 antibody and co-staining with anti-V α 24 and anti-V β 11 antibodies. Among these methods, CD1d-tetramer has some limitations and technical difficulties (20,21), which makes it not very practical in routine clinical use. Therefore, we compared iNKT cell identification by immunostaining using 6B11 or a combination of anti-V α 24 and anti-V β 11 antibodies. We showed that the numbers of iNKT cells detected by 6B11 or V α 24-V β 11 were significantly different, yet there was a strong positive correlation between the two methods. Our studies also point to 6B11 antibody as identifying a higher number of iNKT cells than the combination of anti-V α 24 and anti-V β 11.

Few studies have used different reagents for identification of iNKT cells. In a study by Montoya et al., (22) combination of V α 24-V β 11 detected higher number of iNKT cells compared to CD1d-tetramer or 6B11 but the difference was not significant. In their study, the sample size was just 10 cases and among them, a few cases showed significant differences between V α 24-V β 11 and 6B11. In a study by Kukreja (23), the frequency of iNKT cells identified by V α 24-6b11 and V α 24-V β 11 was not significantly different although the comparison was made based on just four donors.

Consistent with our data, the frequency of iNKT cells from sputum or bronchoalveolar lavage (BAL) of asthmatic patients detected by 6B11 or combination of V α 24-V β 11 antibodies was significantly different and 6B11 antibody detected higher number of iNKT cells than the combination of V α 24 and V β 11 (data were extracted from a graph) (18).

Several reasons may account for variability in detection of iNKT cells based on the choice of antibody. First of all and although in human, the canonical V α 24-J α 18 TCR of iNKT cells pairs almost exclusively with V β 11 chain (1,24). Some other V β chains such as V β 2, 3, 5 and 8 also can pair with V α 24-J α 18 (25,26). This varied pairing of V β chain may contribute to variability in detection of iNKT cells based on 6B11 vs. V α 24-V β 11. Furthermore, another study reported a subset of CD1d restricted NKT cells which do not express V α 24 chain but have V β 11 chain while both V α 24-negative and V α 24-positive clones used J α 18 and paired with V β 11 chain (27). In the same context, it has been shown that V α 24 clones are highly polyclonal and different beta chains can pair with V α 24 chain (28). In addition, some non-iNKT cells can express V α 24 (3,29), and 6B11 antibody can detect some non-CD1d restricted B-cell and T-cell epitopes (17,30). Therefore, it is possible that at least in some cases, 6B11 and V α 24-V β 11 antibodies do not identify the same population of cells because of the difference in the pairing of alpha and beta TCR chains and consequently, the number of V α 24-V β 11-iNKT cells are not the same as 6B11-iNKT cells.

Another possible reason could be variation in the expression of surface markers over time. Although studies have shown that the number of iNKT cells is stable over time (22,31), the sampling that was done in a period of about one month might have affected the frequencies. There is also a body of evidence about the quick disappearance of NKT

cells because of down regulation of the TCR after stimulation even with anti-CD3 monoclonal antibody (32,33). We also used anti-CD3 monoclonal antibodies in our study and although the incubation time was very short, it is still possible that anti-CD3 antibody caused a temporary down-regulation of TCR, leading to a discrepancy between two different methods.

Finally, though iNKT cells express an invariant TCR, some minor changes in the sequence of TCR were reported due to the N region addition or point mutations (26,34). It is also possible that V α 24 chain rearranged to a different J α segment (1) and these minor changes when happen in a critical binding site can affect the affinity and specificity of monoclonal antibodies leading to a discrepancy in the number of iNKT cells identified by different antibodies.

In conclusion, the result of this study showed that the current methods for identification of iNKT cells are different in specificity and sensitivity. However, there is a correlation between 6B11 and V α 24-V β 11. For studies requiring the exact quantification of the number of iNKT cells, it is helpful to have a consensus in the field to allow for comparison between different studies.

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