

# Invariant natural killer (iNK) T cell deficiency in patients with common variable immunodeficiency

D. A. Fulcher,\* D. T. Avery,<sup>†‡</sup>  
N. L. Fewings,\* L. J. Berglund,\*  
S. Wong,\* D. S. Riminton,<sup>§</sup>  
S. Adelstein<sup>¶</sup> and S. G. Tangye<sup>†‡</sup>  
*\*Immunology Unit, Institute of Clinical  
Pathology and Medical Research, Westmead  
Hospital, Westmead, <sup>†</sup>Garvan Institute of Medical  
Research, <sup>‡</sup>Centenary Institute of Cancer  
Medicine and Cell Biology, <sup>§</sup>Concord Hospital,  
and <sup>¶</sup>Royal Prince Alfred Hospital, Sydney, NSW,  
Australia*

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Correspondence: D. A. Fulcher, Immunology  
Unit, Institute of Clinical Pathology and  
Medical Research, Westmead Hospital,  
Westmead, Sydney, NSW 2145, Australia.  
E-mail: david\_fulcher@wmi.usyd.edu.au

## Introduction

Invariant NK (iNK) T cells are thymic-derived T lymphocytes that express V $\alpha$ 24-J $\alpha$ 18/V $\beta$ 11 T-cell receptor (TCR) chains in humans, and are selected to recognize foreign and self-derived lipid antigens presented on CD1d [1,2]. Their ability to produce rapidly a variety of T helper type 1 (Th1) and Th2 cytokines [1–4], as well as interleukin (IL)-21 [5], suggests an important but as yet poorly defined role in immunoregulation. Indeed, iNK T cells have been implicated in the development and prevention of numerous autoimmune and inflammatory conditions, as well as in tumour/immune surveillance [1,2]. A critical function of iNK T cells in immune regulation and homeostasis is supported further by the findings of a near-absence of iNK T cells in patients with X-linked lymphoproliferative disease (XLP) due to mutations in *SH2D1A* and *XIAP* [6–9], and reduced frequencies of iNK T cells in Omenn's syndrome [10], Wiskott–Aldrich syndrome [11], human immunodeficiency virus (HIV)-1 infection [12,13] and possibly hyperimmunoglobulin (Ig)M syndrome [14]. Thus, a lack of iNK T cells in these conditions may contribute to infection susceptibility in affected patients. Common variable immunodeficiency (CVID) has an incidence of approximately 1:25 000, and is manifested primarily by hypogammaglobulinaemia associated with recurrent sinopulmonary

## Summary

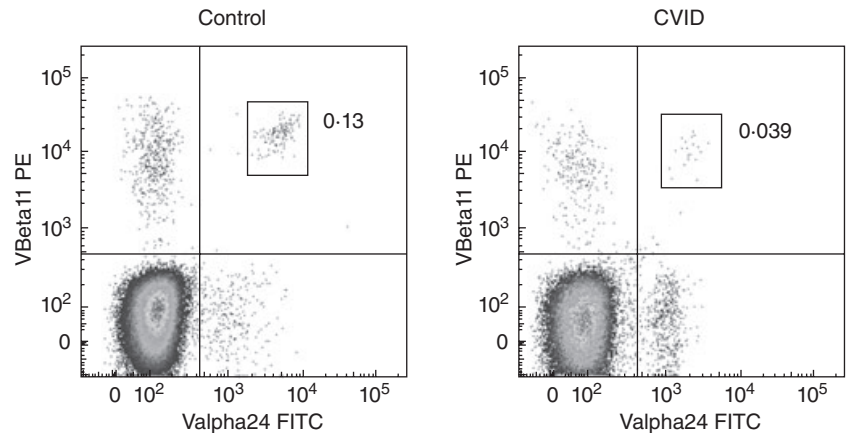
Common variable immunodeficiency (CVID) is a B cell immunodeficiency disorder characterized frequently by failure of memory B cell development and antibody secretion. A unifying cellular pathogenesis for CVID has not been forthcoming, but given the immunoregulatory role of invariant NK (iNK) T cells and their absence in several other immunodeficiencies, we quantified these cells in the blood of 58 CVID patients. There was a marked decrease in the proportion of iNK T cells in CVID patients compared with controls. This was particularly notable in those with low isotype-switched memory B cells, but subset analysis demonstrated no difference when stratified by specific clinical features. We propose that the decreased proportion of iNK T cells in CVID might be linked to the failure of memory B cell generation, which may contribute to reduced antibody production in these patients.

**Keywords:** B cells, common variable immunodeficiency, NK T cells

and gastrointestinal infections. The most common cellular abnormality that accompanies the loss of Ig production is a paucity of isotype-switched memory B cells [15,16]. Despite this, little is known about the regulatory abnormalities underlying this defect. We report that patients with CVID have low proportions of iNK T cells and speculate that this abnormality may underlie some of the humoral irregularities in this condition.

## Methods

Fifty-eight CVID patients and 30 healthy controls were recruited from Westmead, Concord and Royal Prince Alfred Hospitals in Sydney, Australia, following informed consent, according to protocols approved by local ethics review boards. CVID was diagnosed based on a decreased serum IgG level along with a decrease in either serum IgA or IgM, and exclusion of secondary causes of hypogammaglobulinaemia. The clinical phenotype was determined as described by Berglund *et al.* [15]. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque centrifugation; in the initial study commenced in 2005, cells were cryopreserved until used, whereas in the confirmatory study of 2008 cells were stained immediately upon isolation. iNK T cells were identified by staining with



**Fig. 1.** Quantification of invariant natural killer (iNK) T cells by co-expression of T cell receptor (TCR)-V $\alpha$ 24 and TCR-V $\beta$ 11 chains by flow cytometry. Initial gating on lymphocytes by forward and side light scatter was followed by gating on CD3<sup>+</sup> cells. Plots represent the median values from the cohort of control subjects (left), and common variable immunodeficiency (CVID) patients (middle); the right-hand plot depicts a CVID patient with the highest iNK T cell proportion.

anti-CD3 (UCHT1) conjugated to either Pacific Blue (eBioscience, San Diego, CA, USA) or allophycocyanin (APC) (BD Biosciences Pharmingen, San Diego, USA), anti-T cell receptor (TCR)-V $\alpha$ 24-fluorescein isothiocyanate (FITC) (C15; Immunotech, Marseille, France) and anti-TCR-V $\beta$ 11-phycoerythrin (PE) (C21; Immunotech). The percentage of CD3<sup>+</sup> T cells co-expressing TCR-V $\alpha$ 24/V $\beta$ 11 was determined by flow cytometry (Fig. 1), following the collection of 3–10  $\times$  10<sup>5</sup> events. Isotype-switched memory B cells were quantified by staining either cryopreserved cells with anti-CD20-FITC (L27; BD), anti-IgD-PE (IA6-2; BD) and anti-CD27-biotin (0323; eBioscience); or fresh cells with anti-CD20-PE-Cy5 (B9E9; Immunotech), anti-IgD-FITC (IA6-2; BD) and anti-CD27-PE (1A4C27; Immunotech). Proportions of isotype-switched (CD20<sup>+</sup>/CD27<sup>+</sup>/IgD<sup>-</sup>) B cells of < 0.4% of gated lymphocytes were allocated to Freiburg group I and the remainder to group II, whereas patients with total B cell proportions below 1% were excluded from subdivision analysis [16]. The samples were fixed in 1% formaldehyde; data were acquired on a fluorescence activated cell sorter (FACS) Calibur flow cytometer (BD Biosciences Pharmingen) and analysed using FlowJo (Tree Star, Inc., Ashland, OR, USA). PBMCs were stained with PE-labelled  $\alpha$ -GalCer-loaded CD1d tetramers (a kind gift of Dale Godfrey, Department Microbiology and Immunology, University of Melbourne) at a concentration of 0.82  $\mu$ g/ml, along with anti-CD3-Pacific Blue (as above).

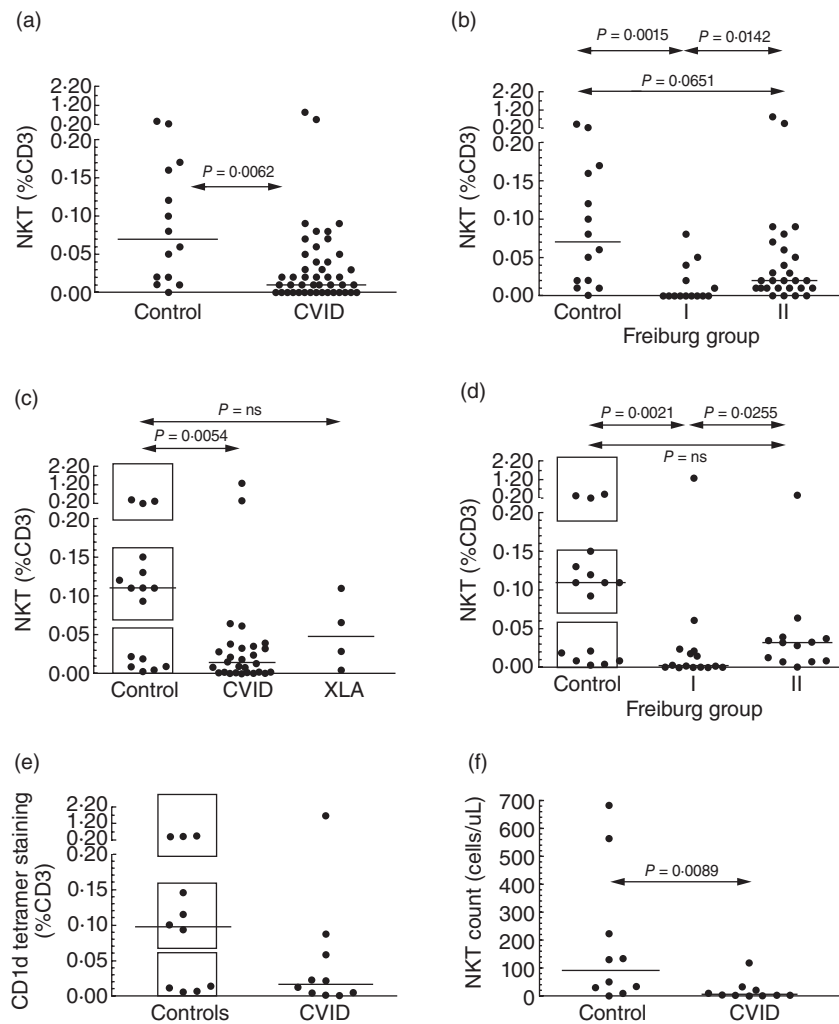
Statistical analysis was performed using Prism version 4.0c for Macintosh (GraphPad Software Inc., La Jolla, CA, USA). The mean difference between continuous variables was analysed using the Mann–Whitney *U*-test, with *P*-values < 0.05 considered significant. For analysis of iNK T cell proportion differences over time, the two-tailed paired Wilcoxon signed-rank test was used.

## Results

CD3<sup>+</sup> T cells present in cryopreserved PBMCs from a cohort of 47 CVID patients and 14 controls were analysed for co-expression of the V $\alpha$ 24 and V $\beta$ 11 TCR chains

(Fig. 1). There was a significant reduction in the proportion of iNK T cells within the CD3<sup>+</sup> T cell population in the CVID cohort [median 0.01%, interquartile range (IQR) 0.0–0.04] compared with controls (median 0.07%, IQR 0.015–0.165) (Fig. 2a). This reduction was most striking in patients with decreased frequencies of isotype-switched memory B cells, referred to commonly as Freiburg group I [16] (Fig. 2b).

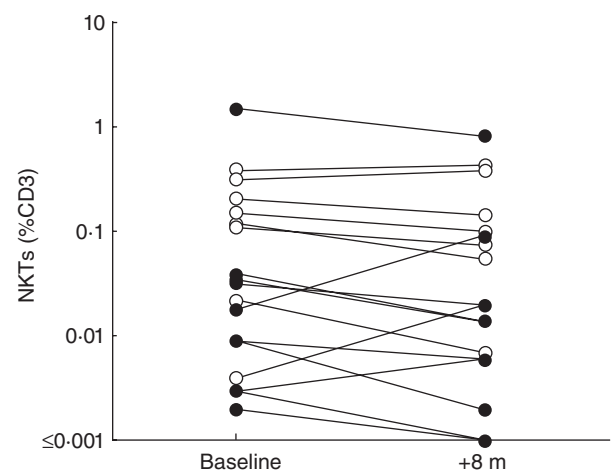
To confirm these findings, and correlate with clinical features, proportions of iNK T cells were then determined in fresh PBMCs from an overlapping cohort of 28 fully phenotyped CVID patients (aged median 46 years, range 23–75) and 16 controls (aged median 41 years, range 22–62). The data from these patients were analysed as a separate group because, in 17 CVID patients analysed by both collection methods, iNK T cell proportions in cryopreserved samples were slightly but significantly higher than in freshly collected samples (Supplementary Fig. S1). As expected, iNK T cell proportions were again decreased markedly in the CVID cohort irrespective of whether the data were expressed as a proportion of total T cells (CVID: median 0.013%, IQR 0.001–0.034, *versus* control: 0.110%, IQR 0.014–0.140) (Fig. 2c) or of PBMCs (data not shown), and again patients from Freiburg group I showed the lowest proportions (Fig. 2d). There was no relationship between iNK T cell proportions and age (data not shown). Interestingly, the proportions of iNK T cells in normal donors seemed to partition into three separate groups – low (< 0.06%), moderate (0.07–0.16%) and high (> 0.2%; Fig. 2c,d) – which would be consistent with the finding that the production of iNK T cells is controlled genetically [17,18]. Unlike CVID patients, there was no difference in the percentage of switched memory B cells in controls with low NK T cells compared with those who had higher NK T cell proportions (data not shown). On the other hand, the great majority of CVID patients fell within the low group (< 0.06%), except for two patients who had unexpectedly high proportions (Fig. 2c). To exclude the possibility that intravenous gammaglobulin replacement therapy itself might be contributing to a reduction in NK T cell



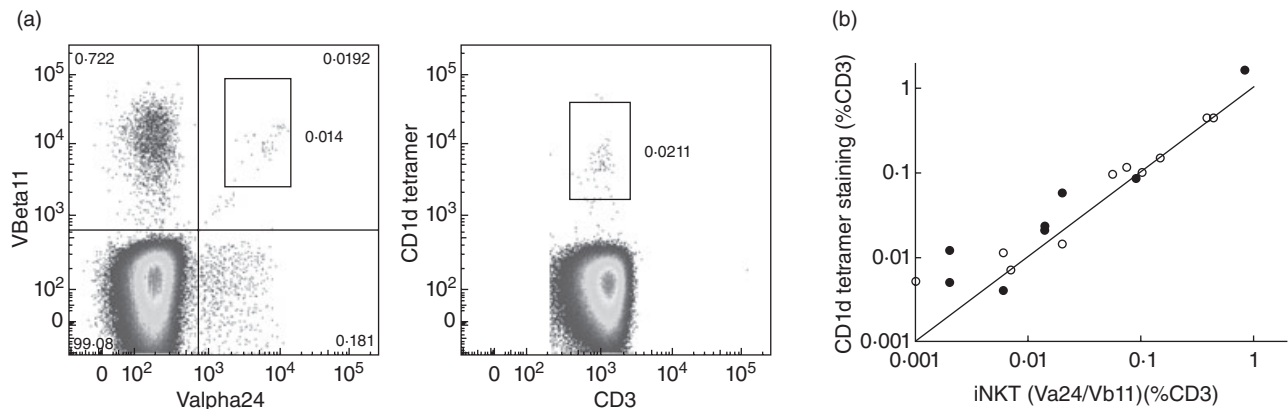
**Fig. 2.** Invariant natural killer (iNKT) T cell proportions expressed as a percentage of CD3<sup>+</sup> cells in control subjects and common variable immunodeficiency (CVID) patients, based on staining of cryopreserved (a, b) cells from the first cohort, or fresh (c–e) peripheral blood mononuclear cells from the second. Graphs represent CVID patients *versus* control subjects (a, c) or the breakdown of CVID patients according to the proportion of isotype-switched memory B cells (b, d), with low proportions designated Freiburg group I and normal proportions, group II. In (c), analysis of four X-linked agammaglobulinaemia (XLA) patients is shown for comparison. In (c, d), boxes have been drawn to indicate the three apparent tiers of iNKT T proportions in control subjects. (e) Proportions of iNKT T cells as detected by CD1d tetramer staining in 10 CVID patients and 10 controls. (f) Absolute counts of iNKT T cells in the peripheral blood of 10 CVID patients and 10 controls.

numbers, four patients with X-linked agammaglobulinaemia (XLA) were analysed, but had iNKT T proportions not different to controls (Fig. 2c). When the second cohort of CVID patients was stratified on the basis of clinical characteristics (splenomegaly, lymphadenopathy, autoimmune disease), there were no significant differences in iNKT T cell proportions in those subsets (data not shown).

The stability of the iNKT T cell proportion over time was examined by reanalysing a subset of 10 CVID patients and 10 controls from the second cohort 8 months after the first analysis. Values were very similar to baseline, with no significant differences in iNKT T proportions between the two time-points (Fig. 3). To validate the V $\alpha$ 24 and V $\beta$ 11 TCR expression, the same subset of 20 subjects was also stained with CD1d tetramers, with similar findings (Fig. 2e) and very close correlation between the two methods (Fig. 4). Finally, when these data were analysed in terms of absolute NK T cell counts, there was again a marked reduction in CVID patients compared with controls (Fig. 2f).



**Fig. 3.** Invariant natural killer (iNKT) T cell proportions expressed as a percentage of CD3<sup>+</sup> cells in 10 control subjects (open circles) and 10 common variable immunodeficiency (CVID) patients (filled circles), analysed at two time-points, 8 months apart ( $P = 0.22$ ).



**Fig. 4.** CD1d tetramer staining in comparison to staining with T cell receptor (TCR)-V $\alpha$ 24 and TCR-V $\beta$ 11. (a) Dot plots from a representative common variable immunodeficiency (CVID) patient stained by the two methods; (b) comparison between the two methods in 10 controls (open circles) and 10 CVID patients (filled circles). The line of equivalence is shown.

## Discussion

We report the finding that CVID is another immunodeficiency disease characterized by depressed or absent iNKT cells, suggesting that iNKT cell deficiency may be a common factor in the pathogenesis of humoral immunodeficiencies. The low iNKT cell phenotype was demonstrated in a large cohort of CVID patients, was reproducible using two distinct identification reagents, was shown to be a stable finding over time and was not associated with intravenous gammaglobulin itself, as it was not noted in patients with XLA. A paucity of iNKT cells was noted particularly in those patients with low or absent switched-memory B cells, suggesting a link between iNKT cells and B cell function, making an intriguing parallel to XLP in which failure of antibody production and absence of both iNKT cells and memory B cells are common characteristics [7,19]. Further support for an association between iNKT cells and humoral immunity comes from two cardinal observations: first, iNKT cells can express CD40L [1,20] and have the capacity to secrete large concentrations of IL-4, IL-10, IL-13 and IL-21 [1–5], which are all capable of inducing isotype switching and antibody secretion by CD40L-stimulated human B cells [21]; and secondly, iNKT cells can provide cognate help to stimulate B cell activation and enhance antigen-specific responses, both *in vivo* in mouse models [22,23] and *in vitro* in co-culture systems utilizing human B cells and autologous iNKT cells [20]. Therefore, it is highly likely that iNKT cells make a significant contribution to basal Ig production *in vivo*, and an absence of iNKT cells can result in the development of hypogammaglobulinaemia as observed in XLP and CVID. On the other hand, it is unlikely that decreased iNKT cell proportions alone are responsible for the entirety of the CVID phenotype, as a subset of healthy control donors also had proportions of iNKT cells that were as low as CVID patients (Fig. 2) and, similarly, some patients with CVID had normal or even high proportions; rather, this characteristic may be a contributing factor in a multi-factorial process.

Reduced iNKT cell proportions in CVID may relate to defective thymic development, similar to other immunodeficiency disorders. Thus, the failure of iNKT cells to develop normally in XLP patients relates presumably to the loss of signalling lymphocyte activation molecule (SLAM)-associated protein (SAP)-mediated signalling downstream of SLAM and NK T-B antigen (NTB-A) (murine Ly108) following homotypic interactions between these molecules on CD4<sup>+</sup>/CD8<sup>+</sup> thymocytes and thymic epithelium [24], thereby preventing positive selection. In Omenn's syndrome, due to hypomorphic mutations in *Rag-1* and *Rag-2*, defective ability to make appropriate VDJ recombinations might prevent the specific rearrangements required to express the invariant TCR. No clear parallel exists for CVID, because a genetic explanation is apparent in < 10% of cases, yet our findings point towards the thymus in the pathogenesis of this condition, in which multiple other defects in conventional T cell biology have been described [25]. However, we cannot exclude the alternate possibility that our findings represent a deficiency in peripheral blood iNKT cells in CVID due to their sequestering to sites outside the circulation.

One further observation in this study was that iNKT cell values in cryopreserved samples were generally higher than in fresh samples taken 3 years later (Supplementary Fig. S1). It is likely that this finding is methodological, as iNKT cell proportions appeared to be constant in CVID patients and controls, at least over an 8-month period (Fig. 3).

In conclusion, deficiency of iNKT cells may have implications for our understanding of antibody failure in CVID and may suggest a common link with other immunodeficiency disorders.

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## Disclosure

The authors have no conflicts of interest to declare.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Comparison of inducible natural killer (iNK) T cell proportions as derived from cryopreserved cells ( $y$ -axis) in comparison to fresh cells ( $x$ -axis) in a subset of 18 common variable immunodeficiency (CVID) patients analysed by both methods at different time-points. Line of best fit is dashed ( $r^2 = 0.9962$ , slope = 1.27), while the  $y = x$  line is shown in grey for comparison.

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