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# Invariant Natural Killer T Cells in Chronic Lymphocytic Leukaemia

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“It was afterwards...when the remedies had already been discovered, that men began to discuss the reasons for them: the Art of Medicine was not a discovery following upon reasoning, but after the discovery of the remedy, the reason for it was sought out”

Aulus Cornelius Celsus, *De Medicina*, ca. 30AD<sup>1</sup>



# Abstract

Invariant natural killer T (iNKT) cells recognise glycolipid antigens, such as the synthetic ligand alpha galactosylceramide ( $\alpha$ -GalCer), in the context of CD1d. Upon recognition of  $\alpha$ -GalCer on CD1d, iNKT cells become activated and rapidly produce cytokines. Recruitment of iNKT cells with  $\alpha$ -GalCer results in potent antitumour activity in some pre-clinical cancer models. Effector mechanisms include direct cytotoxicity of iNKT cells against CD1d-expressing tumours in the presence of  $\alpha$ -GalCer, transactivation of natural killer (NK) cells by iNKT cell-derived interferon gamma (IFN- $\gamma$ ), and iNKT cell-induced maturation and activation of dendritic cells (DCs), leading to enhanced T cell responses to DC-presented peptides.

Chronic lymphocytic leukaemia (CLL) is a clonal malignancy of B lymphocytes. Chemotherapy induces remission in most patients, but most eventually relapse. Allogeneic stem cell transplantation can be curative, but is not available to most patients due to advanced age or co-morbidities. Thus, CLL is an attractive candidate for cancer immunotherapy. This thesis aims to assess the iNKT cell/CD1d axis of patients, and to explore the possibility of exploiting iNKT cells for the immunotherapy of CLL.

Peripheral blood mononuclear cells (PBMCs) were isolated from patients with CLL, and from healthy age-matched controls, and analysed by flow cytometry. Absolute number and phenotype of circulating iNKT cells was similar in patients and controls, although patients exhibited a relative reduction in iNKT cells due to expansion of other T cell populations. iNKT cell frequency did not correlate with disease stage or with subsequent progression-free survival in patients with untreated CLL. Expression of CD1d on dendritic cells and monocytes from patients with CLL was similar to controls.

The cytokine profile of patient iNKT cells was similar to that of controls. *In vitro* proliferation of invariant natural killer T (iNKT) cells from patients with CLL was preserved, and iNKT cell lines generated from patients exhibited cytokine and cytotoxicity profiles similar to those from healthy controls, producing both Th1- and Th2-type cytokines, and lysing a target cell in a CD1d- and  $\alpha$ -GalCer-dependent manner. Lysis of autologous CLL cells by iNKT cell lines was inefficient.

*In vitro* vaccine recall responses were enhanced by  $\alpha$ -GalCer in PBMCs

containing high frequencies of iNKT cells. The treatment of leukaemic cells with  $\alpha$ -GalCer enhanced their ability to stimulate proliferation of allogeneic PBMCs from healthy donors *in vitro*, largely due to iNKT cell proliferation. Leukaemic cells treated with  $\alpha$ -GalCer induced proliferation of autologous iNKT cells, and  $\alpha$ -GalCer treatment also led to enhanced proliferation of 'conventional' T cells.

These results indicate that the iNKT cell/CD1d axis is largely intact in patients with CLL and suggest that if low iNKT cell frequencies can be overcome, the adjuvant activity of iNKT cells might be exploited in cellular immunotherapy of CLL, for example by employing  $\alpha$ -GalCer-pulsed leukaemic cells as a whole tumour vaccine.

To my wife Teresa and son Henry.





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# List of Abbreviations

<b><math>\alpha</math>-GalCer</b>	alpha galactosylceramide
<b>Allo-SCT</b>	allogeneic stem cell transplantation
<b>Auto-SCT</b>	autologous stem cell transplantation
<b>ADCC</b>	antibody-dependent cellular cytotoxicity
<b>AEC</b>	3-amino-9-ethylcarbazole
<b>AIHA</b>	autoimmune haemolytic anaemia
<b>ANOVA</b>	analysis of variance
<b>APC</b>	allophycocyanin
<b>APC</b>	antigen-presenting cell
<b>APRIL</b>	a proliferation-inducing ligand
<b>ATG</b>	anti-thymocyte globulin
<b>BAFF</b>	B-cell activation factor belonging to the TNF family
<b>BCG</b>	Bacille Calmette-Guerin
<b>BCR</b>	B cell receptor
<b>BDCA-1</b>	Blood Dendritic Cell Antigen 1
<b><math>\beta_2</math>M</b>	beta 2 microglobulin
<b>Btk</b>	Bruton's tyrosine kinase
<b>C1R</b>	C1R cell line (HLA-A and -B negative human B cell line)
<b>C1R-CD1d</b>	CD1d-transfected C1R cell line
<b>CAR</b>	chimeric antigen receptor
<b>CCR7</b>	C-C chemokine receptor type 7
<b>CDK</b>	cyclin-dependent kinase
<b>CDR</b>	complementarity determining region
<b>CFSE</b>	carboxyfluorescein succinimidyl ester
<b>cIMDM</b>	complete Iscove's Modified Dulbecco's Medium
<b>CLL</b>	chronic lymphocytic leukaemia
<b>CMV</b>	cytomegalovirus
<b>CTLA-4</b>	Cytotoxic T Lymphocyte Antigen-4
<b>CTO</b>	CellTracker™ Orange (5-(and-6)-(((4-chloromethyl)benzoyl)-amino)tetramethylrhodamine)
<b>DAPI</b>	4',6-diamidino-2-phenylindole
<b>DC</b>	dendritic cell
<b>DiOC6</b>	dihexyloxacarboyanine iodide

<b>DLI</b>	donor lymphocyte infusion
<b>DMSO</b>	dimethyl sulfoxide
<b>DPBS</b>	Dulbecco's phosphate buffered saline
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>ELISpot</b>	enzyme-linked immunosorbent spot
<b>Fab</b>	antigen-binding fragment
<b>FACS</b>	fluorescence-activated cell sorting
<b>FasL</b>	Fas ligand
<b>Fc</b>	crystallizable fragment
<b>FCR</b>	fludarabine, cyclophosphamide and rituximab
<b>FCS</b>	foetal calf serum
<b>FISH</b>	fluorescent in-situ hybridisation
<b>FITC</b>	fluorescein isothiocyanate
<b>FMO</b>	fluorescence minus one
<b>Foxp3</b>	forkhead box P3
<b>GM-CSF</b>	granulocyte-macrophage colony stimulating factor
<b>GVHD</b>	graft versus host disease
<b>GVL</b>	graft versus leukaemia
<b>HEPES</b>	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
<b>HLA</b>	human leukocyte antigen
<b>HMB-PP</b>	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
<b>HRP</b>	horseradish peroxidase
<b>ICS</b>	intracellular cytokine staining
<b>IFN-<math>\gamma</math></b>	interferon gamma
<b>Ig</b>	immunoglobulin
<b>IgG</b>	immunoglobulin G
<b>IgH</b>	immunoglobulin heavy chain
<b>IgVH</b>	immunoglobulin heavy chain variable region
<b>IL</b>	interleukin
<b>IMDM</b>	Iscove's Modified Dulbecco's Medium
<b>IMiD</b>	immunomodulatory drug
<b>iNKT</b>	invariant natural killer T
<b>IPP</b>	isopentyl pyrophosphate
<b>KIR</b>	killer Ig-like receptor
<b>LDH</b>	lactate dehydrogenase
<b>LDT</b>	lymphocyte doubling time

<b>Lin 1</b>	lineage cocktail 1
<b>mAb</b>	monoclonal antibody
<b>MAIT</b>	mucosal-associated invariant T cells
<b>MART-1</b>	melanoma antigen recognised by T cells-1
<b>MBL</b>	monoclonal B cell lymphocytosis
<b>mDC</b>	myeloid dendritic cell
<b>MDSC</b>	myeloid-derived suppressor cell
<b>MHC</b>	major histocompatibility complex
<b>MLR</b>	mixed lymphocyte reaction
<b>moDC</b>	monocyte-derived dendritic cell
<b>MRD</b>	minimal residual disease
<b>NIH</b>	National Institutes of Health
<b>NK</b>	natural killer
<b>NKT</b>	natural killer T
<b>NOD</b>	non-obese diabetic
<b>NY-ESO-1</b>	New York esophageal squamous cell carcinoma-1
<b>NZB</b>	New Zealand Black
<b>OVA</b>	ovalbumin
<b>PBMC</b>	peripheral blood mononuclear cell
<b>PBS</b>	phosphate-buffered saline
<b>PCR</b>	polymerase chain reaction
<b>PD-1</b>	Programmed Cell Death-1
<b>pDC</b>	plasmacytoid dendritic cell
<b>PE</b>	phycoerythrin
<b>PerCP</b>	peridinin-chlorophyll protein complex
<b>PHA</b>	phytohaemagglutinin
<b>PI</b>	propidium iodide
<b>PI3K</b>	phosphatidylinositol-3-kinase
<b>PIM</b>	phosphatidylinositol mannoside
<b>PMA</b>	phorbol 12-myristate 13-acetate
<b>RIC-allo</b>	reduced intensity conditioning allogeneic stem cell transplantation
<b>RPMI</b>	Roswell Park Memorial Institute medium
<b>RT-PCR</b>	reverse transcription quantitative polymerase chain reaction
<b>scFv</b>	single-chain variable fragment
<b>SCID</b>	severe combined immunodeficiency
<b>SEM</b>	standard error of the mean
<b>SFU</b>	spot-forming unit

<b>sIgM</b>	surface IgM
<b>SLL</b>	small lymphocytic lymphoma
<b>SLVL</b>	splenic lymphoma with villous lymphocytes
<b>Syk</b>	spleen tyrosine kinase
<b>TAA</b>	tumour-associated antigen
<b>TACI</b>	transmembrane activator, calcium-modulator, and cyclophilin ligand interactor
<b>TAM</b>	tumour-associated macrophage
<b>TCR</b>	T cell receptor
<b>TGF-<math>\beta</math></b>	transforming growth factor beta
<b>Th1</b>	T helper 1
<b>Th2</b>	T helper 2
<b>TLR</b>	Toll-like receptor
<b>TNF-<math>\alpha</math></b>	tumour necrosis factor alpha
<b>TRAIL</b>	tumour necrosis factor-related apoptosis-inducing ligand
<b>TREC</b>	T cell receptor gene rearrangement excision circle
<b>T<sub>reg</sub></b>	regulatory T cell
<b>VITAL</b>	<i>in vivo/in vitro</i> technique for assessing lysis
<b>WT1</b>	Wilms' tumour 1
<b>ZAP-70</b>	zeta chain-associated protein 70

# Chapter 1

## Introduction

### 1.1 Chronic lymphocytic leukaemia

#### 1.1.1 Definition and Epidemiology

CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) is an indolent B cell malignancy, characterised by an infiltration of mature clonal B lymphocytes in the peripheral blood and bone marrow. The clinical features of CLL include a lymphocytosis, lymphadenopathy, hepatosplenomegaly, constitutional symptoms, propensity to infection, bone marrow failure due to replacement of the haematopoietic compartment with malignant lymphocytes and a predisposition to autoimmune cytopenias.

CLL is distinguished from other indolent B cell malignancies by a characteristic immunophenotype,<sup>2</sup> which allows the diagnosis to be established by flow cytometric analysis of the peripheral blood in the majority of cases.<sup>3</sup> Diagnostic criteria for CLL are listed in table 1.1.

Many healthy individuals harbour a circulating clonal B cell population with an immunophenotype identical to that of CLL, but do not meet the numeric criteria for CLL (the B lymphocyte count is  $< 5.0 \times 10^9/L$ ). This condition is known as monoclonal B cell lymphocytosis (MBL). MBL rises in frequency with age, being identified in over 5% of healthy individuals over 60 years of age,<sup>6,7</sup> and can be considered pre-malignant: progression to CLL requiring treatment occurs at a rate of 1 – 2% per year.<sup>6</sup>

Table 1.1 Diagnostic criteria for CLL<sup>4,5</sup>

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<b>All of the following criteria should be met:</b>
$\geq 5 \times 10^9/L$ B lymphocytes in peripheral blood
$\leq 55\%$ of lymphocytes show atypical morphology
Low surface IgM or IgD expression with $\kappa$ or $\lambda$ restriction
B cell surface antigen expression (CD19, CD23)
CD5 expression

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Small lymphocytic lymphoma (SLL) is an indolent B-cell lymphoma with similar immunophenotypic and cytogenetic characteristics to CLL, and is considered its lymphomatous counterpart. Many patients with CLL develop significant lymphadenopathy during the course of their disease, and many patients initially diagnosed with SLL will enter a leukaemic phase (with  $> 5 \times 10^9/L$  malignant B cells in the peripheral blood) and be re-categorised as having CLL. The term CLL/SLL is often used to emphasise the overlap between these two clinical entities.

CLL is the most common leukaemia in the Western World. It is primarily a disease of the elderly, with a median age at diagnosis of around 70 years.<sup>8,9</sup> CLL has a strong association with age, the relative risk in an individual 70 – 79 years old being 352 times that of an individual  $< 40$  years of age.<sup>8</sup> There is a male predisposition, nearly 60% of cases occurring among males.<sup>8</sup>

The incidence of CLL varies with ethnicity, age-adjusted incidence ratios being approximately 0.75 and 0.23 for Americans of Black or Asian descent compared to White Americans, respectively.<sup>10</sup> The incidence of CLL in the Far East is much lower than in Western countries, although CLL rates appear to be rising in more recent birth cohorts.<sup>11</sup> Asian migrants to the United States retain their lower risk of CLL, implying that the differences in rates by ethnicity are largely genetic or are due to antenatal or early childhood environmental exposures.<sup>12</sup>

A familial predisposition to CLL can be identified: A first-degree relative of a patient with CLL has a relative risk of  $\sim 8.5$  for developing CLL, and of  $\sim 1.9$  for the development of other indolent B cell malignancies.<sup>13</sup> In keeping with this, first-degree relatives of patients with CLL also have an increased chance of developing MBL (from 3.5% to 13.5%).<sup>14</sup> A number of single nucleotide polymorphisms have been associated with increased risk of CLL, including polymorphisms of the genes for the cell cycle regulator cyclin H, the apoptotic-associated protein caspase 8, the chemokine receptor CCR7 and the interleukin IL-16.<sup>15</sup>

Specific environmental factors appear to predispose to CLL. A history of benzene exposure is associated with an increased risk of CLL,<sup>16</sup> as is an occupational history including crop growing and horticulture, textile bleaching and dyeing, or plastics manufacturing.<sup>17,18</sup>

### 1.1.2 Diagnosis

Lymphocytosis, often noted incidentally on a routine blood count, is usually the first indication of CLL. The blood film shows a monomorphic population of small mature lymphocytes with scanty cytoplasm and clumped chromatin. Prolymphocytes, with a prominent nucleolus, are present in a minority of cases. CLL cells frequently become disrupted during preparation of the blood film, leading to characteristic ‘smudge’ cells on routine microscopy. The propensity of CLL cells to form these ‘smudge’ cells has been linked to low expression of the cytoskeletal protein vimentin in some patients. A smudge cell percentage of 30% appears to identify a subset of patients with inferior prognosis.<sup>19</sup>

The surface immunophenotype of CLL cells resembles that of normal mature antigen-experienced B lymphocytes with the exception of aberrant CD5 expression: Alongside



## 1.1. Chronic lymphocytic leukaemia

**Table 1.2 Immunophenotypic CLL scoring system** Scores are summed for expression of each surface marker. A score  $\geq 4$  strongly supports a diagnosis of CLL.<sup>20</sup>

Surface marker	Score, according to surface expression	
	0	1
SmIg <sup>*</sup>	Strong	Weak
CD5	Negative	Positive
CD23	Negative	Positive
FMC7 <sup>†</sup>	Positive	Negative
CD79b	Strong	Weak

\* Surface membrane immunoglobulin

† Antibody clone directed against an epitope of CD20

CD5, CLL cells express B cell markers including CD19 and CD23, and express low levels of surface IgM (sIgM) (with or without IgD), CD79b and the CD20 epitope FMC7. These markers have been combined to create a simple flow cytometric scoring system, which has been prospectively validated and is widely used for the diagnosis of CLL (table 1.2).<sup>20</sup>

Peripheral blood flow cytometry is usually sufficient to secure a diagnosis of CLL. However, in those with a CLL score of  $\leq 3$ , or where SLL is suspected, bone marrow and/or lymph node biopsy may be necessary. Where performed, the bone marrow aspirate is hypercellular, with increased numbers of mature lymphocytes, typically constituting  $> 30\%$  of nucleated cells. On bone marrow trephine histology, CLL infiltrates may be interstitial, nodular or diffuse. Immunohistochemistry can support the flow cytometric diagnosis, CLL cells typically being positive for CD5, CD20 (weak), CD23, CD43 and CD79a, and negative for CD10 and cyclin D1.<sup>21</sup> Lymph node histology in CLL and SLL shows a diffuse infiltration with monomorphic small round lymphocytes. Ill-defined pale nodular regions, known as pseudofollicles, can be appreciated at low magnification. Immunohistochemical findings in the lymph node are as in the bone marrow.<sup>22</sup>

### 1.1.3 Pathophysiology

#### 1.1.3.1 Cell of origin

The malignant cells in CLL are clonal B lymphocytes. Clonality can be demonstrated both by light chain restriction,<sup>23</sup> and by molecular evidence of clonal immunoglobulin heavy chain variable region (IgVH) gene usage. The emergence of this clonal B cell population precedes the clinical development of CLL: in a prospective cohort study, 44 of 45 patients (98%) who subsequently developed CLL had a detectable B cell clone (a MBL) in the peripheral blood at an average of 32 months before diagnosis.<sup>24</sup> These MBL clones frequently exhibit genomic aberrations typical of CLL, such as trisomy 12 and 13q deletion.<sup>6</sup>

Since aberrant CD5 expression is a hallmark of CLL, normal CD5 expressing B cells have

been proposed as the cell of origin for the leukaemia. CD5 is expressed by only 3% of circulating B cells in healthy humans, but is more frequently expressed by B cells in the marginal and mantle zones of lymphatic tissue and in foetal spleen.<sup>25</sup> However, CLL cells do not share other typical immunophenotypic characteristics with marginal zone or mantle zone B cells. Indeed, gene expression profiling indicates that CLL cells are most closely related to memory B cells,<sup>26</sup> a finding which would be consistent with their expression of the memory markers such as CD27.

Several lines of evidence indicate that the leukaemic cells in CLL derive from antigen-experienced B cells. In common with other antigen-experienced B cells, clonal IgM CLL cells are capable of isotype class switching, secreting IgG and IgA upon CD40 ligation *in vitro*.<sup>27</sup> The leukaemic cells of CLL also express a mannosylated form of sIgM, which is associated with antigen exposure.<sup>28</sup>

Studies of the immunoglobulin gene provide evidence of antigen exposure in CLL cells. The use of VH genes in CLL is biased: Certain VH genes are preferentially expressed in CLL, including VH4-34, VH3-07, VH1-69 in Western countries.<sup>29</sup> Interestingly, VH usage varies by ethnicity: compared to cases of CLL from Western countries, cases in Japan, China and Iran use VH4 genes more frequently, and VH1 genes less frequently.<sup>30</sup> Usage of the D and JH genes is also non-random, varying significantly depending on the VH gene used.<sup>29,31</sup> The length and composition of the CDR3 (complementarity-determining region) varies with VH usage, implying that selection has taken place,<sup>29</sup> stereotyped CDR3 sequences accounting for 20% – 30% of cases of CLL.<sup>32-34</sup>

Somatic hypermutation is evident in a proportion of CLL cases, with around 50% of cases demonstrating  $\geq 2\%$  mutation from the germ line sequence. Somatic hypermutation is more frequent in CLL cells than in normal circulating CD5+ or CD5- B cells.<sup>29</sup> Finally, gene expression profiling indicates that CLL cells are most closely related to memory B cells.<sup>26</sup>

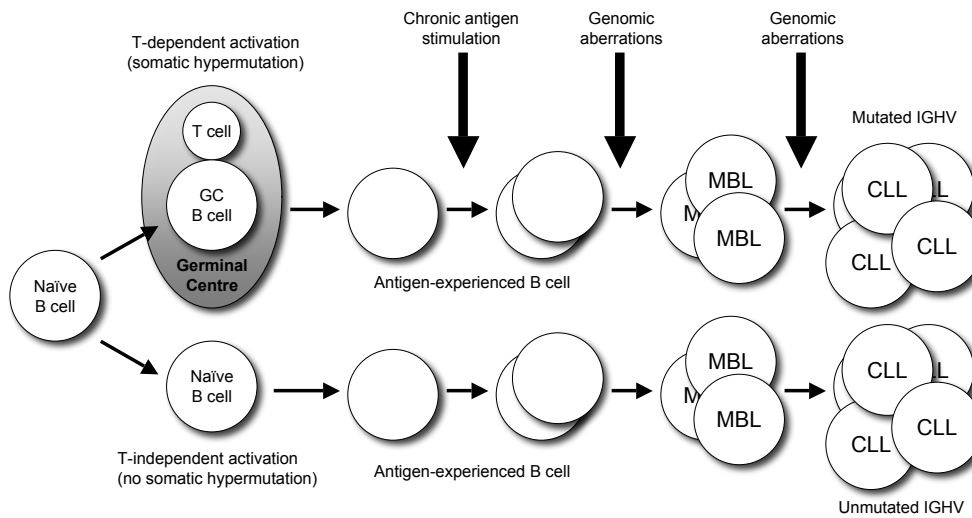
How, then, can the characteristic aberrant CD5 expression of CLL B cells be reconciled with a memory B cell origin? The expression of CD5 on B cells can be induced by chronic antigen stimulation: normal human B cells express CD5 *in vitro* after stimulation with phorbol 12-myristate 13-acetate (PMA),<sup>25</sup> after cross-linking of sIgM,<sup>35</sup> or following chronic stimulation by autoantigens.<sup>36</sup>

Other features of CLL cells also suggest that they are chronically antigen-stimulated *in vivo*. Leukaemic cells directly isolated from patients with CLL express activation markers such as CD23, CD25, CD69 and CD71.<sup>37</sup> CLL cells express low levels of sIgM and the IgM-associated heterodimer, CD79a/CD79b,<sup>37</sup> consistent with sIgM downregulation secondary to antigen exposure, and CLL cells spontaneously re-upregulate sIgM upon *in vitro* culture.<sup>38</sup>

CD5 itself may play a major role in generation of the CLL cell phenotype: CD5 is phosphorylated upon engagement of the B cell receptor (BCR), leading to upregulation of IL-10, transforming growth factor beta (TGF- $\beta$ ) and surface activation markers including CD54, and down-regulation of co-stimulatory antigens such as CD80 and CD86,<sup>39</sup> all phenotypic features of CLL.

Together these lines of evidence suggest that the leukaemic clone in CLL is an antigen-experienced memory B lymphocyte, which upregulates CD5 and CD23 due to chronic

Figure 1.1 Model of CLL pathogenesis, adapted from Klein and Dalla-Favera<sup>48</sup>



antigen stimulation and due to microenvironmental factors. As will be discussed, cases of CLL with IgVH genes that have not undergone somatic hypermutation tend to produce polyspecific autoantibodies, suggesting that the cell of origin in ‘unmutated’ CLL is a B cell that has undergone T cell-independent activation. In contrast, cases with mutated IgVH genes tend to produce antibodies of narrower specificity, and in some cases are class switched, implying that their cell of origin has been activated in a T cell-dependent manner.<sup>40</sup>

### 1.1.3.2 Nature of the antigen

If the leukaemic cells are antigen-experienced, what is the antigen? It has long been recognised that around half of CLL cases produce autoantibodies, which are frequently polyspecific.<sup>41,42</sup> This could be considered consistent with the CD5 positivity of CLL, as CD5+ B cells are often considered characteristic producers of ‘natural antibodies’: In the mouse, CD5+ B cells are termed B-1a cells and produce low affinity, polyreactive IgM natural antibodies which may be directed against microorganisms or against autoantigens.<sup>43</sup>

Antibodies from cases of CLL with both unmutated and mutated IgVH genes can be autoreactive. Antibodies cloned from cases of unmutated CLL are frequently polyreactive, whereas those cloned from cases of mutated CLL exhibit more restricted reactivity. However, when nonpolyreactive antibodies cloned from mutated CLL cases were reverted to their germline sequence, they became polyreactive,<sup>44</sup> suggesting that for both mutated and unmutated CLL forms, the cell of origin is a self-reactive B cell.

Specific antigens identified as targets of CLL cell BCRs include cytoskeletal proteins such as non-muscle myosin heavy chain IIa<sup>45</sup> and vimentin,<sup>46</sup> *Streptococcus pneumoniae* polysaccharides,<sup>46</sup> and oxidised lipids.<sup>46,47</sup>

A schematic model of the pathogenesis of CLL is given in figure 1.1.<sup>48</sup> In this model, naïve

B cells are stimulated by antigen, and depending on the nature of the antigen, undergo activation in a germinal centre with CD4+ T cell help (T-dependent activation), which results in somatic hypermutation and affinity maturation, or outside of the germinal centre without T cell help (T-independent activation), in which case there is no somatic hypermutation. Because the BCR is directed against a common antigen (autoantigen, oxidised lipid or common bacterial antigen), these B cells are subjected to chronic antigenic stimulation. Genetic changes, such as 13q deletion take place and drive clonal proliferation of the B cell, which may result in monoclonal B cell lymphocytosis or early CLL. Eventually, the acquisition of further genetic changes, such as TP53 mutation/deletion or ATM mutation/deletion may result in transformation and the clinical phenotype of CLL.

### 1.1.3.3 CLL kinetics and apoptosis

Anti-apoptotic proteins are markedly overexpressed in CLL, including Bcl-2<sup>49</sup> and the caspase inhibitor, survivin.<sup>50</sup> This may appear difficult to reconcile with the high spontaneous apoptosis rates observed when CLL cells are cultured *in vitro*. However, the spontaneous apoptosis of CLL cells can be inhibited by co-culture with stromal cells,<sup>51,52</sup> IL-4,<sup>53</sup> or by cross-linking of surface IgM.<sup>54</sup> Thus, factors in the tumour microenvironment contribute to a resistance to apoptosis in CLL cells *in vivo*.

Despite *in vivo* resistance to apoptosis and typically slow clinical progression, CLL cannot be considered a static condition: CLL cells undergo measureable degrees of 'birth' and 'death', as determined by *in vivo* incorporation of deuterated water into DNA. In one study, birth rates ranged from 0.1 – 1.0% of circulating CLL cells daily. Death rates were in a similar range, the difference between birth and death rates correlating with changes in the lymphocyte count.<sup>55</sup>

A relatively small proportion of circulating CLL cells actively proliferate, around 99.5% of the circulating population being in the G0/G1 phase of the cell cycle.<sup>56</sup> It has been proposed that the majority of CLL cell proliferation takes place within pseudofollicles in secondary lymphoid tissue,<sup>57</sup> where CLL cells interact with activated CD4+ T cells and upregulate CD38.<sup>58</sup> Consistent with this hypothesis, the majority of the dividing circulating CLL cells are CD38+, perhaps representing a population that has recently emerged from lymphoid tissue.<sup>59</sup>

Together, the above findings give rise to the prevailing view that CLL is an active process involving birth and death of a substantial number of leukaemic cells daily, and that leukaemic cells require appropriate environmental signals in order to survive and proliferate.<sup>60</sup> There is therefore considerable interest in developing therapeutic strategies that deprive CLL cells of their supporting microenvironmental stimuli.

### 1.1.3.4 Clonal evolution

Clonal evolution of CLL after diagnosis has been observed: In longitudinal series, a quarter of patients acquire a new cytogenetic abnormality over five years. 13q- is most commonly acquired, although acquisition of trisomy 12, 17p- and 11q- have also been observed.<sup>61,62</sup> Clonal evolution can also be observed in individual patients at a single time point: in one

study, three out of four patients exhibited differential copy number aberrations between the circulating CD38+ and CD38- CLL cell subsets, one of which was at the TP53 locus, and another of which was at the ATM locus.<sup>63</sup>

Clinical progression of the malignant clone is also well recognised in CLL: by 10 years from diagnosis, around 15% of patients will experience transformation to diffuse large B cell lymphoma (DLBCL), a phenomenon known as Richter's transformation. The risk of Richter's transformation is higher in patients without the good-prognosis 13q- deletion.<sup>64</sup> In approximately 80% of cases of Richter's transformation, DLBCL arises from the CLL clone, as determined by IgVH analysis. The remaining cases represent a new B cell clone. Richter's transformation is frequently associated with acquisition of a TP53 mutation or 17p-.

### 1.1.3.5 Immune dysfunction

Immune dysfunction has long been recognised as a feature of CLL,<sup>65</sup> and includes predisposition to autoimmune cytopenias and to infections.

Autoimmunity is a common feature of CLL,<sup>66</sup> and is usually directed against cells of myeloid origin. Autoimmune haemolytic anaemia (AIHA) is seen in 10 – 25% of patients during the course of their disease and autoimmune thrombocytopenia in 2%. Autoimmune neutropenia and pure red cell aplasia are less frequently seen. Despite the potential of CLL cells to produce autoreactive monoclonal IgM antibodies, the autoimmune cytopenias observed in CLL are usually due to polyclonal IgG antibodies produced by non-clonal B cells.<sup>67</sup>

In keeping with the notion that autoimmune cytopenias are not directly mediated by the leukaemic clone, successful treatment of CLL with radiotherapy<sup>68</sup> or with chemotherapy such as alkylating agents or fludarabine can precipitate rather than ameliorate overt AIHA.<sup>69</sup> Interestingly, chemotherapy with a combination of fludarabine and the alkylating agent, cyclophosphamide, results in a lower rate of AIHA than that seen with fludarabine therapy alone.<sup>69</sup>

One intriguing explanation for the phenomenon of autoimmune cytopenias in CLL is the possibility that leukaemic cells themselves are able to present certain autoantigens to CD4+ T cells. Although CLL cells are poor antigen presenters *in vitro*, they are numerous, and CLL cells from patients with anti-Rh related AIHA have been shown to present Rh autoantigens to autologous T helper cells.<sup>70</sup>

Numerous specific defects of immunological function have been described in CLL, affecting innate immunity, humoral immunity, antigen presenting cell function, T cell function, and levels of circulating cytokines levels. Patients with CLL are particularly prone to bacterial infections including pneumonia, septicaemia, cellulitis and urinary tract infections.<sup>71</sup>

**Innate immunity** Dysfunction of innate immunity has been reported in CLL. Despite increased numbers of circulating NK cells, their capacity to lyse target cells *in vitro* is reduced.<sup>72,73</sup> In some patients with CLL, defects of neutrophil migration, chemotaxis and

oxidative burst generation are observed, and correlate with a history of recurrent infections.<sup>74</sup> Reduced serum levels of complement proteins have been described in patients with CLL, and are lower in patients with more advanced clinical stages.<sup>75</sup>

**Humoral immunity** Hypogammaglobulinaemia is a common clinical feature of CLL, and is associated with an increased risk of bacterial infections in patients with CLL.<sup>65,76</sup> T cells activated *in vitro* in the presence of CLL cells upregulate CD30, exhibit reduced CD40 ligand expression and impair immunoglobulin class switching in non-malignant B cells.<sup>77</sup>

**Antigen Presenting Cells** The numbers of myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) are reduced as a proportion of circulating nucleated cells in patients with CLL,<sup>78</sup> as would be expected due to the large numbers of circulating leukaemic cells. Dysfunction of mDCs has also been described in CLL: One study suggested that CLL DCs exhibit normal function but possess an abnormal cytokine profile, with increased IL-10 and reduced IFN- $\gamma$  production.<sup>79</sup> Another study demonstrated reduced expression of the co-stimulatory molecule CD80, reduced stimulatory ability in allogeneic mixed lymphocyte reactions, and reduced IL-12 secretion by mDCs from patients with CLL.<sup>80</sup> Soluble CLL cell factors emulated these defects in DCs generated *in vitro* from healthy donors, suggesting that soluble factors from the CLL cells themselves are responsible for DC dysfunction.<sup>80</sup> In keeping with this observation, functional monocyte-derived dendritic cells (moDCs) can be generated from patients provided there is no contamination with CLL cells.<sup>81,82</sup>

**T cells** Patients with CLL have markedly increased numbers of T cells.<sup>83,84</sup> This T cell expansion has been attributed to interactions between B-cell activation factor belonging to the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) expressed on the malignant B cells, and transmembrane activator, calcium-modulator, and cyclophilin ligand interactor (TACI) expressed on T cells.<sup>85</sup>

T cell expansion may be a critical component in the pathogenesis of CLL: a xenograft model suggests that T cells are necessary for the maintenance of the leukaemic B cell population.<sup>86</sup> In light of this, it is interesting to note that two of the most effective treatments for CLL, fludarabine and alemtuzumab, both cause profound and prolonged depletion of T cells.

Despite their increased overall number, numerous abnormalities and functional defects of T cells have been described in patients with CLL. These include:

- Numeric abnormalities, such as inversion of the CD4/CD8 ratio, with CD8+ T cells being relatively increased compared to the CD4+ subset,<sup>83,84</sup> and oligoclonal T cell expansions within both the CD4+<sup>87</sup> and the CD8+<sup>88</sup> subsets of T cells. In one instance, a large clonal expansion of CD8+ T cells from a patient with CLL had specificity for the leukemic clone.<sup>89</sup>
- Immunophenotypic abnormalities, such as reduced T cell expression of CD40 ligand<sup>90</sup> (the supernatant from activated CLL cells inhibits activation-induced upreg-

ulation of CD40 ligand on healthy donor T cells<sup>91</sup>). Reduced expression of CD28 and the  $\zeta$  chain of CD3 on T cells is seen in patients with CLL, and is more marked in advanced than in early clinical stages.<sup>92</sup> Increased T cell Cytotoxic T Lymphocyte Antigen-4 (CTLA-4, or CD152) expression has been observed on T cells from patients with CLL. CTLA-4 binds to the co-stimulatory molecules CD80 and CD86, inhibiting T cell activation, and *in vitro*, CTLA-4 blockade significantly enhances T cell proliferative responses to autologous, CD40-activated CLL cells.<sup>93</sup>

- Functional abnormalities, such as impaired *in vitro* T cell expansion in response to mitogenic stimuli,<sup>94</sup> and increased T cell IL-4 production.<sup>95</sup> The IL-4 receptor is expressed by CLL cells, and there is evidence that IL-4 may protect CLL cells from apoptosis.<sup>96</sup> Defective immunological synapse formation between T cells and antigen-presenting cells (APCs) has been described in patients with CLL. This appears to be an effect of the malignant clone, as healthy donor T cells which have previously been cultured with CLL cells fail to generate normal immunological synapses, and effect which is dependent upon cell:cell contact.<sup>97</sup>
- An increased frequency of circulating regulatory T cell ( $T_{reg}$ ) cells is seen in early stage CLL, higher  $T_{reg}$  numbers being associated with a shorter time to treatment.<sup>98</sup> Higher frequencies of circulating  $T_{reg}$ s are associated with reduced CD8+ IFN- $\gamma$  enzyme-linked immunosorbent spots (ELISpots) in response to CLL tumour-associated antigens (TAAs).<sup>99</sup> A possible explanation for the increased  $T_{reg}$  numbers is that CLL cells express high levels of CD200, and ligation of the CD200 receptor on APCs leads to enhanced induction of Tregs.<sup>100</sup>
- The gene expression profile of CD4+ and CD8+ T cells is abnormal in patients with CLL. CD4+ T cells from CLL patients show changes in the JNK and p38 MAPK pathways, which are involved in regulating Th1 and Th2 differentiation. CD8+ T cells from CLL patients exhibit changes in the expression of genes relating to the cytoskeleton and intracellular trafficking, potentially accounting for reduced cytotoxicity. CLL cells are able to induce similar gene expression changes in healthy donor T cells, in a manner which requires cell-cell contact.<sup>101</sup>

**Cytokines and other soluble factors** Abnormalities of plasma and PBMC culture supernatant cytokine levels have been observed in patients with CLL. These include higher plasma TGF- $\beta$  levels in patients with CLL than in healthy controls<sup>102</sup>—CLL cells secrete TGF- $\beta$  upon *in vitro* culture, and TGF- $\beta$  can inhibit DC migration and T cell stimulatory activity.<sup>103</sup>

Patients with CLL have raised serum IL-6 and IL-10 levels, higher circulating levels of these cytokines being associated with an inferior prognosis.<sup>104</sup> IL-6 from CLL cells may result in blunting of T cell activation, and a Th2 skewing of T cell responses.<sup>91</sup> IL-10 inhibits the antigen-presenting capacity of monocytes to abrogate antigen-specific T cell proliferation.<sup>105</sup>

The leukaemic cells in CLL express CD25 (the IL-2 receptor alpha), and may have the capacity to both absorb IL-2 directly,<sup>106</sup> and to secrete soluble CD25<sup>107</sup> which acts as a decoy receptor for IL-2, potentially depriving T cells of an activating and proliferative signal. CLL cells can also release soluble CD40, CD80 and CD83. The soluble forms of these

APC co-stimulatory factors can bind to their ligands on T cells and mediate immunosuppression.<sup>108–110</sup>

### 1.1.3.6 Murine models of CLL

A number of murine models for CLL have been developed, which as well as enabling *in vivo* research, provide some insights into the pathogenic mechanisms underlying CLL. Selected murine models are summarised in table 1.3, and some discussed in more detail.

The New Zealand Black (NZB) mouse spontaneously develops an age-associated monoclonal expansion of autoreactive CD5+ B-1 cells which morphologically and immunophenotypically resembles CLL.<sup>111,112</sup> This predisposition to a CLL-like condition has been attributed to a germline point mutation close to the pre-mir-16-1 micro-RNA gene in NZB mice.<sup>113</sup> Genomic alterations affecting miR-16 expression are present in the around half of CLL patients (those with the 13q deletion). Consistent with a role for miR-16 in the pathogenesis of CLL, mice harbouring a deletion of the murine homologue of the 13q14-minimal deleted region observed in many humans with CLL (MDR mice) also develop an indolent monoclonal B cell disorder resembling CLL.<sup>114</sup>

$E\mu$ -miR-29 transgenic mice, in which overexpression of the micro-RNA miR-29 is governed by an IgVH promoter, spontaneously develop an expansion of CD5+ B cells, which is clonal in a proportion of cases. 20% of  $E\mu$ -miR-29 mice develop a fatal leukaemia at two years of age. Consistent with a role for this micro-RNA gene, miR-29 is overexpressed in patients with indolent, but not with aggressive CLL. The reporters of this model suggest that miR-29 overexpression causes B cell expansion, but that secondary events are necessary for the phenotype of aggressive CLL.<sup>115</sup>

The TCL1 oncogene is expressed in CLL, splenic lymphoma with villous lymphocytes (SLVL) and in T-cell prolymphocytic leukaemia, but not in normal B cells.  $E\mu$ -TCL1 transgenic mice, which overexpress TCL1, accumulate polyclonal CD5+ B cells from around 5 months of age, which then become oligo- or monoclonal. Evidence of disease with leukocytosis, lymphadenopathy and splenomegaly appears at 13 – 18 months.<sup>116,117</sup> Morphologically, the leukaemia in TCL1 transgenic mice resembles prolymphocytic CLL. Together with the finding that among patients with CLL, the highest levels of TCL1 expression are observed in cases with unmutated IgVH genes,<sup>118</sup> has led to the conclusion that TCL1 transgenic mice are a model of aggressive, rather than indolent, CLL.<sup>117</sup>

Insertion of the simian virus 40 T (SV40 T) oncogene into the immunoglobulin heavy chain (IgH) results in mice which develop a monoclonal expansion of CD5+ B cells. In this model, IgVH usage was biased, and both unmutated and mutated IgVH genes were observed in difference mice, resembling the spectrum of IgVH features in patients with CLL. The leukaemia incidence was increased further by concomitant deletion of p53.<sup>119</sup>

Finally, xenograft models of CLL have been developed. In one example,  $Rag2^{-/-} \gamma c^{-/-}$  mice, which lack B cells, T cells and natural killer (NK) cells were injected with the stable human CLL line, MEC1. The transferred MEC1 CLL cells localise to lymph nodes, spleen and peripheral blood, as well as lung, kidney and the peritoneal cavity.<sup>120</sup>



It is notable that three of the murine models of CLL listed in table 1.3 (the miR-29 transgenic, the MDR knockout and the New Zealand Black mouse) rely solely or largely on enhanced or reduced expression of micro RNAs. In all three of these models, penetrance is incomplete, the lymphoproliferative disorder may be described as indolent and the leukaemia passes through an initial polyclonal expansion before becoming clonal. These models may be suitable for studies of CLL pathogenesis or prevention.

Models that utilise oncogenes (SV40 T, TCL1) tend to exhibit higher penetrance, an earlier age of onset of disease and are more clinically aggressive. These models may lend themselves better to the investigation of therapeutic strategies for CLL.

### 1.1.4 Prognostic factors

#### 1.1.4.1 Clinical staging systems

Useful prognostic information can be derived from readily-available clinical and laboratory data. Advanced patient age, male sex, higher absolute lymphocyte count, greater extent of lymphadenopathy and raised serum beta 2 microglobulin ( $\beta_2M$ ) levels are all associated with inferior prognosis on multivariate analysis.<sup>124</sup> The clinically-observed lymphocyte doubling time (LDT) is a strong predictor for overall survival, a doubling time of  $\leq 12$  months being associated with a median survival of three years versus  $>8$  years for a LDT of  $>12$  months.<sup>125</sup>

Two clinical staging systems are in routine use for CLL: the Rai (see table 1.4) and Binet (see table 1.5) systems. Current clinical guidelines recommend the use of both systems to give a combined score.<sup>4</sup>

A prognostic nomogram incorporating age, sex, lymphocyte count,  $\beta_2M$ , extend of lymphadenopathy and clinical stage has been developed, and is highly predictive of overall survival duration.<sup>124</sup>

#### 1.1.4.2 Cytogenetics

Approximately 80% of cases of CLL harbour a genomic abnormality detectable by interphase fluorescent in-situ hybridisation (FISH). The most common abnormalities identified by FISH, together with their frequencies and prognostic implications are listed in table 1.6.

In addition to these cytogenetic abnormalities, a proportion of patients harbour mutations of the TP53 or ATM genes, as determined by gene sequencing, without deletion of 17p or 11q, respectively. Patients with these cytogenetically-silent defects of TP53 or ATM have similar prognoses to those with 17p or 11q deletion, respectively.

Although the two most unfavourable genomic abnormalities (11q deletion and 17p deletion) are found more frequently in unmutated CLL cases, they remain independent prognostic factors on multivariate analysis, even after taking IgVH mutation status into account.<sup>130</sup>

Table 1.3 Selected murine models of CLL Adapted from <sup>121</sup>

Background strain(s)	Transgene	Earliest disease manifestations	Special characteristics	Reference(s)
CBALB/c x FVB/N	TRAFDN/Bcl2	Splenomegaly and lymphadenopathy by 6m	Clonal; 20% survival at 14m	122
FVB/N	mir-29	Splenomegaly from 12m	Clonal in 60% of cases; 20% develop leukaemia by 24m	115
cC56BL/6	SV40 T	Splenomegaly and lymphadenopathy from 4 – 6m	Clonal; biased igVH usage; unmutated and mutated IgVH genes seen; median survival 6m	119
FVB/N	Deletion of mir-15a/16-1 and DLEU2	Peripheral blood from 15 – 18m	Clonal; 42% penetrance	114
CB6C3	TCL1	Peripheral blood from 6 months; splenomegaly from 8m	Clonal; prolymphocytic morphology; model of aggressive/unmutated CLL	116
NZB	None (point mutation near mir-16)	Peripheral blood and spleen from 4 – 6m	Clonal; associated with autoimmune haemolytic anaemia	111,112
cC57/Bl6	TNFSF13 (APRII)	Mesenteric lymph nodes and Peyer's patches	Clonality not reported	123
Rag2 <sup>-/-</sup> γc <sup>-/-</sup>	None (xenograft model)	Spleen, liver, peripheral blood, kidney, liver, peritoneal cavity	Death at day 40 post-transfer of human MEC1 CLLs	120

Table 1.4 Rai clinical staging system<sup>126</sup>

Stage	Criteria	5 year overall survival (%)
0	Absolute lymphocytosis > 15 x10 <sup>9</sup> /L	98
I	as stage 0 + lymphadenopathy	95
II	as stage 0 + hepato- or splenomegaly +/- lymphadenopathy	88
III	as stage 0 + anaemia (Hb <110 g/L) +/- organomegaly +/- lymphadenopathy	< 5
IV	as stage 0 + thrombocytopenia (< 100 x10 <sup>9</sup> /L) +/- organomegaly +/- lymphadenopathy	0

Table 1.5 Binet clinical staging system<sup>127</sup>

Stage	Criteria	Median survival (months)
A	0, 1 or 2 lymph node areas *	> 120
B	3, 4 or 5 lymph node areas *	61
C	Any areas with Hb < 100 g/L or platelets < 100 x10 <sup>9</sup> /L	32

\* one lymph node area = lymph nodes >1cm in neck, axilla, groin or spleen or liver enlargement

Table 1.6 Principal cytogenetic changes in CLL Adapted from Zenz et al. <sup>128</sup>

Cytogenetic finding	Frequency	Prognostic implications	Median overall survival (months)	Candidate gene(s) affected
13q deletion	55%	Better prognosis. More frequent in mutated IgVH CLL	133	miR-15a and miR-16-1 micro-RNAs <sup>129</sup>
Trisomy 12	10%	Standard prognosis	111	Unknown
11q deletion	15%	Inferior prognosis. Rapid progression & bulky adenopathy. More frequent in unmutated IgVH CLL	79	ATM
17p deletion	7%	Poorest prognosis. More frequent in unmutated IgVH CLL	32	TP53

#### 1.1.4.3 IgVH mutation status

Somatic hypermutation of the BCR is a normal part of the process of affinity maturation of antigen-stimulated B cells. Analysis of the clonal IgVH sequence from patients with CLL indicates that in around half of cases, somatic hypermutation has taken place (defined as  $\geq 2\%$  deviation from the germ line sequence). <sup>131</sup> At diagnosis, patients with CLL harbouring a mutated IgVH have a superior prognosis, with a median survival 310 months compared to 119 months in those with an unmutated IgVH. <sup>132</sup>

#### 1.1.4.4 Expression of ZAP-70

Determining IgVH mutation status requires access to high-throughput DNA sequencing, facilities for which are not routinely available in many hospital laboratories. Intracellular levels of the zeta chain-associated protein 70 (ZAP-70) within CLL cells correlates with IgVH mutational status: patients with raised intracellular ZAP-70 are more likely to have an unmutated IgVH gene and to have a poorer prognosis. <sup>133</sup> ZAP-70 can be readily measured by intracellular flow cytometry in most haematology laboratories. However, the widespread adoption of ZAP-70 as a prognostic marker has been limited by difficulties in standardisation of this quantitative flow cytometric assay. <sup>134</sup> Some of these difficulties may be overcome by using RT-PCR to determine the level of ZAP-70 transcripts within CLL cells. <sup>135</sup>

Table 1.7 Indications for treatment of CLL<sup>4</sup>

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<b>At least ONE of:</b>
Progressive marrow failure
Massive or symptomatic splenomegaly
Massive lymphadenopathy (at least 10 cm in longest diameter), or progressive or symptomatic lymphadenopathy
Progressive lymphocytosis with an increase of more than 50% over a two month period, or a lymphocyte doubling time of <6 months*
Autoimmune anaemia or thrombocytopenia that is poorly responsive to corticosteroids or other standard therapy
Constitutional symptoms (unintended weight loss of $\geq 10\%$ within previous 6 months; significant fatigue, fevers $> 38^{\circ}\text{C}$ for $\geq 2$ weeks without evidence of infection; night sweats for $> 1$ month without evidence of infection

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\* It is recommended that in patients with an initial lymphocyte count of  $< 30 \times 10^9/\text{L}$ , LDT is not used as the sole criterion for initiation of treatment. Other factors contributing to lymphadenopathy or lymphocytosis (eg infection) should be excluded.

### 1.1.4.5 CD38 expression

CD38 is frequently expressed on CLL cells, and correlates with inferior prognosis independently of IgVH mutational status.<sup>136</sup> Unlike IgVH mutation status, CD38 expression is dynamic, changing with time and depending upon contact with CD4+ T cells.<sup>58</sup>

### 1.1.4.6 Others

Many additional prognostic markers for CLL have been proposed, but are not in widespread clinical use. These include serum biomarkers, proliferative indices, telomere length, immunophenotypic characteristics, gene expression profiles, epigenetic characteristics and microRNA expression, and have been reviewed elsewhere.<sup>137</sup>

## 1.1.5 Treatment

### 1.1.5.1 Treatment indications

Randomised controlled trials have demonstrated that treatment of early, asymptomatic CLL with alkylating agents does not prolong survival,<sup>138</sup> leading to recommendations that evidence of CLL progression or symptoms be established before treatment is initiated.<sup>4</sup> Established indications for treatment are listed in table 1.7.

### 1.1.5.2 Choice of treatment

Chemotherapy is the mainstay of therapy for CLL. Combined chemotherapy using the purine analogue fludarabine with the alkylating agent cyclophosphamide leads to better response and progression free survival rates than either the alkylating agent chlorambucil or fludarabine alone.<sup>139</sup> Although CD20 expression on CLL cells is weak, the humanised anti-CD20 monoclonal antibody improves outcome further: The German CLL8 study group reported a benefit in overall survival for patients with CLL with the addition of rituximab to fludarabine and cyclophosphamide.<sup>140</sup> This establishes fludarabine, cyclophosphamide and rituximab (FCR) as the current standard of care for first-line treatment of CLL.

Although prospective randomised trials of ‘risk-adapted’ therapy for CLL are lacking, laboratory and clinical data indicate that patients with deletion of 17p or with mutations of p53 are resistant to standard FCR chemoimmunotherapy.<sup>140</sup> Some authors advocate alternative first-line treatment in these high-risk individuals, typically using the anti-CD52 antibody alemtuzumab with or without high-dose corticosteroids, followed by early allogeneic stem cell transplantation (allo-SCT) for eligible patients.<sup>5</sup>

### 1.1.5.3 Monoclonal antibodies

Most chemotherapeutic agents induce apoptosis of target cells via a pathway involving the p53 and ATM proteins. Monoclonal antibodies have the potential to induce CLL cell death in a manner independent of this pathway, so may have a particular role in the treatment of CLL with 17p deletions (affecting the p53 gene), or with 11q deletions (affecting ATM). Indeed, clinical experience suggests that the application of either rituximab (anti-CD20) or alemtuzumab (anti-CD52) can overcome the adverse prognostic influence of the 11q deletion in CLL.<sup>141</sup> The use of rituximab in the first-line treatment of CLL is now well established.

Alemtuzumab, a humanised anti-CD52 monoclonal antibody, is licensed for relapsed and refractory CLL, leading to partial response rates of around 50% in this setting.<sup>142,143</sup> As well as targeting CLL cells, alemtuzumab leads to a profound T cell depletion, requiring routine antimicrobial prophylaxis. In addition, high rates of cytomegalovirus (CMV) reactivation demand routine CMV monitoring during alemtuzumab therapy and prompt treatment with appropriate antiviral agents.<sup>144,145</sup> The rate of infectious complications may be lower when low-dose subcutaneous alemtuzumab is used.<sup>146</sup>

Alternative monoclonal antibodies are in clinical trials. Ofatumumab is an anti-CD20 antibody that binds to different CD20 epitopes to rituximab, and has an enhanced ability to engage complement-mediated lysis of CLL cells *in vitro*.<sup>147</sup> Ofatumumab appears to be effective in combination with fludarabine and cyclophosphamide in treatment-naïve patients,<sup>148</sup> and has activity as monotherapy in fludarabine-refractory CLL.<sup>149</sup> An anti-CD23 monoclonal antibody, lumiliximab, has modest single-agent activity; the results of phase 3 clinical trials in combination with FCR are awaited.<sup>150</sup>

### 1.1.5.4 Corticosteroids

Like monoclonal antibodies, corticosteroids have the capacity to induce CLL cell apoptosis independently of the p53 and ATM proteins, so have a particular role in the management of chemorefractory CLL.<sup>151</sup> The combination of high dose methylprednisolone with rituximab or alemtuzumab results in high response rates in patients refractory to fludarabine.<sup>152</sup>

### 1.1.5.5 Haematopoietic stem cell transplantation

Haematopoietic stem cell transplantation entails myelosuppressive and/or immunosuppressive chemo- or radiotherapy ('conditioning'), followed by an infusion of autologous or tissue type-matched allogeneic bone marrow or CD34+ rich peripheral blood mononuclear cells, which reconstitutes the host's haematopoietic and immune system compartments.

**Autologous** Autologous stem cell transplantation (auto-SCT) can be performed in CLL with low treatment-related mortality (<10%). Response rates are high, although no randomised trials have been performed.<sup>153</sup> However, a survival plateau is not observed after auto-SCT, suggesting that it is not a curative procedure in CLL. Moreover, a high incidence of myelodysplasia after auto-SCT for CLL has been observed.<sup>154</sup> Together, these factors limit the utility of auto-SCT for CLL, and indications for auto-SCT have not been clearly established in this disease.<sup>153</sup>

**Allogeneic** Unlike auto-SCT, allogeneic stem cell transplantation (allo-SCT) offers the possibility of a graft versus leukaemia (GVL) effect by the grafted donor immune system, and may result in long-term cure. Levels of minimal residual disease (MRD), as assessed by polymerase chain reaction (PCR) for the disease-specific rearranged IgH, are lower after allo-SCT than after auto-SCT. Moreover, the decline in MRD levels occurs later after allo-SCT than after auto-SCT, suggesting that this is primarily an immunological effect rather than an effect of the conditioning regimen.<sup>155</sup>

Registry data and clinical experience provide further evidence for a GVL effect in CLL: the presence of chronic graft versus host disease (GVHD) is associated with reduced risk of relapse; T cell depletion of the graft results in higher relapse risks; and treatment of relapse with infusion of additional lymphocytes from the donor (donor lymphocyte infusion, or DLI) can result in clinical responses.<sup>154</sup> Consistent with these clinical observations of a GVL effect, cytotoxic T cell lines with specificity for minor histocompatibility antigens and TAAs can be identified after allo-SCT for CLL, their presence correlating with clinical response.<sup>156</sup>

Allo-SCT carries substantial morbidity and mortality, due primarily to toxicities of the conditioning regimen, infection, and donor lymphocyte immunity directed against normal host tissues (GVHD). Older age and the presence of co-morbidities are strong predictive factors for mortality following allogeneic stem cell transplantation. Therefore, because

of the high median age of patients with CLL, the role of myeloablative allo-SCT has been limited to all but the very youngest patients with refractory or aggressive disease.

The reduced treatment-related morbidity and mortality associated with less intensive conditioning regimens, which do not irreversibly ablate host haematopoiesis, have resulted in significant interest in reduced intensity conditioning allogeneic stem cell transplantation (RIC-allo) for CLL. A five year overall survival of 50%, and five-year disease-free survival of 39% has been reported for RIC-allo in fludarabine-refractory patients.<sup>157</sup>

The European Blood and Marrow Transplantation working group has drawn up consensus guidelines on the indications for allograft in CLL, concluding that allo-SCT is a reasonable option for younger patients who are fludarabine-refractory (primary resistance, or relapse within 12 – 24 months of treatment), and for patients with p53 abnormalities including 17p deletion.<sup>158</sup>

### 1.1.5.6 Other therapies

A number of other medical treatments are licensed for use in CLL, or are at advanced stages in clinical trials.

**Bendamustine** Bendamustine is an alkylating agent with a purine-like group, and has efficacy against CLL either alone or in combination with rituximab. At present, there is insufficient evidence to recommend bendamustine over FCR for the routine first-line treatment of CLL, but bendamustine-containing combinations have a useful role in refractory CLL, in older patients and in patients with renal impairment, in whom high response rates have been reported.<sup>159</sup>

**Lenalidomide** Lenalidomide, a derivative of thalidomide, is a member of the class of drugs called immunomodulatory drugs (IMiDs), and is in routine clinical use in the treatment of the mature B cell malignancy, myeloma. Lenalidomide can reverse the defective T cell immunological synapse formation observed in CLL.<sup>97</sup> Despite its value in myeloma, the use of lenalidomide in CLL is less well established, because of modest response rates as monotherapy in refractory disease,<sup>160</sup> and a phenomenon of ‘tumour flare’, which can be fatal.<sup>161</sup>

**Cyclin-dependent kinase inhibitors** Cyclin-dependent kinases (CDKs) regulate cell cycle progression, and CDK inhibition can induce apoptosis of tumour cells. Flavopiridol, a CDK inhibitor, induces apoptosis of CLL cells *in vitro* independently of the p53 pathway,<sup>162</sup> and may therefore play a particular role in the treatment of *TP53* mutated (or 17p deleted) cases of CLL. Flavopiridol monotherapy results in modest response rates in refractory CLL,<sup>163</sup> including some durable responses in patients with bulky disease. The CDK inhibitor dinaciclib sensitizes primary CLL cells to vinblastine,<sup>164</sup> and is currently in clinical trials.



**BCL2 inhibitors** The antiapoptotic (pro-survival) protein Bcl-2 (encoded by *BCL2*) is over-expressed in CLL. Oblimersen is an antisense oligonucleotide directed against *BCL2* mRNA. In a phase II trial, addition of oblimersen to fludarabine and cyclophosphamide for the re-treatment of patients with CLL resulted in modestly improved complete response rates.<sup>165</sup> Activation of BH3-only proteins such as Bim, Bad and Noxa leads to the inactivation of pro-survival proteins such as Bcl-2. The BH3 mimetic ABT-737 shows *in vitro* activity against primary CLL cells, and *in vivo* activity against lymphomas and solid malignancies.<sup>166,167</sup> In a phase 1 clinical trial, the BH3 mimetic navitoclax (ABT-263) showed a 35% partial response rate in relapsed or refractory CLL, including responses among patients carrying the 17p deletion.<sup>168</sup>

**Inhibitors of kinases involved in B cell receptor signalling** As discussed in section 1.1.3.1, activation of the B cell receptor (BCR) contributes to the pathophysiology of CLL. Bruton's tyrosine kinase (Btk), mutation of which leads to Bruton's agammaglobulinaemia, is required for BCR signalling. The Btk inhibitor ibrutinib (PCI-32765) blocks BCR signalling in human B cells,<sup>169</sup> induces primary CLL cell apoptosis,<sup>170</sup> and has been associated with overall response rates of over 70% in patients with CLL, including those with adverse cytogenetic features.<sup>171</sup> The phosphatidylinositol-3-kinase (PI3K) inhibitor GS-1101 (formerly CAL-101) inhibits B cell receptor signalling in primary CLL cells, and shows promising activity in combination with ofatumumab, especially in patients with bulky lymphadenopathy.<sup>172,173</sup> Spleen tyrosine kinase (Syk) is essential for BCR signalling. In a phase I study, the Syk inhibitor fostamatinib resulted in a partial response in six of eleven treated patients with CLL.<sup>174</sup>

**T cell redirection** Redirection of T cells has resulted in promising clinical responses in CLL using either T cells transfected with chimeric antigen receptors specific for CD19 or a bispecific antibody directed against CD3 and CD19 (blinatumumab). These treatment strategies will be discussed in section 1.3.2.

### 1.1.5.7 Effect of CLL treatments on immune function

**Myeloid cells** Many of the treatments used for CLL cause a dose-dependent myelosuppression. Grade 3 or 4 febrile neutropenia occurs in 34% of patients treated with FCR chemotherapy.<sup>140</sup> In contrast to this, monocyte numbers are relatively preserved after FCR chemotherapy.<sup>175</sup>

Bendamustine has both purine analog and alkylator properties, and when given with rituximab was associated with grade 3 or 4 neutropenia in 37% of patients with relapsed indolent B cell lymphomas.<sup>176</sup>

Immunotherapy with alemtuzumab and corticosteroids is associated with grade 3 or 4 neutropenia in the majority of patients.<sup>177</sup> In a randomized trial comparing rituximab and alemtuzumab in combination with fludarabine and cyclophosphamide, rates of grade 3 and 4 neutropenia were significantly higher in the alemtuzumab arm.<sup>178</sup>

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The PI3K inhibitor GS-1101 has been associated with grade 3 or 4 neutropenia in 24% of patients, while neutropenia has been reported in < 10% of ibrutinib-treated patients<sup>174</sup>

Antigen presenting cells may also be affected by CLL cell treatment: circulating mDCs express CD52,<sup>179</sup> and alemtuzumab results in depletion of circulating DCs.<sup>180</sup>

**T cells** First-line chemoimmunotherapy for CLL with FCR results in a profound decline in circulating T cell numbers, including CD4+, CD8+ and  $\gamma\delta$ T cells. The decline in circulating T cells is prolonged, absolute numbers remaining low three years after chemoimmunotherapy.<sup>175</sup> Recent fludarabine treatment is associated with poor antibody responses to subsequent vaccination, suggesting that CD4+ T cell help to B cells is functionally impaired.<sup>181</sup> Numbers of circulating T<sub>reg</sub> cells are disproportionately reduced following fludarabine treatment, suggesting that T<sub>reg</sub> cells may be particularly sensitive to fludarabine.<sup>182</sup>

The effect of bendamustine on T cell subsets is not well-documented, but CMV reactivation following treatment has been reported.<sup>176,183</sup>

Like fludarabine, the anti-CD52 monoclonal antibody alemtuzumab is also associated with profound T cell depletion.<sup>184</sup> Infections are common after alemtuzumab, CMV reactivation occurring in 23% of CLL patients treated with alemtuzumab and corticosteroids.<sup>177</sup> When given in combination with fludarabine and cyclophosphamide, alemtuzumab results in lower CD4+ T cell counts than rituximab, and higher rates of infection.<sup>178</sup> The effect of alemtuzumab on T cells is prolonged, lasting at least three years, although CD8+ T cell recovery occurs earlier than CD4+ T cell recovery.<sup>185</sup>

In contrast to the prolonged T cell depletion that follows fludarabine and alemtuzumab-containing treatments, the treatment of CLL with lenalidomide is associated with a very transient reduction in T cell numbers, lasting only three weeks.<sup>186</sup> In patients with CLL treated with lenalidomide, greater T cell activation as determined by CD69 upregulation, and greater CLL cell activation as determined by CD80 upregulation, are both associated with more severe clinical manifestations of cytokine release syndrome.<sup>186</sup> This is consistent with the *in vitro* finding that lenalidomide leads to normalisation of immunological synapse formation between T cells and autologous CLL cells.<sup>97</sup>

**NK cells** NK cells decline after FCR chemotherapy, although are relatively preserved compared to T cells.<sup>175</sup> Compared with healthy donors, *in vitro* NK cell cytotoxic function in the presence of IL-2 is impaired in CLL patients before FCR chemotherapy and at the end of treatment, but normalises by 12 months after FCR chemotherapy.<sup>175</sup>

In contrast with the relative sparing of NK cell numbers and activity, alemtuzumab induces a marked and prolonged depletion of NK cells, which express CD52.<sup>184</sup>

**B cells** As would be expected, the anti-CD20 monoclonal antibody rituximab leads to a rapid depletion of B cells, both as monotherapy and in the context of FCR chemoimmunotherapy.<sup>175,187</sup> In contrast to T cell depletion, recovery of normal B cell numbers after

FCR chemotherapy is relatively rapid, B cell numbers normalising within 12 months of treatment.<sup>175</sup> Depletion of B cells with rituximab is associated with impaired antibody responses to subsequently-administered vaccinations in patients with B cell malignancies.<sup>181,188</sup>

The anti-CD20 monoclonal antibody ofatumumab also leads to rapid and profound B cell depletion.<sup>189</sup> B cells express CD52, and alemtuzumab induces a depletion of normal B cells lasting at least 9 months.<sup>184</sup>

The kinase inhibitors showing promising activity in CLL, such as the Btk inhibitor ibrutinib, might be expected to cause depletion of normal B cells, but preliminary reports of ibrutinib have not indicated normal B cell depletion or significant hypogammaglobulinaemia.<sup>174</sup> Published data on the effects of PI3K and other kinase inhibitors on normal B cells are lacking.

## 1.2 Invariant natural killer T cells

### 1.2.1 Antigen presentation by CD1 molecules

‘Conventional’ T cells express a T cell receptor (TCR) that recognises a specific peptide in the context of a specific major histocompatibility complex (MHC)—also known in humans as human leukocyte antigen (HLA). CD4<sup>+</sup> T cells recognise peptides presented by MHC Class II, and CD8<sup>+</sup> cells recognise peptides on MHC Class I. The recognition of specific peptides on specific MHC molecules by variably recombined TCRs lends enormous range to the adaptive immune response.

More recently, the existence of subsets of T cells that are not restricted by MHC has been demonstrated. Such T cells possess functional T cell receptors that recognise antigens in the context of nonclassical MHC molecules such including CD1, HLA-E,<sup>190</sup> and MR1.<sup>191</sup>

The CD1 molecules are conserved  $\beta_2$ M-associated MHC class I-like molecules that present lipids rather than peptides.<sup>192</sup> Unlike MHC, CD1 molecules are minimally polymorphic.<sup>193</sup> Indeed, CD1 molecules are so conserved that mouse CD1d molecules can present glycolipid antigens to human CD1d-restricted T cells and vice versa.

Hydrophobic pockets in CD1 molecules accommodate the fatty acid tails of various self- and microbial-derived glycolipids, lipopeptides and phospholipids. The polar heads of these lipid antigens emerge from the surface of the CD1 molecule, and are recognised by the TCR of CD1-restricted T cells.<sup>194</sup>

CD1 molecules have been divided into two groups on the basis of DNA sequence homology: Group 1 CD1 molecules include CD1a, CD1b, CD1c and CD1e; CD1d is a group 2 molecule. Group 1 CD1-restricted T cells recognise a number of mycobacterial cell wall components, and may be important in the generation of immunity to mycobacteria.<sup>195,196</sup> Group 2 CD1-restricted (i.e. CD1d-restricted) T cells recognise glyco- and phospholipids.

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Usage of CD1 genes varies between species: Humans possess and express all five CD1 genes; cattle express only the group 1 CD1 molecules CD1a, CD1b and CD1e with no CD1c or CD1d;<sup>197</sup> mice do not express any group 1 CD1 molecules, possessing only two copies of the CD1d gene. A transgenic mouse which expresses the human group 1 CD1 molecules has been developed, and may prove a useful tool for the analysis of these T cells.<sup>196</sup>

The tissue expression, known antigens and reported responding T cells of the various CD1 isoforms are summarised in table 1.8.

### 1.2.2 iNKT cells

The archetypal lipid-reactive T cell is the iNKT cell, which recognises glycolipids, such as the synthetic  $\alpha$ -GalCer, in the context of CD1d. Of all CD1-reactive T cells, iNKT cells have been studied in most detail for several reasons. In some mouse strains, iNKT cells constitute a very high proportion of circulating T cells (comprising up to 50% of liver T cells),<sup>208</sup> and are potent and rapid producers of cytokines. Potent and specific synthetic iNKT cell agonists are available, including  $\alpha$ -GalCer, and can be used for iNKT cell stimulation, or for iNKT cell detection. Finally, iNKT cells express a canonical ('invariant') TCR $\alpha$  chain, enabling their detection and isolation using specific anti-TCR antibodies.<sup>205</sup>

The presentation of peptide and glycolipid antigens to conventional CD4+ and CD8+ T cells and to iNKT cells is illustrated in figure 1.2.

#### 1.2.2.1 Nomenclature

The nomenclature of invariant natural killer T (iNKT) cells has evolved in a piecemeal fashion, leading to some inconsistencies. The origin of the term natural killer T (NKT) arose from their initial description in the C57BL/6 mouse strain, where a population of T cells expressing intermediate levels of TCR alongside the murine NK cell marker, NK1.1, were identified. These cells attracted interest, as they were noted to be potent producers of T helper 1 (Th1) and T helper 2 (Th2) cytokines, and to be restricted by CD1d rather than by MHC. Following recognition that the vast majority of these NKT cells use an invariant TCR $\alpha$  chain (V $\alpha$ 14J $\alpha$ 18 in mice), the term iNKT cells was used.<sup>209</sup> However, it should be emphasised that despite their expression of certain molecules classically associated with natural killer cells, iNKT cells are unequivocally thymic-derived  $\alpha\beta$  T cells.

In humans, the majority of circulating T cells that co-express NK markers such as CD16, CD56 or CD161, the human homologue of NK1.1, are not iNKT cells. Thus, in human literature, the term 'NKT cell' has been used both to refer to conventional MHC-restricted T cells which co-express NK cell markers, and to the much narrower population of iNKT cells, which express an invariant TCR- $\alpha$  chain (V $\alpha$ 24J $\alpha$ 18 in humans), and which are restricted by CD1d.

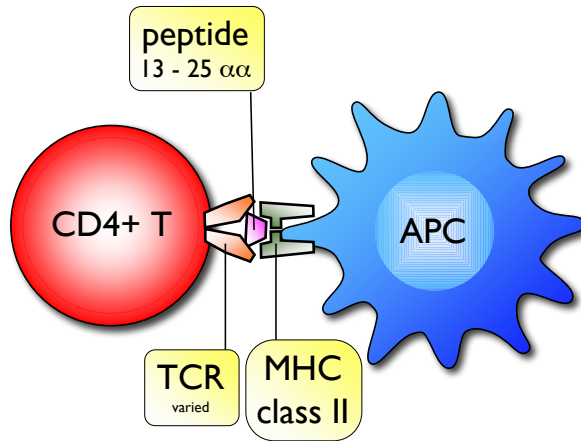
A further distinction has been made between type I and type II NKT cells: Type I NKT cells, also termed iNKT cells, possess an invariant TCR- $\alpha$  chain, exhibit CD1d restriction and recognise  $\alpha$ -GalCer. These iNKT cells are found in both mice and humans, although their frequency is substantially lower in humans than in certain mouse strains. iNKT cells

Table 1.8 CD1 isoforms: tissue expression, known antigens, and responding T cell populations

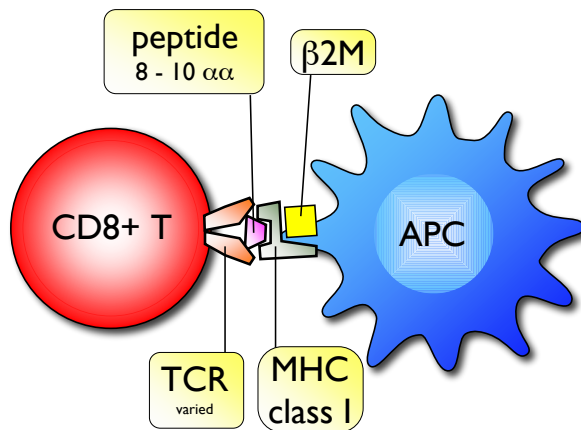
CD1 Isoform	Tissue expression	Known antigens	Responding T cell population(s)
CD1a	Langerhans cells, mDCs, Thymocytes	Self-lipids, <sup>198</sup> Lipopeptides <sup>199</sup>	$\alpha\beta$ T cells, including skin-homing Th22 cells, <sup>198</sup> duodenal $\gamma\delta$ T cells <sup>200</sup>
CD1b	DCs, thymocytes	Mycobacterial lipids and glycolipids (eg mycolic acids, lipoglycan lipoarabinomannan, phosphatidylinositol mannoside), self-lipids (eg ganglioside GM1)	$\alpha\beta$ T cells, <sup>201</sup> $\gamma\delta$ T cells <sup>202</sup>
CD1c	mDCs, Marginal zone B cells, Thymocytes	Phospholipids (including mycobacterial-derived)	$\alpha\beta$ T cells, <sup>203</sup> $\gamma\delta$ T cells <sup>200</sup>
CD1d	mDCs, B cells, epithelium, hepatocytes, vascular smooth muscle, haematopoietic cells, thymocytes	Glycolipids (e.g. $\alpha$ -GalCer), Phosphotidylinositols <sup>204</sup> Lysophospholipids, sulfatide Phospholipids	iNKT cells <sup>205</sup> (semi-invariant TCR) Type II NKT cells <sup>206</sup> (variable TCR usage) $\gamma\delta$ T cells <sup>200</sup>
CD1e	DCs (within Golgi and lysosomes)	Glycolipids which require intracellular processing e.g. phosphatidylinositol mannoside (PIM) <sub>4</sub>	None—involved in lipid processing <sup>207</sup>

Figure 1.2 Comparison of peptide and glycolipid presentation (a) CD4+ T cells recognise peptides on MHC Class II. (b) CD8+ T cells recognise peptides on MHC Class I. (c) iNKT cells recognise glycolipids on CD1d

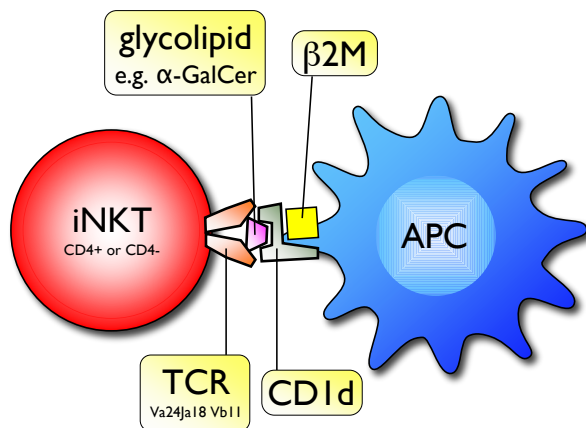
(a)



(b)



(c)



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## 1.2. Invariant natural killer T cells

typically constitute a median of 0.05% of T cells in the blood of healthy humans,<sup>210,211</sup> but 1% of T cells in BALB/C or C57 mice. iNKT cells are widely distributed within the lymphoreticular compartment in the mouse, being detectable in blood, bone marrow, spleen, lymph nodes and thymus. iNKT cells are particularly enriched in the liver, where they constitute 25 – 50% of T cells in some mouse strains,<sup>208</sup> and 0.5% of T cells in humans.<sup>212</sup>

Type II NKT cells have also been referred to as ‘non-classical’ NKT cells. They are CD1d-restricted, but in contrast to iNKT cells (type I NKT cells), exhibit more varied TCR chain usage and show no reactivity to  $\alpha$ -GalCer.<sup>209</sup> Instead, type II NKT cells respond to sulfatide or lysosphingolipid antigens presented on CD1d, and are frequently immunosuppressive.<sup>213</sup> There is evidence for the presence of type II NKT cells in humans: A population of T cells with diverse TCR usage was found to bind to CD1d dimers loaded with lysophospholipids, but not to CD1d dimers loaded with  $\alpha$ -GalCer. These cells were potent producers of the immunosuppressive cytokine IL-13.<sup>206</sup> CD1d-reactive T cells with diverse TCR usage have been identified in human bone marrow and in the placenta, and suppress mixed lymphocyte reactions.<sup>214,215</sup>

Finally, the heterogeneous population of NK-like T cells (that is, T cells which express NK markers, but which are not CD1d restricted and do not respond to  $\alpha$ -GalCer) are sometimes referred to as ‘type III NKT cells’.

This thesis primarily concerns type I NKT cells (CD1d-restricted T cells expressing the V $\alpha$ 24-J $\alpha$ 18 TCR- $\alpha$ chain and responding to  $\alpha$ -GalCer, which I refer to throughout as iNKT cells for brevity. I use the term NK-like T cells to refer to T cells co-expressing classical NK markers (type III NKT cells). Note that by typical immunophenotypic criteria (CD3+CD16/56+ or CD3+CD161+), the NK-like T cell subset includes the majority of iNKT cells. Figure 1.3 illustrates the various NKT cell subsets, and table 1.9 summarises the synonyms, immunophenotype and antigen presentation systems for the various subsets.

### 1.2.2.2 Other invariant T cell populations

Invariant natural killer T cells are not the only T cell population showing limited diversity of TCR repertoire and a lack of MHC restriction. Two other notable populations include V $\gamma$ 9V $\delta$ 2+ gammadelta T cells and mucosal-associated invariant T cells (MAIT) cells, both of which share some characteristics with iNKT cells.

**V $\gamma$ 9V $\delta$ 2+ gammadelta T cells** Gammadelta ( $\gamma\delta$ ) T cells constitute up to 10% of circulating nucleated cells in humans, and a higher proportion in some epithelial surfaces. A major subpopulation expresses the invariant V $\gamma$ 9V $\delta$ 2 TCR, and can comprise up to 50% of circulating gammadelta T cells during certain infections. V $\gamma$ 9V $\delta$ 2+ cells recognise phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which lead to TCR-dependent activation. They are also indirectly activated by aminobisphosphonates, including pamidronate and zoledronate, via production of the phosphoantigen isopentyl pyrophosphate (IPP). Activated V $\gamma$ 9V $\delta$ 2+ are able to produce both Th1-

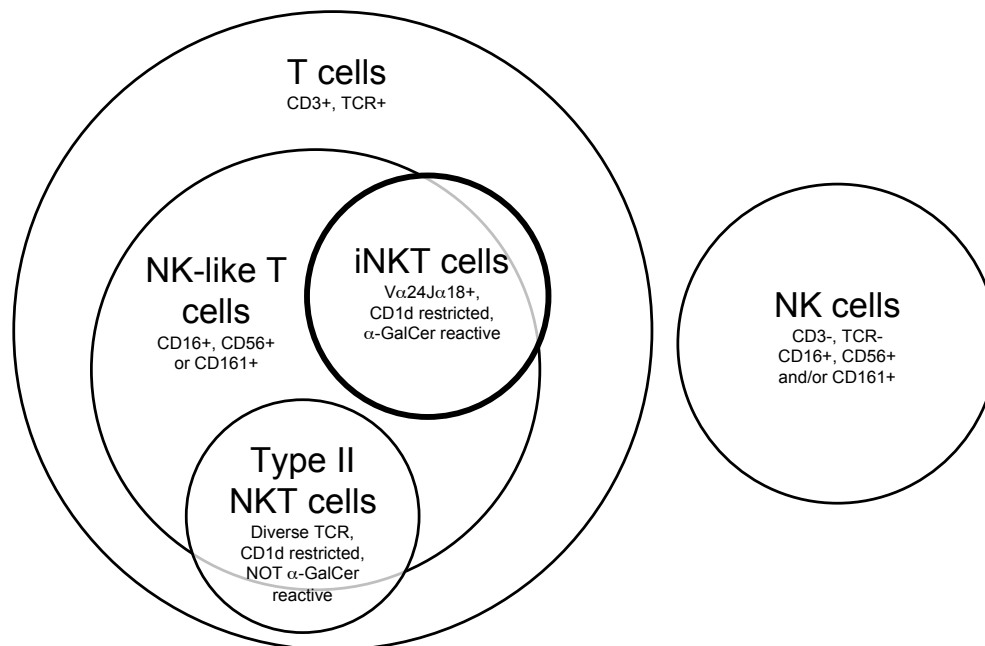
Table 1.9 Nomenclature of NKT cells

Cell type	Synonyms	Immunophenotype (in humans)	Presenting molecule	Known antigens
iNKT	Type I NKT, NKT, Classical NKT	CD3+, Vα24Jα18+, usually Vβ11+ α-GalCer loaded CD1d tetramer+, CD161+ (usually)	CD1d	α-GalCer and analogues, microbial glycolipids, iGb3
Type II NKT	Non-classical NKT	CD3+, Lysosphingolipid- or sulfatide-loaded CD1d tetramer+, Variable TCR usage	CD1d	Lysosphingolipids, sulfatide
NK-like T	Type III NKT, NK T, NKT	CD3+, CD16+, CD56+ or CD161+, Variable TCR usage	MHC Class I or II *	Peptides *
T		CD3+, variable TCR usage	MHC Class I or II *	Peptides *
NK		CD3-, CD16+, CD56+ or CD161+	n/a	n/a

\* NK-like T and T cell subsets include subpopulations of other non-classical T cells, such as γδ T cells and T cells restricted to group 1 CD1 molecules, HLA-E and MRI



**Figure 1.3 Nomenclature of NKT cells** Venn diagram illustrating the overlaps between T, NK, NK-like T, iNKT (also known as type I NKT) and type II NKT cells.



and Th2-type cytokines and are able to lyse some tumour cell lines in a granzyme- and NKG2D-dependent manner.<sup>216</sup>

**MAIT cells** MAIT cells express the invariant TCR $\alpha$  chain iV $\alpha$ 7.2-J $\alpha$ 33 (iV $\alpha$ 19-J $\alpha$ 33 in mice), which pairs with a restricted repertoire of V $\beta$  chains. These cells are found in the lamina propria of the gut, the liver and the blood, and are restricted by the MHC Class I-like molecule MR1, expressed on B cells. Like CD1, MR1 is highly conserved between species. The ligand for MR1 is not known, but as MAIT cells are activated by a wide range of gut microbes, is assumed to be a conserved microbial compound. MAIT cells express high levels of CD161 alongside CCR6, and rapidly produce IFN- $\gamma$ , tumour necrosis factor alpha (TNF- $\alpha$ ), IL-17 and granzymes upon stimulation. MAIT cells express NK receptors such as NKG2D, suggesting that NK signals may also play a role in their activation.<sup>217</sup>

### 1.2.2.3 Subsets

A number of subsets within the iNKT cell compartment have been described. In humans and mice, iNKT cells can be divided on the basis of their CD4 expression. In humans, both CD4+ and CD4- iNKT cells are potent producers of IFN- $\gamma$ , but CD4+ iNKT cells also produce Th2-type cytokines such as IL-4 and IL-13.<sup>218,219</sup> The CD4- iNKT cell subset expresses more perforin.<sup>220</sup> In mice, the gene expression profile of CD4+ iNKT cells differs from that of their CD4- counterparts.<sup>221</sup> In mice, the CD4-, but not the CD4+, subset of iNKT cells is able to reject an inoculated sarcoma.<sup>222</sup> In humans, but not in mice, a

small proportion of iNKT cells also express CD8, the functional significance of which is not clear.

The proportion of CD4+ iNKT cells changes with age: In ageing humans, as well as a reduction in the overall frequency of iNKT cells, a bias towards increased numbers of CD4+, Th2 cytokine-producing iNKT cells occurs.<sup>223</sup>

The majority of circulating human iNKT cells possess an effector memory phenotype: CD45RO+, CCR7-, CD62L-.<sup>211</sup> Indeed, even iNKT cells from umbilical cord blood exhibit a memory phenotype, in contrast with conventional T cells, which are naïve in neonates. This suggests that iNKT cells are exposed to a naturally-occurring ligand *in utero*.<sup>224</sup> The immunophenotypic and functional properties of iNKT cells and of naïve, central memory, and effector memory T cells are compared in table 1.10.

In humans, the majority of iNKT cells express the NK marker CD161 (the human homologue of NK1.1), whereas only 10% express the NK marker CD56.<sup>218</sup> There is evidence that the NK receptors expressed by iNKT cells, including CD161, are functional. In mice, blockade of the NK activating receptor NKG2D prevents a CD1d-dependent NKT-mediated hepatitis.<sup>225</sup> Murine iNKT cells also express the NK inhibitory receptor NKG2A, blockade of which exacerbates an iNKT-dependent Con A-induced hepatitis.<sup>226</sup> A proportion of human iNKT cells also express NKG2A.<sup>219</sup> In humans, ligation of CD161 (also known as NKR-P1A, a C-type lectin) enhances TCR-mediated iNKT cell activation.<sup>227</sup> Finally, human iNKT cells preferentially proliferate in response to allogeneic rather than autologous DCs, a finding attributed to iNKT cell upregulation of activating killer Ig-like receptors (KIRs) in response to TCR signalling.<sup>228</sup>

A functional difference between the CD56+ and CD56- subsets of human iNKT cells has been described: CD56+ iNKT cells express higher levels of perforin, and exhibit greater cytotoxicity against myeloid leukaemia cells in the *absence* of  $\alpha$ -GalCer. Cytotoxicity in the *presence* of  $\alpha$ -GalCer was equivalent between the two subsets.<sup>229</sup> This may indicate that the CD56+ subset of iNKT cells exhibits enhanced NK-receptor directed cytotoxicity, but that once a sufficiently strong TCR signal is present (for example following  $\alpha$ -GalCer-treatment of the target cells), both subsets are able to lyse the target.

### 1.2.3 CD1d ligands

First reported to specifically activate murine iNKT cells in a CD1d- and TCR-restricted fashion in 1997,  $\alpha$ -GalCer, a synthetic glycolipid derived from the marine sponge *Agelas mauritanus*, is the archetypal iNKT cell agonist.<sup>205</sup> The high frequency of iNKT cells, their conservation across species, and their effector memory phenotype, however, imply that naturally-occurring CD1d ligands exist, either endogenous or derived from ubiquitous microbes.

#### 1.2.3.1 Endogenous

Activation of iNKT cells by CD1d-expressing APCs in the absence of exogenous glycolipids provides indirect evidence of the presentation of endogenous ('self') lipids by CD1d. Consistent with this, glycolipid and phospholipid fractions of cell extracts are able to

Table 1.10 Comparison of the phenotype of T cells and iNKT cells

T cell subset	CD45RA	CD45RO	CCR7	CD62L	Response to antigen (or anti-CD3)	Proliferative potential	Cytokines produced	Tissue distribution	Notes
Naïve	+	-	+	+	+/-*	+++	IL-2	Lymph nodes	Can differentiate into T <sub>CM</sub> and T <sub>EM</sub>
T <sub>CM</sub>	-	+	+	+	++	++	IL-2	Lymph nodes	Can differentiate into T <sub>EM</sub>
T <sub>EM</sub>	-	+	-	+/-	+++	+	IFN- $\gamma$ IL-4 IL-5 (rapid)	Peripheral tissues	CD8+ T <sub>EM</sub> express perforin. Differentiate into T <sub>EMRA</sub>
T <sub>EMRA</sub>	+	-	-	-	+++	-	IFN- $\gamma$ IL-4 IL-5 (rapid)	Peripheral tissues	Terminally differentiated
iNKT cells	-	+	-	-	+++	++	IFN- $\gamma$ IL-4 IL-5 (rapid)	Peripheral tissues	

\* Costimulatory signals (i.e. CD28) are required

**Table 1.11 Lipids eluted from secreted CD1d from a transfected human lymphoblastoid cell line<sup>230</sup>**

Class	Molecules
Glycerophospholipids	Diacylglycerols
	Plasmalogens
	Lysophospholipids
	Cardiolipins
Sphingolipids	Sphingomyelins
	Glycosylated sphingolipids

stimulate iNKT cells when presented by plate-bound recombinant CD1d. Analysis of lipids eluted from CD1d purified from cell cultures reveals a range of polar lipids. Table 1.11 summarises the lipids eluted from a CD1d secreted by a CD1d-transfected human cell line in one study.<sup>230</sup>

Particular interest has focussed on a few specific potential endogenous ligands. Isoglobotrihexosylceramide (iGb3) was proposed as a major endogenous ligand for iNKT cells, but it is now clear that although iGb3 functions as a CD1d ligand, its expression is not required for iNKT cell development or maintenance.<sup>231</sup> The ganglioside GD3 is expressed on melanoma cells, and can activate iNKT cells in a CD1d-restricted fashion.<sup>232</sup> The myelin-derived glycolipid sulfatide is recognised by a population of immunosuppressive type II iNKT cells, and administration of sulfatide can prevent experimental autoimmune encephalitis in a CD1d-dependent manner.<sup>233</sup>

### 1.2.3.2 Microbial

Some microbial-derived glycolipids are able to bind CD1d and to be recognised by iNKT cells. Table 1.12 lists selected microbial CD1d ligands; the role of iNKT cells in the protection against infection will be discussed later (section 1.2.7.1).

The glycolipid  $\alpha$ -GalCer, the structure of which is illustrated in figure 1.4, is a potent iNKT cell agonist, but the mixed Th1- and Th2-type cytokine responses it elicits have prompted searches for alternative ligands capable of generating more selective responses. Modifications to the sphingosine base, the length or saturation of the acyl chains, the composition of the glycosidic linkage or to the structure of the carbohydrate moiety can all result in  $\alpha$ -GalCer derivatives with iNKT cell stimulating activity.<sup>231,234,235</sup> For instance, a glycolipid with a modified fatty acid chain, 7DW8-5, is reported to exhibit higher CD1d binding affinity, improved induction of iNKT cell cytokine production and enhanced adjuvant activity compared to  $\alpha$ -GalCer.<sup>234</sup>

In the mouse, some  $\alpha$ -GalCer derivatives, such as its sphingosine-truncated analog OCH (figure 1.5), exhibit greater Th2 stimulating capacity,<sup>236</sup> whereas others, such as  $\alpha$ -C-GalCer (figure 1.5), induce greater Th1 cytokine production.<sup>231</sup> Using human iNKT clones and a library of  $\alpha$ -GalCer derivatives, the Th2-type cytokine IL13 was induced by weak ag-

Table 1.12 Microbial-derived CD1d ligands adapted from Venkataswamy & Porcelli<sup>231</sup>

Organism(s)	CD1d ligand	Activity
<i>Mycobacterium bovis</i>	PIM <sub>4</sub>	IFN- $\gamma$ secretion from a subset of iNKT cells
<i>Borrelia burgdorferi</i>	$\alpha$ -galactosyl diacylglycerols	IFN- $\gamma$ secretion from a subset of mouse iNKT cells and a human iNKT cell line
<i>Sphingomonas</i> spp <i>Ehrlichia</i> spp	$\alpha$ -glucuronosylceramides	Th1 and Th2 cytokine production from human and mouse iNKT cells
<i>Leishmania donovani</i>	Lipophosphoglycan	IFN- $\gamma$ secretion from a fraction of mouse iNKT cells
<i>Entamoeba histolytica</i>	Lipopeptidophosphoglycan	IFN- $\gamma$ secretion from mouse iNKT cells

Figure 1.4 Structure of  $\alpha$ -GalCer

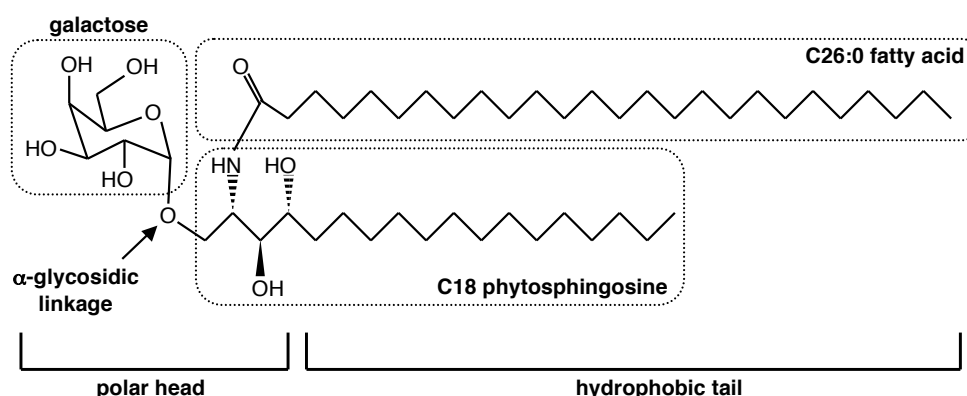


Table 1.13 Cytokines and chemokines produced by iNKT cells<sup>240,241</sup>

Th1-type	Th2-type	Other	Chemokines
IFN- $\gamma$	IL-4	IL-17	RANTES
TNF- $\alpha$	IL-5	IL-21	Eotaxin
	IL-6	TGF- $\beta$	Lymphotoxin
	IL-10	GM-CSF	MIP-1 $\alpha$
	IL-13	IL-2	MIP-1 $\beta$
		IL-3	

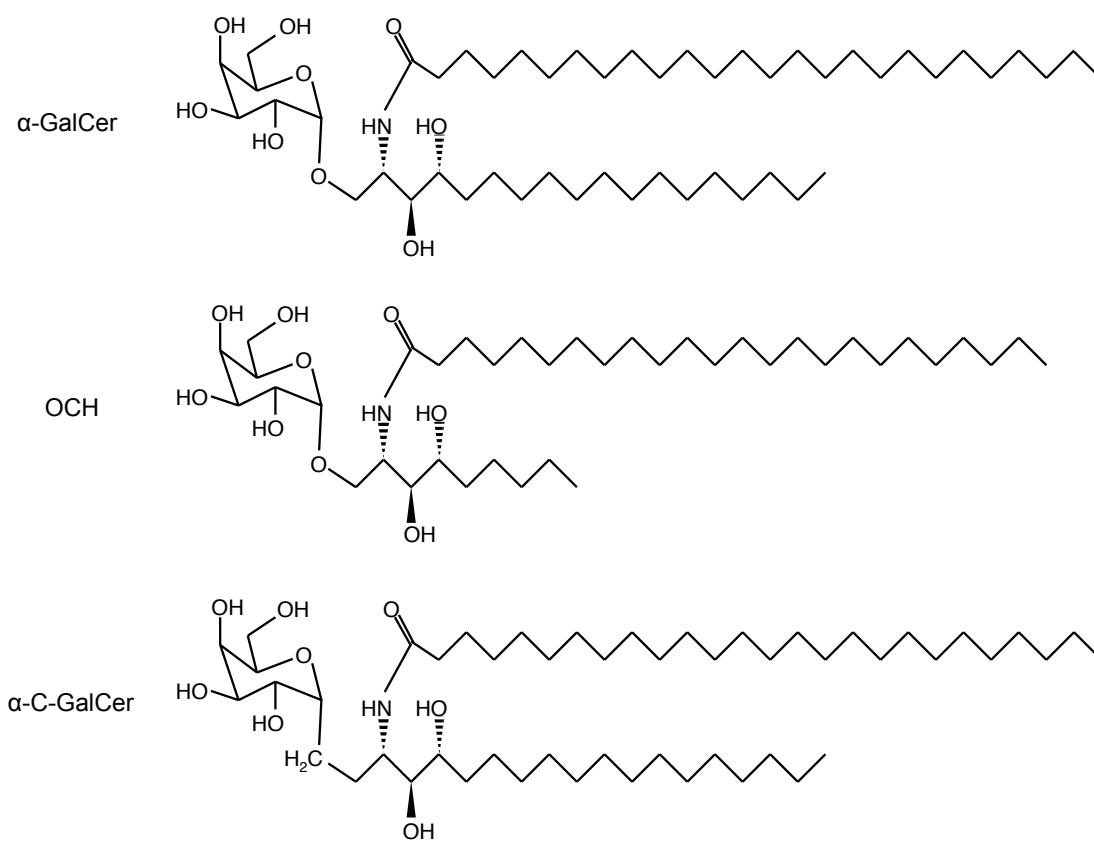
onists at modest concentrations or by strong agonists at very low concentrations, whereas IFN- $\gamma$  induction and transactivation of NK cells required strong iNKT agonists at modest concentrations or weak agonists at high concentrations.<sup>237</sup> This polarisation of cytokine response according to avidity of the agonist is also observed with ‘conventional’ T cells: CD4+ T cells are polarised towards production of Th2-type cytokines in the presence of weak agonists, while strong agonists induce Th1-type cytokines.<sup>238,239</sup>

### 1.2.4 Cytokine production

In keeping with their effector memory phenotype, iNKT cells rapidly produce cytokines upon stimulation. Reported iNKT cell-derived cytokines are listed in table 1.13. Both CD4+ and CD4- iNKT cell subsets produce Th1-type cytokines, but the production of the Th2-type cytokines is largely restricted to the CD4+ iNKT cell subset.<sup>218,220</sup>

A number of factors influence the cytokine profile of stimulated iNKT cells:

Figure 1.5 Structure of selected  $\alpha$ -GalCer derivatives



- **Relative proportion of iNKT cell subsets:** Human CD4<sup>+</sup> iNKT cells exhibit greater production of Th2-type cytokines than their CD4<sup>-</sup> counterparts.<sup>218,220</sup> In mice, the NK1.1<sup>+</sup> subset of iNKT cells produce less IL-4 and more IFN- $\gamma$  than NK1.1<sup>-</sup> iNKT cells.<sup>241</sup>
- **Organ of origin:** In mice, thymic iNKT cells produce more Th2 cytokines, and liver iNKT cells produce more Th1 cytokines than those from the periphery.<sup>241</sup>
- **Structure and concentration of iNKT cell agonist:** The stimulation of iNKT cells with weak agonists, such as the truncated analogue of  $\alpha$ -GalCer, OCH, or with very low concentrations of strong agonists, results in preferential production of Th2-type cytokines. In contrast, strong agonists such as  $\alpha$ -C-GalCer, or high concentrations of weak agonists, tend to induce the production of Th1-type cytokines by iNKT cells.<sup>231,237</sup>
- **Antigen presenting cell:** Optimal IFN- $\gamma$  production by iNKT cells requires both DC-derived IL-12 and a CD40/CD40L interaction,<sup>242,243</sup> and TLR ligand-activated DCs are particularly strong inducers of IFN- $\gamma$  production by iNKT cells.<sup>244</sup> In contrast, the presentation of  $\alpha$ -GalCer by non-professional APCs such as B cells or Schwann cells results in Th2-skewed iNKT cell cytokine production.<sup>245,246</sup>
- **Exogenous cytokines:** Whether derived directly from DCs, or added to culture media, the presence of IL-12 favours IFN- $\gamma$  production by iNKT cells. In contrast, IL-2 in combination with weak TCR stimulation favours the production of Th2-type cytokines by human iNKT cells.<sup>247</sup>
- **Metabolic factors:** Tryptophan catabolites skew iNKT cells toward a Th2 profile, while inhibition of indoleamine 2,3-dioxygenase, which degrades tryptophan, favours a Th1 profile.<sup>248</sup>

Interleukin-17-producing iNKT cells have been described in mice, and have been implicated in the induction of airway neutrophilia<sup>249</sup> and collagen-induced arthritis<sup>250</sup> in experimental models. However, human iNKT cells have not been conclusively shown to produce IL-17:<sup>251</sup> Although one study showed that human NK-like T cells can produce IL-17 upon stimulation, the cell population studied was not restricted to iNKT (type I NKT) cells.<sup>252</sup>

### 1.2.5 Cytotoxicity

iNKT cells can lyse target cells, including moDCs, upon recognition of  $\alpha$ -GalCer on CD1d.<sup>253</sup> The degree of cytotoxicity exhibited by iNKT cells correlates positively with target cell CD1d expression and iNKT cell agonist potency.<sup>254</sup> As discussed above, iNKT cell cytotoxicity also appears to be modulated by NK receptor signals.<sup>225,226</sup>

iNKT cells express granzyme B and perforin, can upregulate Fas ligand (FasL) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), and can produce TNF- $\alpha$ . There is evidence for involvement of each of these cytotoxicity pathways in iNKT cell lysis of CD1d-expressing target cells.

Perforin and granzyme B are required for  $\alpha$ -GalCer-induced iNKT cell lysis of primary



human myelomonocytic leukemia cells.<sup>229</sup> Concanamycin A inhibits the lysis of target cell lines by human umbilical cord blood-derived iNKT cells, suggesting a perforin-dependent mechanism.<sup>255</sup>

Upon activation, iNKT cells upregulate FasL,<sup>220</sup> which can induce apoptosis of target cells expressing the Fas receptor.  $\alpha$ -GalCer-pulsed B cells are killed by iNKT cells in wild type but not in FasL-deficient mice, suggesting that this pathway plays a role in iNKT cell cytotoxicity.<sup>254</sup> Both Fas- and FasL-deficient mice are resistant to  $\alpha$ -GalCer induced hepatocellular damage.<sup>256</sup>

Human iNKT cells express TRAIL on activation. Co-culture of leukemic blasts from patients with acute myelomonocytic leukemia with TRAIL-expressing iNKT cells leads to leukemia cell apoptosis, an effect blocked by a TRAIL neutralising antibody.<sup>257</sup>

Finally, blocking studies indicate a contribution of TNF- $\alpha$  to the killing of target cells by human iNKT cells,<sup>229,255</sup> although both of these reports found that the major contributor to cytotoxicity was the perforin/granzyme B pathway.

To summarise, iNKT cells integrate signals from their TCR, their activating and inhibitory NK receptors and their environment to determine target cell killing. A potent TCR stimulus (i.e. high target cell CD1d expression in association with a strong iNKT cell agonist) and a balance of activating NK signals will favour target cell lysis. Several mechanisms of cytotoxicity may be involved, but the perforin/granzyme B and Fas/FasL systems appear to be the most important.

### 1.2.6 Interactions with other cells of the immune system

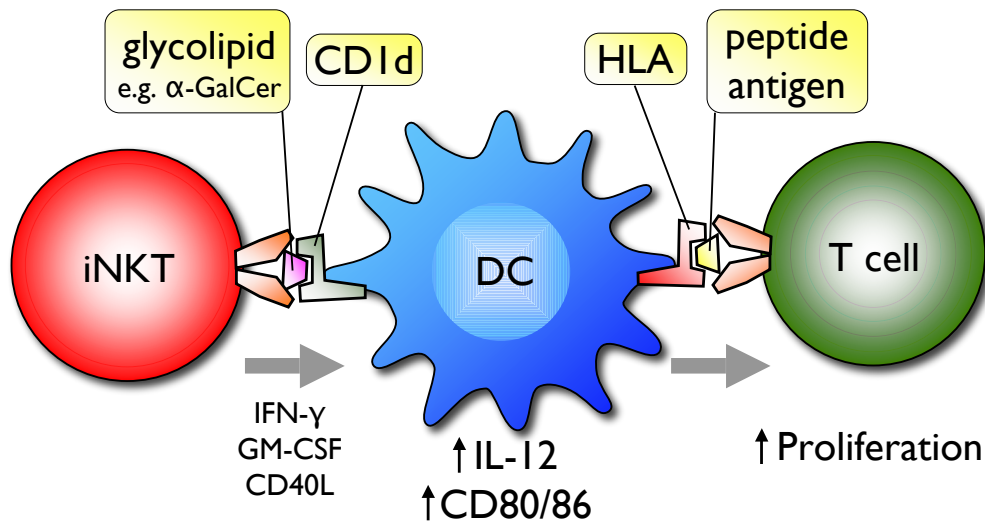
#### 1.2.6.1 Dendritic cells

Myeloid dendritic cells express CD1d, and can present  $\alpha$ -GalCer to iNKT cells. In response to this TCR stimulation, iNKT cells release cytokines including TNF- $\alpha$  and IFN- $\gamma$ , and upregulate costimulatory molecules such as CD40L. This results in DC upregulation of the co-stimulatory markers CD80 and CD86, and production of IL-12, which can feed back on iNKT cells.<sup>242,258</sup> The reciprocal interaction between iNKT cells and DCs in the presence of  $\alpha$ -GalCer enhances responses to DC-presented protein antigens by 'conventional' T cells.<sup>258-260</sup> A diagram of the interaction between DC, iNKT and T cells is presented in figure 1.6.

Under some circumstances, the interaction of iNKT cells with DCs can result in immunosuppression or induction of tolerance. The outcome of an interaction between DCs and iNKT cells appears to depend on a number of factors:

- **Exposure to iNKT cell agonist:** Both  $\alpha$ -GalCer and iNKT cells are required to turn immature DCs into effective producers of IL-12. In the absence of  $\alpha$ -GalCer, iNKT cells fail to induce full maturation of DCs.<sup>261</sup>
- **Contact of iNKT cells with non-professional APCs:** Although iNKT cells can induce DC maturation and enhance Th1 responses when added to  $\alpha$ -GalCer pulsed immature human moDCs, the presence of iNKT cells throughout the period of monocyte culture has the opposite effect: moDCs generated in the presence of iNKT cells

Figure 1.6 Interaction between DC, iNKT and T cells



have an impaired capacity to produce IL-12 and to elicit Th1-type T cell responses, whether  $\alpha$ -GalCer is present or not. Thus, the  $\alpha$ -GalCer/iNKT cell stimulus operates differentially on monocytes (DC precursors) and DCs themselves.<sup>262</sup>

- **Route of  $\alpha$ -GalCer administration:** While  $\alpha$ -GalCer presented exclusively by DCs can induce DC maturation, the systemic administration of  $\alpha$ -GalCer (e.g. by intravenous injection), results in a skewing of DCs towards a tolerogenic phenotype in non-obese diabetic (NOD) mice.<sup>263</sup>

Thus, presentation of  $\alpha$ -GalCer to iNKT cells by DCs results in DC maturation, upregulation of co-stimulatory markers, improved T cell responses to protein antigens, and enhanced anti-tumour immunity. In contrast, premature exposure of monocytes to iNKT cells and  $\alpha$ -GalCer, exposure of DCs to iNKT cells in the absence of  $\alpha$ -GalCer, and  $\alpha$ -GalCer delivered in a non-directed fashion can all result in the generation of DCs with poor stimulatory capacity, or even tolerogenic properties. The timing and mode of delivery of  $\alpha$ -GalCer are therefore critical if immune responses are to be augmented.

### 1.2.6.2 T cells

Activation of iNKT cells with  $\alpha$ -GalCer results in the enhancement of peptide-specific CD4+ and CD8+ T cell responses.<sup>258,260</sup> Much of this effect appears to be due to iNKT cell enhancement of DC maturation, as discussed above, because the transfer of DCs from  $\alpha$ -GalCer- and peptide-treated mice leads to transfer of the enhanced immunity,<sup>260</sup> and because CD40 expression on DCs is essential for  $\alpha$ -GalCer enhancement of T cell responses.<sup>259</sup>

Under certain circumstances, iNKT cells may also be able to inhibit T cell responses. Culture of iNKT cells in the presence of TGF- $\beta$  results in expression of forkhead box P3 (Foxp3) on a subset of iNKT cells. Similar to Foxp3+ T cells, these iNKT cells suppress T cell responses in a contact-dependent manner.<sup>264</sup>

T<sub>reg</sub> cells also interact with iNKT cells in a bi-directional fashion: T<sub>reg</sub> cells can inhibit the proliferation, cytokine production and cytotoxicity of iNKT cells through a cell-cell contact mechanism. In the opposite direction, iNKT cells can promote T<sub>reg</sub> generation by inducing tolerogenic DCs as outlined in section 1.2.6.1 above. Finally, iNKT cell-derived IL-2 can promote T<sub>reg</sub> proliferation.<sup>265</sup>

### 1.2.6.3 B cells

**iNKT cell effect on antibody production** Co-administration of protein immunogens with  $\alpha$ -GalCer results in higher antibody titres and improved protection against infection. Moreover, combining protein vaccination with  $\alpha$ -GalCer results in IgG responses in MHC class II knockout mice, implying that iNKT cells can substitute for antigen specific CD4+ T cell help in the generation of B cell immunity.<sup>266</sup> Chimeric mouse studies indicate that CD1d expression on B cells themselves is necessary for this effect.<sup>267</sup>

There is also evidence that iNKT cells can also provide help to B cells to produce antibodies directed against glycolipids themselves—this interaction requires CD40L, CD80/CD86 and IFN- $\gamma$ .<sup>268</sup>

**Role of B cells in maintenance of iNKT cells** B cells express CD1d, and have been reported to be important for the maintenance of iNKT cells. Depletion of CD19+ B cells from human PBMCs resulted in impaired *in vitro* iNKT cell proliferation and reduced iNKT cell cytokine production, and  $\alpha$ -GalCer-loaded B cells are able to induce iNKT cell proliferation.<sup>269</sup> In pre-clinical studies, marginal zone B cells are required for the full activation of iNKT cells by  $\alpha$ -GalCer *in vivo*, an effect which appears to be mediated by DCs.<sup>270</sup>

### 1.2.6.4 Natural killer cells

Systemic administration of  $\alpha$ -GalCer to mice leads to activation and expansion of NK cells and enhancement of NK cytotoxicity.<sup>271,272</sup> Indeed, NK cell-derived IFN- $\gamma$  is required for the antimetastatic effect of  $\alpha$ -GalCer in a preclinical melanoma model.<sup>271</sup>

The transactivation of NK cells following  $\alpha$ -GalCer administration appears to be cytokine-mediated—it can be reduced by simultaneously blocking both IFN- $\gamma$  and IL-12.<sup>273</sup> Murine iNKT cells are also capable of IL-21 production, a cytokine with mixed effects on NK cell function, including enhanced NK cell maturation and cytotoxicity as well as early NK cell death.<sup>274</sup> An immunotherapy strategy comprising  $\alpha$ -GalCer-pulsed DCs followed by systemic IL-21 administration resulted in improved NK cell cytotoxicity and regression of established metastases in several mouse models of cancer.<sup>275</sup>

There is *in vitro* evidence that human NK and iNKT cells can co-operate in a similar fashion. The addition of *in vitro*-expanded iNKT cells and  $\alpha$ -GalCer to PBMC cultures resulted in enhanced NK cell cytotoxicity, an effect which was augmented by IL-21. Transwell experiments demonstrated that the transactivation of human NK cells by iNKT cells was dependent on soluble factors.<sup>276</sup>

### 1.2.6.5 Myeloid cells

Administration of intravenous  $\alpha$ -GalCer to mice leads to increased numbers of haematopoietic progenitor cells in the spleen and an increase in circulating mature neutrophils, an effect consistent with the production of IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) by iNKT cells.<sup>277</sup>

The effects of iNKT cells on neutrophil recruitment to tissues can be variable: in murine studies, Th-17-producing iNKT cells contributed to airway infiltration by neutrophils in one model,<sup>278</sup> whereas IL-4 secreting iNKT cells suppressed neutrophil IFN- $\gamma$  secretion and lung inflammation in another.<sup>279</sup>

The production of Th2 cytokines and eotaxin by *in vitro* stimulated iNKT cells is consistent with the finding that several mouse models of asthma indicate a role for iNKT cells in the eosinophil recruitment to the airways.<sup>280</sup> Evidence of role for iNKT cells in human asthma is less clear, in part because methodological difficulties have complicated the detection of iNKT cells in human respiratory samples.<sup>281</sup>

Myeloid cells with suppressive activity, such as tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) have been identified within tumours, and themselves express CD1d. TAMs, which support tumour cell growth through IL-6 production, can be killed by iNKT cells, leading to suppression of tumour growth in a neuroblastoma xenograft model.<sup>282</sup> iNKT cells can also control MDSC numbers: iNKT cell-deficient mice have increased numbers of MDSCs, and adoptive transfer of iNKT cells into  $J\alpha 18^{-/-}$  (iNKT-cell deficient) mice led to a reduction of MDSC numbers and alleviated MDSC-mediated immunosuppression.<sup>283</sup>

### 1.2.7 Role in health and disease

#### 1.2.7.1 Infection

A number of infections are exacerbated in iNKT cell-deficient mice (both in  $CD1d^{-/-}$  and  $J\alpha 18^{-/-}$  animals), suggesting that iNKT cells are of relevance in controlling the pathogenic organisms or the immune response. The impact of iNKT cell deficiency on selected infection models is summarised in table 1.14.

Indirect evidence suggests that iNKT cells may play a role in mycobacterial infections in humans. Circulating iNKT cells have an activated phenotype in patients with tuberculosis.<sup>285</sup> Patients with both tuberculosis and leprosy have a higher proportion of CD4+ iNKT cells than healthy controls.<sup>286</sup> Incubation of human monocytes with liposomes bearing *Mycobacterium leprae* cell wall antigens results in upregulation of CD1b and CD1d, and enhanced IFN- $\gamma$  secretion within the NK-like T cell population.<sup>287</sup>

Infections may activate iNKT cells via several mechanisms. As discussed in section 1.2.3.2, microbial-derived iNKT cell agonists have been isolated, and may be presented to iNKT cells by APCs. Infections may result in changes of cellular glycolipid composition, resulting in altered expression of self glycolipids to iNKT cells. Finally, microbial danger signals acting on other immune system cells may lead to changes in circulating cytokines

Table 1.14 Selected infections exacerbated in iNKT deficient mice adapted from Kinjo et al.<sup>284</sup>

Organism type	Species	Effect of iNKT deficiency on infection	
		CD1d <sup>-/-</sup>	J $\alpha$ 18 <sup>-/-</sup>
Parasitic	<i>Leishmania major</i>	—	Exacerbated
	<i>Trypanosoma cruzi</i>	Not exacerbated	Exacerbated
Fungal	<i>Cryptococcus neoformans</i>	—	Exacerbated
Bacterial	<i>Borrelia burgdorferi</i>	Exacerbated	—
	<i>Pseudomonas aeruginosa</i>	Exacerbated	—
	<i>Streptococcus pneumoniae</i>	—	Exacerbated
	<i>Sphingomonas</i> spp.	Exacerbated	Exacerbated
Viral	<i>Herpes simplex</i>	Exacerbated (HSV-2)	Exacerbated (HSV-1)

or altered expression of co-stimulatory molecules on APCs, resulting secondary iNKT cell activation.<sup>284</sup>

### 1.2.7.2 Allergy and autoimmunity

The effect of iNKT cells on allergy and autoimmunity varies according to the model used.

In some models, iNKT cell activation exacerbates allergy and autoimmunity. For instance, the systemic administration of  $\alpha$ -GalCer abrogates oral tolerance in mice, an effect mediated by DCs in the lamina propria and involving the activation of small intestine iNKT cells.<sup>288</sup> In some animal models of allergic asthma, iNKT cell-deficient mice are resistant to allergen challenge.<sup>289</sup> A bacterial-induced autoimmune hepatitis is dependent upon iNKT cells.<sup>290</sup> Deficiency of iNKT cells ameliorates collagen-induced arthritis, and CD1d blockade early in the course of the arthritis mitigates the disease.<sup>291</sup> Finally, in humans, activated iNKT cells are reported to account for a high proportion of the cellular infiltrate in contact dermatitis.<sup>292</sup>

In contrast, other studies indicate that iNKT cells can provide protection against allergy and autoimmunity. The Th2-polarising iNKT cell ligand OCH prevents the development of experimental autoimmune encephalitis in an IL-4 dependent manner.<sup>293</sup> In non-obese diabetic mice, repeated intraperitoneal injections of  $\alpha$ -GalCer suppress type 1 diabetes by recruiting tolerogenic DCs,<sup>263</sup> and the adoptive transfer of iNKT cells suppresses diabetes, while CD1d knockout exacerbates it.<sup>294</sup> The Th1-polarising iNKT cell agonist  $\alpha$ -C-GalCer is protective against experimental autoimmune uveitis.<sup>295</sup> Finally, in a spondyloarthritis model which involves gut and joint inflammation, deficiency of iNKT cells aggravated the disease.<sup>296</sup>

Studies of mouse models of GVHD are particularly informative: host iNKT cells are required for protection against acute GVHD.<sup>297</sup> This tolerance requires host recognition of CD1d on donor marrow cells<sup>298</sup> and involves IL-4 produced by host iNKT cells acting upon donor T<sub>reg</sub> cells.<sup>299</sup> In contrast, early administration of  $\alpha$ -GalCer after mismatched transplant results in DC- and IL-12-dependent transactivation of NK and T cells, hyperacute GVHD and early death.<sup>300</sup> Interestingly, the  $\alpha$ -GalCer variant C20:2, which unlike  $\alpha$ -GalCer fails to induce NK cell transactivation, inhibits GVHD.<sup>300</sup>

### 1.2.7.3 Cancer

Experimental and observational evidence suggests that iNKT cells play a role in immune surveillance against spontaneous malignancies.

Mice deficient in iNKT cells have an increased susceptibility to methylcholanthrene-induced sarcoma, and tumour resistance can be restored by adoptive iNKT cell transfer.<sup>301</sup> In a mouse model of spontaneous prostate adenocarcinoma, the absence of iNKT cells was associated with earlier onset of cancer and shorter survival.<sup>302</sup>

Patients with advanced solid malignancies and acute myeloid leukaemia have reduced numbers of circulating iNKT cells compared to healthy controls.<sup>210,303-305</sup> There is some

evidence that numerical and functional defects of iNKT cells are selective for this subset. Omental iNKT cell numbers are reduced in patients with colorectal carcinoma, while other omental T cell subsets are retained.<sup>306</sup> In advanced prostate cancer, reduced iNKT cell IFN- $\gamma$  production has been observed, despite normal IFN- $\gamma$  production by bulk T cells.<sup>304</sup> Finally, impaired iNKT cell production of IFN- $\gamma$  has been reported in patients with progressive myeloma, despite normal IFN- $\gamma$  responses to influenza and staphylococcal enterotoxin A, and despite normal circulating T and NK cell numbers.<sup>307</sup>

The number of circulating iNKT cells may also be of prognostic relevance. In patients with advanced prostate cancer, the reduction in iNKT cell numbers compared to healthy controls was similar to the reduction seen in T cells and NK cells, but only iNKT cell numbers correlated with survival.<sup>308</sup> In patients with acute myeloid leukaemia, although the number of bone marrow T and NK-like T cell populations correlated with survival on univariate analysis, only peripheral blood iNKT cell number predicted survival on multivariate analysis.<sup>305</sup>

There are several mechanisms by which iNKT cells might help to eradicate malignancy, including direct cytotoxicity against CD1d-expressing tumour cells, transactivation of other effector cell types, such as NK cells, and augmentation of 'conventional' T cell responses to TAAs by promotion of DC function. Each of these mechanisms has been demonstrated preclinically *in vivo*.

- **Direct cytotoxicity:** An  $\alpha$ -GalCer-pulsed CD1d-transfected B16 melanoma cell line was lysed by iNKT cells *in vivo*, resulting in significant protection against metastasis.<sup>254</sup> *In vivo* iNKT cell cytotoxicity against tumour cells is dependent on the expression of CD1d on the tumour and is strongly enhanced by the presence of  $\alpha$ -GalCer.<sup>309,310</sup>
- **Transactivation of NK cells:** The production of IFN- $\gamma$  by NK cells is necessary for  $\alpha$ -GalCer-induced protection against metastatic malignancy.<sup>271</sup> The combination of  $\alpha$ -GalCer and IL-21 resulted in potent antitumour activity, which was dependent upon NK cells, perforin and IFN- $\gamma$ .<sup>272</sup>
- **Activation of DCs:** The administration of  $\alpha$ -GalCer in conjunction with tumour antigen resulted in dendritic cell maturation and enhanced 'conventional' CD4+ and CD8+ T cell responses against subsequent tumour challenge.<sup>258,259,311</sup>

In contrast, there is also evidence that iNKT cells can suppress immune responses to cancer, with deficiency of iNKT cells improving outcomes in some murine tumour models. For instance, responses of a 4T1 mammary carcinoma to radiotherapy and anti-CTLA-4 monoclonal antibody (mAb) were augmented in  $V\alpha 14J\alpha 18^{-/-}$  iNKT cell-deficient mice.<sup>312</sup> Mice deficient in iNKT cells (both  $CD1d^{-/-}$  and  $J\alpha 18^{-/-}$ ) were more resistant to an inoculated CD1d-transfected T cell lymphoma than wild type mice. Following tumour inoculation, wild type mice had much higher levels of circulating IL-13 than the iNKT cell deficient mice, suggesting that iNKT cell-derived or -induced IL-13 may be involved in iNKT cell-mediated immunosuppression.<sup>313</sup>

In some models, CD1d-dependent tumour tolerance appears to be mediated by type II NKT cells rather than iNKT cells:  $CD1d^{-/-}$ , but not wild type, mice rejected an immunogenic fibrosarcoma.  $J\alpha 18^{-/-}$  mice also failed to reject the tumour, indicating that type II NKT cells were responsible for tumour tolerance in the wild type and  $J\alpha 18^{-/-}$  mice.<sup>314</sup>

The administration of sulfatide, a type II NKT cell agonist, resulted in an increase in the number of metastases from an inoculated colon carcinoma cell line, an effect which could be attributed to CD4+ type II NKT cells. Moreover, in this latter model, the immunosuppressive effect of sulfatide-mediated type II NKT cell stimulation was dominant over the tumour protective effect of  $\alpha$ -GalCer-mediated iNKT cell stimulation.<sup>315</sup>

### 1.2.8 Directing the iNKT cell response

The findings in models of infection, allergy, autoimmunity and cancer, outlined in sections 1.2.7.1 to 1.2.7.3, indicate that iNKT cells are capable of both immunostimulatory and immunosuppressive effects. This could be considered consistent with the capacity of iNKT cells to produce a broad range of cytokines, as discussed in section 1.2.4, and with their interactions with multiple cell types, as discussed in section 1.2.6.

Effective application of iNKT cell-directed immunotherapies requires an understanding of the factors which influence the outcome of iNKT cell stimulation. Considering the factors which influence the outcome of iNKT cell activation in different *in vivo* and *in vitro*, settings, common themes emerge.

**Potency of iNKT cell ligand** Activation of iNKT cells with potent synthetic iNKT cell ligands, such as  $\alpha$ -GalCer or  $\alpha$ -C-GalCer tends to favour the induction of Th1-type cytokine production by iNKT cells, and rejection of tumour. Activation with weaker iNKT cell ligands, such as endogenous glycolipids, or with stronger ligands at limiting concentrations, or with strong ligands at very low concentrations, tends to favour production of Th2-type cytokines by iNKT cells, or results in activation of type II NKT cells, which can result in tolerance.

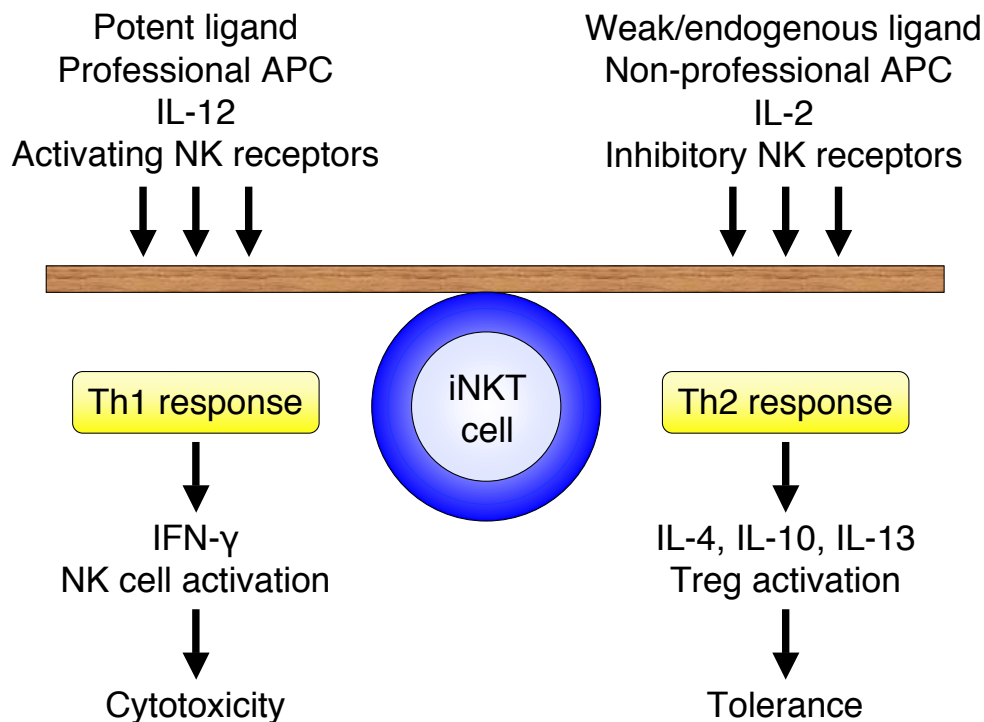
**Type of antigen-presenting cell** The presentation of a potent iNKT cell ligand on CD1d by professional APCs, such as DCs, tends to favour iNKT cell-induced rejection of tumour. Presentation on non-professional APCs, such as monocytes, tends to favour tolerance.

**Cytokine environment** The presence of IL-12 during iNKT cell stimulation, which may be derived from mDCs during iNKT cell stimulation *in vivo*, favours Th1-type responses and tumour rejection. In contrast, the presence of IL-2 can favour the induction of tolerance.

**Natural killer receptor signals** Invariant natural killer T cells express functional NK receptors and, like NK cells, integrate signals from activating and inhibitory NK receptors. A balance of activating NK receptor signals, such as may be elicited by allogeneic cells or by tumour cells which have downregulated MHC Class I, will favour a Th1-type cytokine response and GVHD. In contrast, the presence of strong inhibitory NK receptor signals, for instance elicited by an autologous MHC Class I- or HLA-G-expressing tumour, would favour tolerance.



Figure 1.7 Factors influencing the outcome of iNKT cell stimulation



**Phenotype of responding natural killer T cell** The CD4<sup>+</sup> subset of iNKT cells produces the potentially immunosuppressive Th2-type cytokines, whereas the CD4<sup>-</sup> subset does not. While iNKT cells can reject tumour, type II NKT cells (which are CD1d-restricted but are non-invariant, and which do not respond to  $\alpha$ -GalCer), are associated with tolerance induction and tumour suppression.<sup>316,317</sup>

To summarise, stimulation of iNKT cells with high concentrations of potent synthetic ligands such as  $\alpha$ -GalCer presented by professional APCs, in combination with stimulation of activating NK receptors, and/or IL-12, favours a Th1-type cytokine response, eradication of malignancy and infection, and, in some models, autoimmunity.

In contrast, stimulation of iNKT cells (or type II NKT cells) with weak or endogenous ligands presented by non-professional APCs, in conjunction with an inhibitory NK signal, and the presence of suppressive cytokines, favours a Th2-type cytokine response and tolerance induction.

Figure 1.7 illustrates some stimuli which may influence iNKT cell cytokine production and effector responses.

### 1.3 Cellular immunotherapy

As opposed to humoral immunotherapy or conventional chemotherapy, cellular immunotherapies aim to elicit long-lasting T cell immunity against malignant cells, with the goal of eradicating or controlling the tumour.

In this section, I shall discuss the evidence that CLL is susceptible to immunological control. I shall then review strategies for adoptive immunotherapy and discuss the clinical experience of adoptive immunotherapy of CLL to date. Finally, I shall review methods of incorporating iNKT cell engagement into the adoptive immunotherapy of cancer.

### 1.3.1 Immunological control of CLL

A number of clinical observations suggest that CLL is susceptible to immunological control, and may therefore be a suitable candidate malignancy for adoptive immunotherapy.

Experience of allo-SCT for CLL indicates that allogeneic T cells are capable of eradicating CLL. Allo-SCT can result in durable clinical remissions ('cures'), and in allogeneic transplantation registry studies, T cell depletion of the graft is associated with higher relapse risk, and development of chronic GVHD is associated with lower relapse risk.<sup>154</sup> After allo-SCT, a graft-versus-leukaemia effect against CLL can be observed, with disease remission following the infusion of additional unselected donor lymphocytes to patients who have relapsed after allo-SCT.<sup>318</sup> Interestingly, the rates of long-term disease-free survival after syngeneic transplantation are higher than those observed after autologous transplantation, suggesting that T cells can control CLL cells even without the assistance of minor histocompatibility antigen or NK receptor mismatches.<sup>319</sup>

Autologous T cells may also be able to eradicate CLL. Spontaneous remissions of CLL, although rare, are well documented,<sup>320,321</sup> and may be associated with the development of autoimmune cytopenias.<sup>322</sup> Leukaemia-reactive T cells can be detected in the peripheral blood of some patients with CLL, and can increase in number after administration of autologous tumour vaccines<sup>323,324</sup> or a DC vaccine.<sup>325</sup> Supporting the notion of control of leukaemia by autologous T cells, patient-derived activated T cells were able to protect against leukaemia development after autologous CLL challenge in a chimeric mouse model.<sup>326</sup>

Finally, early clinical experience with T cell redirection using bispecific antibodies against B cells and CD3 and with chimeric T cell receptors directed against B cells suggests that T cells can eradicate CLL, even in patients with a heavy disease burden.<sup>327,328</sup>

Together, these findings imply that T cells have the potential to mediate long-lasting immunity against CLL, and that at least some patients with CLL have a T cell population capable of recognising their own leukaemic cells. Nonetheless, the fact that patients have developed CLL could be construed as *de facto* evidence that barriers to T cell control of autologous CLL cells exist.

There are many potential reasons that immunological control of CLL might fail, resulting in disease progression.

**Recruitment of suppressive myeloid cells** The principal of immunosuppression by myeloid cells within the tumour microenvironment is well-established in solid malignancies, where MDSCs, a heterogeneous population of myeloid cells, accumulate in tumours and lymph nodes and potently suppress T cell function.<sup>329</sup> In CLL, the term 'nurse-like cells'

has been used to refer to myeloid-derived cells that attract CLL cells in culture and support CLL cell survival. Although derived from CD14<sup>+</sup> precursors, these nurse-like cells have an immunophenotype distinct from monocytes.<sup>330</sup> In addition, monocytes themselves can promote survival of CLL cells through release of soluble CD14.<sup>331</sup>

**Recruitment of regulatory T cells** T<sub>reg</sub> cells exert immunosuppression via both contact-dependent mechanisms and through the release of soluble inhibitors. The number of Tregs is increased in CLL, and higher numbers are associated with a poorer prognosis.<sup>98,332</sup> CLL-derived TGF- $\beta$  or CLL-induced IL-10 may be responsible for T<sub>reg</sub> recruitment.

**Induction of dendritic cell dysfunction** Numeric,<sup>78</sup> immunophenotypic and functional<sup>80</sup> defects have been reported in the circulating DC compartment of patients with CLL. These defects are dependent on CLL cells, as moDCs generated from the same patients in the absence of CLL cells have normal phenotype,<sup>333</sup> and addition of allogeneic CLL cells to healthy donor moDCs results in abnormal DC maturation and function<sup>80</sup>

**Secretion of inhibitory factors** Levels of the immunosuppressive cytokine IL-10 are increased in patients with CLL, and correlate inversely with survival,<sup>104</sup> although the cell of origin is not clear.<sup>334</sup> Tumour cell-derived IL-6 has been proposed as a mediator of CLL immunosuppression; serum IL-6 levels are raised in CLL patients, and higher levels are associated with an inferior prognosis.<sup>91</sup> Soluble forms of the APC costimulatory molecules CD40, CD80 and CD83 exhibit immunosuppressive properties *in vitro*, presumably by binding their ligands on T cells. Levels of soluble CD40, CD80 and CD83 are raised in patients with CLL, and correlate inversely with prognosis.<sup>108-110</sup> CLL cells also produce TGF- $\beta$ ,<sup>335</sup> which suppresses effective DC presentation of tumour antigens.<sup>103</sup>

**Diversion of immunostimulatory factors** CLL cells frequently express CD25, the IL-2 receptor alpha, and may deprive T and NK cells of a proliferative signal by themselves absorbing IL-2<sup>106</sup>

**Resistance to natural killer cell killing** HLA-G is a non-classical HLA class Ib antigen, and is tolerogenic: HLA-G expression on target cells inhibits NK cytotoxicity by binding to NK inhibitory receptors.<sup>336</sup> HLA-G can also suppress CD8<sup>+</sup> cytotoxic T cell responses, and when expressed on antigen presenting cells skews CD4<sup>+</sup> responses towards tolerance. Expression of HLA-G in health is usually restricted to immune-privileged organs including the placenta, thymus, cornea and erythroblasts. CLL cells express surface HLA-G, and blocking antibodies to HLA-G enhance the susceptibility of CLL cells to NK cell killing.<sup>337</sup> Additionally, soluble forms of ligands of the activating NK receptor NKG2D, can reduce NK cell cytotoxicity by inducing downregulation of NKG2D on NK cells. Levels of soluble NKG2D ligands are raised in patients with CLL and correlate inversely with prognosis.<sup>338</sup>

**Restriction of T cell repertoire** Although the diversity of the CD4+ T cell repertoire is well maintained in healthy humans up to 60 years of age, it declines dramatically beyond the age of 70,<sup>339</sup> the median age of patients with CLL at diagnosis. Patients with CLL have evidence of oligoclonal T cell expansions, and exhibit a restricted T cell repertoire as determined by TCR V $\beta$  spectratyping,<sup>326,340</sup> even in comparison with age-matched controls.<sup>87</sup> CLL treatment with alemtuzumab results in a further exacerbation of this CDR3 restriction, although this recovers over time.<sup>341</sup> Thus, there is a possibility that, because of advanced age, chemo- or immunotherapy, or the leukemia itself, patients with CLL do not possess a sufficiently diversified T cell repertoire to be able to counter their own malignant clone.

The majority of the mechanisms of immune evasion of CLL outlined above are dependent on presence of the leukemic clone. Therefore, depletion of leukaemic cells before immunotherapy may enhance immunological responses. This may be one reason that patients who are in remission before undergoing RIC-allo have a significantly better long-term disease-free survival than those who enter the procedure with active disease.<sup>342</sup>

### 1.3.2 Strategies for cellular immunotherapy of CLL

Allo-SCT is the only form of cellular immunotherapy currently in routine clinical use for the treatment of haematologic malignancies, but despite its proven efficacy, the application of allo-SCT remains limited due to high treatment-related morbidity and mortality, cost and a lack of suitable stem cell donors. Cellular immunotherapy strategies with the potential for lower toxicity are therefore attractive.

Many cellular immunotherapy strategies rely on a common series of events that mimic the normal induction of immunity: TAAs are taken up by APCs and presented to T cells. As a result, T cells specific for the TAAs are activated and expand, and directly or indirectly lyse the target tumour cells.

Direct vaccination of a patient with TAAs, such as with whole tumour cells, tumour-derived proteins or peptides, or with tumour DNA or RNA (which is transcribed and/or translated to tumour protein) is simple, and may result in immunological responses against residual tumour within the patient. However, this strategy relies on intact APC and T cell function within the tumour-bearing host.

To overcome potential impairments of patient APC function, some groups have used DC vaccination, in which autologous or allogeneic moDCs are generated *in vitro* under optimal conditions, are pulsed with whole tumour cell, tumour peptide or protein or tumour DNA or RNA, and are subsequently administered as a cellular vaccine, to generate anti-tumour T cell responses *in vivo*.

As DC vaccination may not overcome defects of T cell activation and proliferation in patients with cancer, some groups go further by selecting and expanding tumour-reactive T cells *in vitro*, for subsequent reinfusion. Even this 'adoptive immunotherapy' approach has theoretical limitations, as it presupposes that patients with cancer possess a sufficient T cell repertoire to eradicate the tumour. Administration of allogeneic T cells, or autologous T cells bearing a synthetic TCR or a chimeric antigen receptor directed against a tumour

antigen may overcome this limitation. Finally, patients' own T cells may be redirected against tumour using bispecific antibodies against T cell CD3 and a TAA.

A number of these cellular immunotherapy strategies are illustrated schematically in table 1.15. The next section will discuss each of these strategies, with specific reference to their use in the treatment of CLL.

### 1.3.2.1 Peptide vaccination

Studies of mRNA expression indicate that certain TAAs are expressed in the leukaemic cells of CLL, but not in normal cells. These are potential candidate antigens for peptide, protein, RNA or DNA vaccination strategies. Selected CLL-associated antigens reported in the literature are detailed in table 1.16. Individual or multiple peptide antigens can be administered as vaccines, although even when a single antigen is used, the phenomenon of antigen spreading can result in antitumour responses against tumour-specific peptides other than those vaccinated against.<sup>343</sup>

A phase I clinical trial peptide vaccination in CLL utilised a HLA-A2 restricted RHAMM-derived epitope (the 'R3 peptide') together with GM-CSF and incomplete Freund's adjuvant. This vaccination strategy resulted in proliferation of antigen-specific CD8+ T cells, as detected by tetramer and by ELISpot. White cell counts were lower after the four vaccine doses than before vaccination in four out of six patients, although no patients met criteria for a clinical partial response.<sup>3,344</sup>

Heteroclitic peptides are those that have been modified to bind to MHC with higher affinity, while leaving the TCR recognition residues intact. Heteroclitic peptides can lead to enhanced induction of T cell responses against both the heteroclitic and the native peptide. Cytotoxic lymphocytes generated using heteroclitic Ig-derived peptides exhibited enhanced cytotoxicity against primary CLL cells *in vitro*, compared to those generated using native peptide.<sup>345</sup>

### 1.3.2.2 Tumour cell vaccination

As B cells express MHC class II as well as MHC class I, and are effective, albeit non-professional APCs, CLL cells themselves may be employed as a vaccine. This approach has some appeal, as leukaemic cells are readily available from peripheral blood, and are sufficiently common that minimal or no purification steps are required.

**Unmodified CLL cells** In a phase I study, autologous irradiated leukemic cells were administered intradermally to patients with early CLL with or without the Bacille Calmette-Guerin (BCG) bacterium as an adjuvant. Reductions in the leukocyte count were observed in five of seventeen patients, and a prolongation of the lymphocyte doubling time in seven of seventeen patients. There was no evidence of an improvement in vaccine efficiency with the addition of BCG.<sup>356</sup>

Another clinical trial consisted of repeated intramuscular vaccinations with heat-shocked, ultraviolet light-treated and oxidised autologous whole blood (which included a high

Table 1.15 Methods of generating T cell anti-tumour immunity

Immunotherapeutic strategy	Generation of TAA	APC uptake of TAA	TAA presentation to T cells	T cell expansion	Tumour killing
Allogeneic stem cell transplantation	<i>In vivo</i>				
Whole tumour cell vaccination	<i>In vivo</i>				
Peptide or protein vaccination	<i>In vitro</i>		<i>In vivo</i>	<i>In vivo</i>	<i>In vivo</i>
DC vaccination	<i>In vitro</i>			<i>In vivo</i>	<i>In vivo</i>
Adoptive T cell transfer	<i>In vitro</i>				
Transgenic T cell transfer		—		<i>In vitro</i>	<i>In vivo</i>
T cell redirection		—			<i>In vivo</i>

Table 1.16 Selected CLL tumour-associated antigens

CLL-associated antigen	Frequency of expression	T cell responses	Reference(s)	Notes
RHAMM *	77%	3/6 patients showed RHAMM R3 peptide-specific ELISPOTs after restimulation. Expansion of R3-specific CD8+ T cells seen in 2/3 cases	346	Clinical trial of R3 peptide vaccination reported <sup>3,344</sup>
Fibromodulin	63 - 100%	Fibromodulin-specific CD8+ T cells detected by ELISPOT and tetramer after restimulation	346,347	
Id †	100%	Antigen differs for each patient. 67% of patients have Id-peptide reactive T cells	348,349	
hTERT ‡	76%	hTERT-specific cytotoxic T cells expanded from 6/7 telomerase CLL patients	350	A second study showed no hTERT expression in CLL <sup>346</sup>
ROR1	100%	Anti-ROR1 antibodies detected in sera of 3/6 patients who received iv autologous CD40L-transfected CLL cells	351,352	
Ly9 (CD229)	100%	Expansion of CD229-specific T cells observed in 5/5 cases after 2x restimulations	353	
MDM2 §	78 – 85%	T cell responses demonstrated by ELISPOT and tetramer staining	354	
CD23	100%	T cell responses demonstrated by ELISPOT and tetramer staining	355	

\* receptor for hyaluronic acid mediated motility; CD168

† idiotype (specific CLL cell BCR)

‡ human telomerase reverse transcriptase

§ human homologue of murine double-minute 2

proportion of CLL cells). Clinical partial responses were seen in 5 out of 18 patients with CLL.<sup>357</sup>

**CD40L-activated CLL cells** Although they express MHC Class II, unmodified CLL cells are poor inducers of allogeneic T cell responses.<sup>358</sup> However, CLL cells express CD40, and upon treatment with CD40 ligand (CD40L, or CD154), upregulate costimulatory and adhesion molecules, and become potent presenters of antigen to both CD4+ and CD8+ allogeneic T cells.<sup>359</sup>

The effect of CD40L-activated CLL cells may be somewhat different in the autologous setting: CD40L-activated CLL cells led to expansion of CD8+ cytolytic T cells in the allogeneic setting, but in the autologous setting resulted in expansion of a population of CD4+ Th1-like (IFN- $\gamma$  producing) T cells.<sup>360</sup> A separate study also showed impaired CD8+ responses to CD40L-activated CLL cells in the autologous setting.<sup>361</sup>

An alternative approach to providing a CD40L signal to CLL cells is to induce CLL cells to express CD40L themselves. Transfection of human CLL cells with an adenoviral vector encoding mouse CD154 (CD40L) resulted in crosslinking of CD40 on the transduced, and on bystander CLL cells. This resulted in upregulation of costimulatory molecules on the CLL cells and improved allogeneic and autologous T cell responses to the leukaemic cells *in vitro*.<sup>362</sup> Co-transfer of both CD40L and the additional accessory molecule OX40 ligand (OX40L) to CLL cells may further enhance and enable expansion of autologous cytotoxic CD8+ cells.<sup>363</sup>

One concern regarding CD40 ligand-stimulated B cells is that CD40L treated CLL cells are resistant to apoptosis. However, despite increased resistance to chemotherapy-induced apoptosis, CD40L-treated, peptide-pulsed CLL cells remain sensitive to killing by autologous T cells.<sup>364</sup>

Phase I clinical trials of administration of autologous CD40 activated CLL cells have been reported. In one study, eleven patients were treated with autologous CLL cells transfected with mouse CD40L.<sup>324</sup> This resulted in at least a doubling of T cell numbers and a transient reduction in CLL cell count in most patients. Interestingly, reductions in lymph node size were more marked and prolonged. An increase in T cell responses to autologous CLL cells was observed by ELISpot and mixed lymphocyte reaction (MLR). A further study using CLL cells transfected with human CD40L yielded similar results, including regression of lymphadenopathy and a prolongation of lymphocyte doubling time in around half of patients.<sup>365</sup>

In a clinical trial CD40L- and IL-2 transfected CLL cells were co-administered as a subcutaneous vaccine to nine patients with CLL. *In vitro* T cell responses against autologous CLL cells were observed in seven patients, but no reduction in circulating CLL cells was seen, and only three patients exhibited a transient reduction in lymphadenopathy.<sup>366</sup> An increase in CD4+CD25+ T cells, a phenotype associated with T<sub>reg</sub> activity, was seen after vaccination, raising the possibility that the IL-2 transfected CLL cells promoted T<sub>reg</sub> proliferation.



**B-7-, ICMA-1- and LFA-3- transfected CLL cells** The transfection of CLL cells with a viral vector encoding the co-stimulatory molecules B7-1, ICAM-1 and LFA-3 resulted in a similar degree of augmentation of autologous T cell responses to transfection with CD40L *in vitro*.<sup>367</sup>

**Toll-like receptor ligand-treated CLL cells** CLL B cells express the Toll-like receptors (TLRs) TLR-1, TLR-2, TLR-6, TLR-7 and TLR-9. TLR stimulation can result in production of inflammatory cytokines including IL-6 and TNF- $\alpha$ , upregulation of costimulatory molecules such as CD86, enhanced immunogenicity in MLRs and can increase CLL cell susceptibility to cytotoxic chemotherapies. A case report describes regression of a cutaneous CLL deposit with topical imiquimod, a TLR-7/8 agonist.<sup>357</sup> However, a subsequent phase I trial of the imidazoquinoline TLR-7 agonist 852A given intravenously as monotherapy to patients with relapsed CLL did not result in clinical responses.<sup>368</sup> A note of caution should also be sounded, as TLR ligands can trigger CLL cell proliferation.<sup>369–371</sup> Nonetheless, interest in TLR ligands in combination with chemotherapies, or as adjuvants to vaccination strategies, remains.

**Epigenetically-modified CLL cells** CLL cells treated with a combination of a demethylation and histone deacetylase inhibitor upregulated cancer testis antigens and costimulatory molecules, becoming more immunogenic in an allogeneic assay *in vitro*.<sup>372</sup>

**Trioma vaccine** ‘Trioma’ cells are produced by fusing tumour cells with an anti-CD64 mouse hybridoma. CD64 is the high affinity IgG receptor (Fc $\gamma$ RI), and is expressed on macrophages and monocytes. Trioma CLL cells possess TAAs and express anti-CD64, which targets them to macrophages and monocytes. A CLL trioma vaccine was tested for T cell activating activity *in vitro*, and resulted in expansion of T cells against antigens overexpressed on the original leukemic cells.<sup>373</sup> No clinical studies of this approach have been published to date.

#### 1.3.2.3 Dendritic cell vaccination

As defects of DC function have been described in CLL, a rational approach may be to generate functional DCs *in vitro*, pulse these with tumour antigen, and deliver tumour-loaded DCs as a vaccine. In support of this approach, patient moDCs loaded with CLL cell lysates can stimulate proliferation of tumour-specific T cells *in vitro*.<sup>374</sup>

Various sources of TAAs for DCs have been studied, including apoptotic tumour cells themselves, whole cell lysates, peptide eluates or tumour RNA. A phase I trial used autologous moDC loaded with cell lysates, and resulted in reductions in CLL cell burden in five of twelve patients.<sup>356,375</sup> A phase 1 study of vaccination of patients with low-grade B cell malignancies with autologous moDC loaded with eluted peptide included two patients with CLL: neither patient experienced a clinical response.<sup>376</sup> *In vitro* studies suggest that in CLL, apoptotic tumor cells provide stronger autologous T cell responses than tumour cell lysate or RNA.<sup>377</sup> An alternative strategy is to fuse tumour cells with DCs, although

in one study, CLL/DC fusion hybrids exhibited a lower antigen presenting capability than DCs loaded with apoptotic CLL cells.<sup>378</sup>

Despite occasional clinical responses, the adoption of autologous DC vaccination for CLL has been limited by difficulties in generating moDCs from CLL patients. Yields of moDC are often lower in CLL patients than in healthy donors.<sup>376</sup> This may be in part because monocytes constitute a smaller proportion of circulating nucleated cells when circulating leukemic cells are frequent. In addition, CLL cells can directly interfere with moDC generation.<sup>81</sup> Immunomagnetic selection of CD14+ monocytes can overcome these problems,<sup>379</sup> and a clinical-scale process for this has been described.<sup>380</sup>

One way of overcoming practical difficulties in the generation of moDC from patients with CLL, and bypassing any defective moDC function would be to make use of allogeneic rather than autologous moDC as antigen presenters. A phase I clinical trial of DC vaccination with allogeneic moDCs, loaded with whole cell lysates or apoptotic bodies<sup>325</sup> resulted in transient reductions in white cell counts in some patients, but no durable objective clinical responses.

### 1.3.2.4 Adoptive T cell transfer

**Activated autologous T cells** In one study, five of twenty patients with CLL had circulating T cells exhibiting *in vitro* cytotoxicity against autologous CLL cells. When these patients' T cells were activated by CD3/CD28 engagement and co-administered to mice alongside CLL cells in a xenograft model, CLL cells were eradicated. Unactivated T cells, and activated T cells from patients which did not show *in vitro* cytotoxicity, did not eradicate CLL.<sup>326</sup>

A phase I/II clinical trial of CD3/CD28 activated autologous T cells for CLL has been performed. Final results have not been published, but a preliminary abstract indicated that the treatment led to reductions in lymphadenopathy, but no reduction in circulating CLL cell numbers.<sup>381</sup>

**Allogeneic T cells** The experience of allo-SCT indicates that allogeneic T cells have the capacity to eradicate leukaemia cells in CLL. In one notable case report, the administration of CD3/CD28-activated donor T cells resulted in a complete clinical response in a patient with persistent CLL after allo-SCT.<sup>382</sup>

The major disadvantages of using allogeneic T cells are the need for tissue-type matching and the risk of GVHD in the recipient. One approach that may overcome both of these risks is the generation of allo-restricted antigen-specific T cells.

MoDCs from healthy HLA-A\*0201-negative donors were engineered to express HLA-A\*0201 presenting a peptide from CD20 (a B cell antigen). These moDCs were cultured with autologous T cells. T cells specific for the CD20 peptide presented on HLA-A\*0201 expanded, and could be detected and purified using a pentamer. *In vitro*, these allo-restricted CD20-specific T cells were able to specifically lyse CLL lines from HLA-A\*0201 patients. HLA-A\*0201 negative cells and CD20 negative cells were not lysed.<sup>383</sup>

A potential disadvantage of this approach is that allo-restricted antigen-specific T cells generated in this way would be susceptible to rejection by the leukaemia-bearing host.

**Transgenic TCR** Transfection of autologous T cells with a transgenic TCR specific for a tumour antigen holds the promise of overcoming deficiencies in the patient's T cell repertoire. Preclinical and clinical studies suggest that this is a promising approach.

In a xenograft model, T cells from patients with myeloid leukaemias were transfected to express a TCR specific for the Wilms' tumour 1 (WT1) tumour antigen. These T cells were able to eradicate a human leukaemia cell line in NOD/severe combined immunodeficiency (SCID) mice.<sup>384</sup>

Lymphodepleting chemotherapy followed by adoptive transfer of autologous T cells transfected with a TCR specific for the tumour-associated antigen NY-ESO-1 led to objective clinical responses in five of eleven patients with metastatic melanoma and four of six patients with synovial cell carcinoma.<sup>385</sup> Clinical responses have also been reported following adoptive transfer of autologous T cells bearing transgenic TCRs specific for the tumour-associated antigen MART-1 in patients with metastatic melanoma.<sup>386</sup>

A major disadvantage of the use of transgenic TCR T cells is that the marked polymorphism of the HLA locus means that the generation and validation of antigen-specific TCRs to cover a comprehensive range of tissue types and malignancies would be a large undertaking. There are also theoretical concerns that heterodimerisation of a transgenic TCR- $\alpha$  or  $\beta$  chain with a native TCR chain might generate a new TCR with undesired (for instance, autoreactive) specificity.<sup>387</sup> Finally, tumour cells may downregulate MHC, reducing their susceptibility to TCR-transduced T cells.<sup>388</sup>

**Chimeric antigen receptors** Chimeric antigen receptors (CARs) comprise a single chain antibody fragment linked to the CD3  $\zeta$  chain of the T cell receptor. In some constructs, the CD3  $\zeta$  chain is also linked to costimulatory molecules such as CD28 or CD137 to provide a co-stimulatory signal. Upon recognition of the CAR cognate antigen, T cell signalling is triggered, and effector functions take place, thus CARs confer specificity without tissue-type restriction.

T cells transfected with a CAR against CD19 or against the CLL-associated antigen ROR1 can lyse autologous CLL cells *in vitro*.<sup>389,390</sup> An anti-CD23 CAR showed promising efficacy *in vitro* and in a xenograft model *in vivo*.<sup>391</sup>

The efficacy of CD19-directed CAR T cells for follicular lymphoma in a phase I clinical trial was limited, perhaps owing to short persistence of the CAR-transfected T cells.<sup>392</sup> However, preliminary results from subsequent trial which incorporates T cell depletion before, and high dose IL-2 after, infusion of CAR T cells indicate that this treatment has the potential to control follicular lymphoma.<sup>393</sup>

Finally, a phase I clinical trial of CD19-directed CAR T cells as a treatment of CLL has shown positive preliminary results, with complete remission of a patient with refractory CLL and persistence of chimeric T cells six months after infusion.<sup>328</sup>

**Risks of adoptive T cell transfer** As with all treatments, adoptive T cell transfer is not without risk: in an animal study of adoptive T cell transfer combined with a long peptide vaccine, >1000 fold expansion of the transferred CD8+ T cells was observed. Subsequent repeated vaccination caused a cytokine storm and multi-organ failure<sup>394</sup>

In a phase I clinical trial of CAR T cells for CLL, one patient died of a sepsis-like syndrome, which began 20 hours after the infusion of autologous CAR T cells following cyclophosphamide chemotherapy.<sup>395</sup> The death was not definitively attributed to CAR T cell infusion, but modifications to the infusion protocol were subsequently made.

Clinical trials of gene therapy for severe combined immunodeficiency have demonstrated the potential for retroviral vectors to cause malignancies, by causing over-expression of proto-oncogenes.<sup>396</sup> One way around this is to transfect T cells with mRNA for a CAR. T cells transfected with the mRNA for an anti-CD19 were able to lyse target B cells up to 8 days after transfection *in vitro*.<sup>397</sup>

Finally, autoimmunity is a potential risk for all immunotherapies.<sup>398</sup> On-target autoimmunity is particularly well described in clinical trials of transgenic TCR therapy for melanoma: vitiligo was frequently observed, 25% of patients required treatment for anterior uveitis (melanocytes are present in the eye), and around a third developed mild hearing loss (melanocytes exist in the inner ear).<sup>399</sup> Similarly, clinical trials of CD19-directed CAR T cells result in a profound depletion of normal, polyclonal B cells,<sup>397</sup> the long-term impacts of which are not yet known. Finally, off-target autoimmunity may occur, either through cross-reactivity of the transfected receptor, epitope spreading, or through activation of a clone of transfected T cells which express a native TCR with autoreactive potential.

### 1.3.2.5 T cell redirection

Bispecific T cell engagers consist of two single-chain antibodies fused together. One antibody is directed against a T cell antigen, usually CD3, and the other against the target cell. The most advanced in clinical studies is blinatumomab, which has specificity for both CD19 and CD3, re-directing T cells against B cells. In a dose-finding study in non-Hodgkin lymphoma, complete or partial responses were seen in all seven patients treated at the highest dose level. Two patients with CLL were treated, both at lower dose levels, one of whom had a partial response.<sup>400</sup> Many of the clinical responses observed have proved durable, raising the possibility that this form of T cell engagement may be able to recruit endogenous tumour-specific T cells capable of long-term disease control.<sup>327</sup>

A related agent makes use of a single chain antibody fused to HLA. Expansion of CMV-reactive CD8+ T cells is observed in many patients with CLL. A streptavidin-linked anti-human CD20 single-chain variable fragment (scFv), coupled to biotinylated HLA class I loaded with CMV peptide, is used to target CMV-reactive T cells to autologous B cells. This reagent redirected CMV reactive CD8+ T cells to recognise and kill CLL cells *in vitro*.<sup>401</sup>

### 1.3.3 Incorporating iNKT cells into immunotherapy

As discussed in section 1.2.2, iNKT cells possess a semi-invariant TCR capable of recognising glycolipid antigens in the context of the MHC Class I-like molecule CD1d. In response to TCR engagement, iNKT cells produce Th1- and Th2-type cytokines and upregulate surface co-stimulatory molecules such as CD40L. Together, these soluble and contact-dependent signals can transactivate other cell types, such as NK cells and DCs.

The interactions between iNKT cells and other cells of the innate and adaptive immune system have been discussed in section 1.2.6. Specific capabilities of iNKT cells that might usefully be exploited for tumour immunotherapy include direct cytotoxicity against CD1d-positive target cells in the presence of  $\alpha$ -GalCer,<sup>310</sup> induction of DC maturation and consequent enhancement of CD4+ and CD8+ T cell responses to DC-presented antigens,<sup>258</sup> transactivation of NK cells and enhancement of NK cytotoxicity,<sup>402</sup> provision of T cell help for the generation of B cell immunity,<sup>266</sup> and depletion of MDSCs and TAMs.<sup>282,283</sup>

Other lymphocyte populations, such as conventional CD4+ and CD8+ T cells or NK cells, can exhibit many of these effects, but iNKT cells possess a unique combination of features.

- **Universal agonist:** T cells bearing the specific iNKT cell TCR can be detected most individuals, if at a low frequency.<sup>220</sup> Thus, a population of iNKT cells capable of responding to  $\alpha$ -GalCer in the context of CD1d is usually present, irrespective of tissue type or of the constitution of rest of the T cell repertoire.
- **Universal presenting molecule:** Unlike HLA, the CD1d locus is minimally polymorphic.<sup>193</sup> This allows the specific activation of iNKT cells by  $\alpha$ -GalCer on CD1d to be exploited in all individuals, without tailoring the therapy to the tissue type.
- **Rapid activation upon first stimulation:** iNKT cells are potent cytokine producers, and like effector memory cells, do not require priming—cytokine release occurs upon the first stimulation with  $\alpha$ -GalCer.

Together with the observations of increased cancer susceptibility in some iNKT cell-deficient mouse models (discussed in section 1.2.7.3), and experimental evidence that iNKT cell activation can protect against inoculated tumours, these factors have prompted extensive investigation of the potential for iNKT cells in tumour immunotherapy.

Broadly speaking, three principal tumour immunotherapy strategies exploiting iNKT cells have been employed:

1. Simple activation of iNKT cells, resulting in cytokine release and consequent transactivation of other immune system cells, such as NK cells. Methods of achieving this include systemic administration of  $\alpha$ -GalCer, or administration of unloaded APCs that have been pulsed with  $\alpha$ -GalCer.
2. *Ex vivo* expansion of iNKT cells for use as effector cells, which can lyse CD1d-positive target tumour cells in the presence of  $\alpha$ -GalCer.
3. Administration of  $\alpha$ -GalCer in conjunction with tumour antigen, as an adjuvant. Methods of achieving this include  $\alpha$ -GalCer pulsing of tumour-loaded DCs,  $\alpha$ -GalCer

pulsing of whole tumour cell vaccines, or co-administration of  $\alpha$ -GalCer with a protein or peptide vaccine.

In the following sections, I shall review preclinical and clinical studies of iNKT cell-directed tumour immunotherapy approaches for the treatment of malignancy.

### 1.3.3.1 Activation of iNKT cells

**Systemic  $\alpha$ -GalCer administration** An improvement in the survival of mice inoculated with subcutaneous B16 melanoma tumours following intravenous  $\alpha$ -GalCer was one of earliest observations of the biological activity of  $\alpha$ -GalCer,<sup>403</sup> and preceded the discovery of iNKT cells. Numerous studies have since confirmed the protective effect of intravenous or intraperitoneal  $\alpha$ -GalCer.

Intravenous  $\alpha$ -GalCer has been administered to patients in clinical trials. In a phase 1 dose-finding study,  $\alpha$ -GalCer was delivered intravenously once weekly for three weeks at each of seven escalating dose levels to patients with a variety of solid cancers. Doses of 50 – 4800  $\mu\text{g}/\text{m}^2$  were administered, and were generally well tolerated. Intravenous  $\alpha$ -GalCer led to a rapid and profound reduction in circulating iNKT cell numbers within 24 hours, which persisted for at least a week. Serum cytokine responses were restricted to patients with the highest numbers of circulating iNKT cells pre-treatment. No clinical responses were observed.<sup>303</sup>

A phase I/II trial of intravenous  $\alpha$ -GalCer for chronic hepatitis B confirmed its safety and capacity to produce a rapid decrease in circulating iNKT cells in humans. Significant reductions in viral yield were not observed.<sup>404</sup>

Thus, despite initial promise in preclinical models, intravenous administration of  $\alpha$ -GalCer has not yet proved effective. Several factors may account for this failure. The systemic administration of  $\alpha$ -GalCer to mice results in an initial expansion of iNKT cells followed by anergy. These anergic iNKT cells are refractory to subsequent  $\alpha$ -GalCer administration, failing to produce IFN- $\gamma$ . Moreover, in mice which have received a single dose of  $\alpha$ -GalCer one month previously, the administration of  $\alpha$ -GalCer alongside tumour no longer protects against melanoma metastasis.<sup>405</sup> Repeated systemic  $\alpha$ -GalCer administration leads to a Th2 bias in whole restimulated splenocytes.<sup>406</sup> Finally, circulating iNKT cell numbers are typically much lower in humans than in mice.

The impressive effects of systemic  $\alpha$ -GalCer in early preclinical tumour models may be reconciled with the disappointing results in clinical trials: A single dose of systemic  $\alpha$ -GalCer administered to a mouse with high numbers of circulating iNKT cells leads to a dramatic but short-lived increase in pro-inflammatory cytokines, including IFN- $\gamma$ . This transiently activates effector cells, such including NK cells. Provided  $\alpha$ -GalCer is given at, or near, the time of tumour inoculation, transactivated NK cells may eradicate the tumour. After initial activation, however, iNKT cells become refractory to further stimulation, and lose their cytokine-producing and cytotoxic functions:  $\alpha$ -GalCer is no longer protective against tumour challenges.

**$\alpha$ -GalCer-pulsed unloaded dendritic cells** Unlike intravenous  $\alpha$ -GalCer, which results in a rapid but brief rise in serum cytokines,  $\alpha$ -GalCer loaded onto DCs results in a delayed and more sustained rise in serum IL-12, IL-4 and IFN- $\gamma$ . Moreover, compared to free intravenous  $\alpha$ -GalCer,  $\alpha$ -GalCer-loaded DCs provide superior protection against lung metastases of B16 melanoma in mice.<sup>407,408</sup>

A number of phase I clinical studies of  $\alpha$ -GalCer pulsed DCs for the treatment of cancer have been undertaken, and are summarised in table 1.17. It is notable that none of these studies used DCs that had also been pulsed with a tumour-specific antigen—the DCs were employed as a means of activating iNKT cells rather than to present tumour antigens. It is also important to note that only two of the studies used-mature moDC; the remainder used either immature moDC or GM-CSF treated PBMCs as APCs. Apart from the study by Kunii et al,<sup>409</sup> in which iNKT cells and APCs were co-administered, the most significant immunologic and clinical responses appeared to occur following treatment with ‘professional’ DCs (i.e. moDC).

#### 1.3.3.2 Use of iNKT cells as effectors

As the frequency of circulating iNKT cells is reduced in many patients with cancer, some authors have proposed the *ex vivo* expansion of iNKT cells for use as effectors or as adjuvants in subsequent cellular immunotherapy. Preclinical studies lend support to this approach: The adoptive transfer of iNKT cells to iNKT-deficient mice provides relative protection against methylcholanthrene-induced sarcoma.<sup>301</sup>

A mouse model demonstrates the capacity of *ex vivo* expanded iNKT cells to eradicate a CD1d-transfected B cell malignancy *in vivo*.<sup>310</sup> The effect was CD1d-specific, as tumour lines transfected with CD1c were not killed. This is consistent with a similar report in a T cell malignancy,<sup>309</sup> and suggests that target cell CD1d expression is a prerequisite for iNKT cell cytotoxicity.

iNKT cells could prove useful effector cells against leukaemias: Human myelomonocytic leukemia and T cell acute lymphoblastic leukaemia cells express CD1d and are sensitive to killing by iNKT cells.<sup>229,416</sup> Importantly CD1d is expressed on CLL cells, and  $\alpha$ -GalCer pulsed human CLL cells can be lysed by iNKT cells *in vitro*.<sup>417</sup>

The feasibility and safety of adoptive iNKT cell transfer was demonstrated in phase I clinical study of *in vitro* expanded iNKT cells in six patients with advanced and recurrent non-small cell lung cancer.<sup>418</sup> However, despite transient increases in circulating iNKT cells, no clinical responses were seen in this setting.

Transfer of iNKT cells has also been used in a hybrid approach: In a phase I clinical study in head and neck cancer,  $\alpha$ -GalCer loaded APCs were administered submucosally, followed by intra-arterial injection of IL-2 and  $\alpha$ -GalCer-treated PBMCs, which were rich in iNKT cells. This resulted in a substantial increase in circulating iNKT cell numbers in seven out of eight patients, and tumour regression in three.<sup>409</sup>

Table 1.17 Clinical trials of  $\alpha$ -GalCer-pulsed DCs for cancer immunotherapy

APC	Route	Patients (n)	Tumours	Immunological responses	Clinical responses	References
GM-CSF/IL-2 PBMCs	Intravenous	11	Non-small cell lung cancer	1/11 patients showed marked increase in iNKT cells and IFN- $\gamma$ production	None	410
GM-CSF/IL-2 PBMCs	Intravenous	17	Non-small cell lung cancer	Transient increase in iNKT nos in 6/17. Increased $\alpha$ -GalCer induced IFN- $\gamma$ ELISPOTs in 10/17	None	411
Immature modDCs	Intravenous	12	Various	Decline, then increase in iNKT cells in 12/12. Increased IFN- $\gamma$ , in 12/12. NK activation.	'Biochemical response' in 5/12	412
Mature modC*	Intravenous	5	Various (including 3 myeloma)	Increased iNKT cells in 5/5. Increased IL12p40, IP-10 + MIP-1 $\beta$ . Increased CMV-specific CD8 + T cells	Two partial responses	413
GM-CSF/IL-2 PBMCs	Nasal submucosa	9	Head and neck cancer	Increased iNKT cells in 4/9, and IFN- $\gamma$ , ELISPots in 8/9	One partial response	414
GM-CSF/IL-2 PBMCs plus 'iNKT' <sup>†</sup>	Nasal submucosa (iNKTs intra-arterial)	8	Head and neck cancer	Increased iNKT cells in 7/8. Increased IFN- $\gamma$ ELISPots in 7/8	Three partial responses	409
Mature modC* plus lenalidomide	Intravenous	6	Myeloma	Reduced circulating iNKT cells. Increased NK cells at day 7 <sup>‡</sup> . Increase in CD16+ monocytes. <sup>‡</sup>	Paraprotein fell in 3/4 assessable <sup>‡</sup>	415

\* Maturation cocktail: IL-1 $\beta$ , IL-6, TNF- $\alpha$ , PGE<sub>2</sub>

<sup>†</sup> IL-2 and  $\alpha$ -GalCer-activated PBMCs

<sup>‡</sup> May represent a response to concomitant lenalidomide



### 1.3.3.3 iNKT cells as adjuvants

**$\alpha$ -GalCer-pulsed loaded dendritic cells** The clinical trial results presented in table 1.17 imply that simply activating patient iNKT cells with  $\alpha$ -GalCer pulsed DCs is not sufficient to eradicate tumour. As iNKT cells are able to induce DC maturation, a rational step is to recruit iNKT cells to DCs that are presenting tumour peptides. That is, to present  $\alpha$ -GalCer and tumour peptide on the same DC.

In mice, optimal enhancement of peptide-specific CD4+ and CD8+ responses by  $\alpha$ -GalCer requires presentation of antigen and  $\alpha$ -GalCer on the same DC.<sup>258,419</sup> This co-operation extends to DCs loaded with whole tumour: addition of  $\alpha$ -GalCer to tumour-loaded as opposed to unloaded DCs results in enhanced tumour protection.<sup>420</sup>

Studies performed on human cells *in vitro* also support this concept: moDCs transfected to over-express IL-12 and pulsed with  $\alpha$ -GalCer and a TAA peptide led to better expansion of TAA-specific CD8+ T cells than did non- $\alpha$ -GalCer pulsed ones.<sup>276</sup>

Interestingly,  $\alpha$ -GalCer can even enhance the efficacy of non-professional APCs in some settings. B cells pulsed with  $\alpha$ -GalCer and peptide upregulate CD86 after administration to mice, and produce enhanced CD8+ T cell responses to the peptide compared to B cells without  $\alpha$ -GalCer, including tumour protection. The responses observed in response to  $\alpha$ -GalCer-pulsed B cells were nearly as good as those seen with  $\alpha$ -GalCer-pulsed DCs.<sup>421</sup> This is particularly pertinent in the case of CLL, a B cell malignancy, as it implies that  $\alpha$ -GalCer-pulsed tumour cells may be an effective immunogen.

**$\alpha$ -GalCer-pulsed tumour cells** Generation of clinical-grade moDCs is a costly exercise, typically requiring several cell purification steps and a period of *in vitro* culture.<sup>380</sup> An alternative strategy is to use whole tumour cells as a vaccine, using these to carry both  $\alpha$ -GalCer and tumour antigen to host resident DCs.

Preclinical studies demonstrate that  $\alpha$ -GalCer-pulsed irradiated tumour cells delivered as a whole tumour vaccine can produce durable antitumour immunity. In this model, resident APCs take up the  $\alpha$ -GalCer-loaded tumour cells and present TAAs alongside  $\alpha$ -GalCer. Presentation of  $\alpha$ -GalCer to iNKT cells results in DC maturation and the local production of cytokines, which together lead to enhanced CD4+ and CD8+ T cell-dependent antitumour immunity.<sup>422</sup> This effect was enhanced when the target tumour cells (B16 melanoma cells) were transfected to express high levels of CD1d. Importantly, vaccination with CD1d-transfected B16 cells protected against subsequent challenge with untransfected B16 cells. The efficacy of this vaccination approach has also been demonstrated in the therapeutic setting:  $\alpha$ -GalCer-pulsed B16 melanoma cells administered three days after a challenge with unmodified B16 melanoma cells protected against development of metastases.<sup>423</sup>

Preclinical studies have explored the use of  $\alpha$ -GalCer pulsed tumour cells as vaccines for haematologic malignancies, many of which express CD1d.

- The non-immunogenic murine B cell lymphoma cell line A20 expresses CD1d at a low level. Lymphoma cells pulsed with  $\alpha$ -GalCer led to iNKT cell activation and expansion, and proved a potent prophylactic and therapeutic tumour vaccine *in*

*vitro*. The tumour protection elicited by  $\alpha$ -GalCer-pulsed lymphoma cells was CD4+ T cell-dependent.<sup>424</sup>

- Plasmacytoma cells were irradiated and pulsed with  $\alpha$ -GalCer before intravenous administration. The dying plasmacytoma cells were taken up by splenic DCs, leading to maturation of the DCs, and generation of protective immunity against subsequent tumour challenge. This protection was tumour-specific, and required both CD4+ and CD8+ T cells.<sup>425</sup>
- Pulsing of the WEHI-3B myelomonocytic leukaemia cell line with  $\alpha$ -GalCer before intravenous inoculation protected against development of tumour upon rechallenge. In contrast, a T cell leukaemia cell line (EL-4) had to be transfected with CD1d to observe protection by  $\alpha$ -GalCer pulsing.<sup>422</sup>

Administration of  $\alpha$ -GalCer-pulsed tumour cells thus has the potential to offer a simple and effective immunotherapeutic strategy, at least for tumour cells which express CD1d.

There may be a barriers to the effective clinical administration of autologous  $\alpha$ -GalCer-pulsed tumour cells, including lack of availability of tumour tissue, concerns about the induction of metastasis, and for some malignancies, lack of CD1d expression on the tumour. One novel solution has been to transfect allogeneic fibroblasts with mRNA encoding TAAs. These fibroblasts, which express CD1d, are then pulsed with  $\alpha$ -GalCer and administered as a tumour vaccine.<sup>426</sup>

**Co-administration of antigen with  $\alpha$ -GalCer** The role of  $\alpha$ -GalCer as an adjuvant is not restricted to whole tumour cell vaccines: Responses to peptide, protein and DNA vaccination can also be enhanced with  $\alpha$ -GalCer.

As has already been discussed, the co-administration of the ovalbumin (OVA) with  $\alpha$ -GalCer results in enhanced OVA-specific T cell responses, an effect mediated by  $\alpha$ -GalCer matured DCs. As with intravenous  $\alpha$ -GalCer administration, the  $\alpha$ -GalCer needs to be delivered at the same time as OVA to yield an optimal effect, at least in the intravenous setting.<sup>258</sup>

In the infectious disease field, preclinical models have demonstrated the efficacy of  $\alpha$ -GalCer as an adjuvant when co-administered with vaccines of diverse types. Responses were augmented when  $\alpha$ -GalCer was co-administered with vaccines comprised of irradiated malaria sporozoites,<sup>427</sup> herpes simplex glycoprotein,<sup>428</sup> inactivated influenza A virus,<sup>429</sup> and HIV DNA.<sup>430</sup>

### 1.3.3.4 Other approaches

Other approaches that utilise iNKT cells for cancer immunotherapy have been proposed or investigated.

One novel approach has been to generate a fusion protein comprising an  $\alpha$ -GalCer-loaded soluble CD1d molecule linked to a scFv with anti-tumour specificity. This simultaneously

targets iNKT cells to the tumour and activates them. In a preclinical study, this fusion protein inhibited the growth and metastasis of a B16 melanoma.<sup>431</sup>

A role for iNKT cells after allo-SCT has been proposed. Studies in mice suggest that iNKT cells can co-operate with Tregs to suppress GVHD, apparently without abrogating the graft versus tumour effect.<sup>432</sup> Delayed iNKT cell reconstitution after haploidentical allo-SCT for children with haematologic malignancies has been associated with increased relapse risk, leading to the suggestion that donor iNKT cells could be expanded *ex vivo*, and returned to the recipient.<sup>433</sup>

Finally, responses to iNKT cell-based immunotherapies can be enhanced by combining them with other adjuvants or with strategies to suppress negative regulation of immune responses. As well as causing upregulation of costimulatory markers, Toll-like receptor agonists enhance glycolipid presentation by human APCs and result in increased iNKT cell IFN- $\gamma$  production.<sup>434</sup> Abrogation of the Programmed Cell Death-1 (PD-1) pathway results in enhanced tumour responses.<sup>435</sup> Finally, depletion of T<sub>reg</sub> populations enhances antigen-specific CD8+ T cell responses to tumour-loaded,  $\alpha$ -GalCer pulsed DCs.<sup>420</sup>

## 1.4 Conclusions

CLL is a common malignancy, caused by the proliferation of clonal mature B lymphocytes in blood, bone marrow and the lymphoreticular system. The malignant cells are antigen-experienced, chronically-stimulated memory B cells, typically carrying an autoreactive IgM B cell receptor (BCR). Constant stimulation of the BCR by autoantigens or ubiquitous microbial antigens gives rise to the characteristic CD5+ CLL phenotype. Although CLL cells undergo spontaneous apoptosis *in vitro*, interactions between the malignant cells and T, myeloid and stromal cells, sustain the malignant clone *in vivo*. Over time, additional genomic changes occur within the clone, resulting in a more clinically-aggressive phenotype. Effective treatments exist, the mainstay being chemoimmunotherapy with a purine analogue, alkylating agent and the anti-CD20 antibody rituximab. However, the only curative treatment, allo-SCT is only available to a minority of patients because of advanced age or co-morbidities.

CLL appears to be a promising candidate for adoptive immunotherapy, because prolonged remissions after allo-SCT and after T cell redirection therapies indicate that CLL is susceptible to T cell killing, because a proportion of patients possess circulating T cells with specificity against their leukaemic clone, the number of these T cells increasing after therapeutic tumour vaccination, and because spontaneous remissions, although rare, are well documented.

Invariant natural killer T cells, a rare subset of T cells, possess a semi-invariant T cell receptor which confers reactivity to glycolipid antigens such as  $\alpha$ -GalCer presented by the MHC Class I-like molecule, CD1d. Functionally, iNKT cells combine properties of T cells, such as the capacity to induce DC maturation and to provide B cell help, with characteristics reminiscent of NK cells such as cytotoxicity, rapid cytokine release and even NK receptor-mediated alloreactivity. Patients with some malignancies have reduced iNKT cell numbers or exhibit impaired iNKT cell function, suggesting that iNKT cells may

play a role in tumour surveillance or control. Consistent with this, the activation of iNKT cells promotes anti-tumour immunity in a number of mouse models.

Numerous adoptive immunotherapy strategies have been proposed or tested in CLL, including peptide vaccination, whole tumour vaccination, DC vaccination, adoptive T cell transfer and T cell redirection.

Since CD1d is minimally polymorphic, and because iNKT cells are detectable in most individuals, the CD1d/iNKT cell axis can be readily exploited for immunotherapies without paying heed to tissue type or NK receptor profile. Potential roles for iNKT cells in tumour immunotherapy include:

- Non-specific activation of immune system cells through  $\alpha$ -GalCer administration and consequent production of proinflammatory cytokines by patient iNKT cells
- An adjuvant effect elicited by co-administration of  $\alpha$ -GalCer with free tumour antigen, with a CD1d-expressing whole tumour cell, or with a DC which is bearing tumour antigen
- Direct cytotoxicity against CD1d-positive target tumour cells in the presence of  $\alpha$ -GalCer

As CLL cells express CD1d, and appear susceptible to immunotherapeutic approaches, the investigation of iNKT cell numbers and function in patients with CLL is warranted, with a view to exploiting the iNKT cell/CD1d axis in future immunotherapies for CLL.

### 1.5 Aims

Chronic lymphocytic leukaemia is an attractive candidate for adoptive immunotherapy, and iNKT cells have the potential to act as useful adjuvants in tumour vaccination or as effector cells targeting CD1d expressing tumour cells such as CLL. However, numerous defects of the immune system have been described in patients with CLL, including functional and immunophenotypic defects of DCs, abnormal T cell numbers and function, and a humoral immunodeficiency. Investigation of the iNKT/CD1d axis in CLL is therefore warranted to inform future trials of iNKT cell-directed immunotherapy for this and related diseases.

The aims of the experimental programme outlined in this thesis are as follows:

1. To determine the frequency and immunophenotype of iNKT cells from patients with CLL,
2. To investigate CD1d expression patterns on APCs in patients with CLL,
3. To analyse the function of iNKT cells from patients with CLL, and
4. To explore potential roles of iNKT cells in adoptive immunotherapy of CLL

# Chapter 2

## Methods

### 2.1 General Materials and Methods

#### 2.1.1 Culture media and buffers

**Dulbecco's phosphate-buffered saline (DPBS)** Calcium- and magnesium-free DPBS was purchased from Invitrogen (Auckland, New Zealand), comprising 200 mg/L KCl, 200 mg/L  $\text{KH}_2\text{PO}_4$ , 8 g/L NaCl and 2160 mg/L  $\text{Na}_2\text{HPO}_4 - 7\text{H}_2\text{O}$ .

**Flow cytometry buffer** 10 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St Louis, MO), 0.01% sodium azide (Sigma-Aldrich) and 2% foetal calf serum (FCS; SAFC Biosciences, Lenexa, KS) were added to 1 L DPBS.

**Immunomagnetic bead buffer** 0.1% bovine serum albumin (ICP Bio, Auckland, New Zealand) and 2 mM EDTA (Sigma-Aldrich) were added to DPBS, and the pH adjusted to 7.4. The buffer was filtered through 0.2  $\mu\text{m}$  Acrodisc™ syringe filters (Pall Life Sciences, Hamilton, New Zealand) before use.

**Iscoe's Modified Dulbecco's Medium (IMDM)** IMDM supplemented with GlutaMAX™, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and 3.024 g/L  $\text{NaHCO}_3$  was purchased from Invitrogen.

**Complete IMDM (cIMDM)** IMDM was supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 55  $\mu\text{M}$  2-mercaptoethanol (2-ME) and 5% human group AB serum (Invitrogen).

**Roswell Park Memorial Institute (RPMI-1640)** Phenol red- and HEPES-free RPMI containing 2 g/L  $\text{NaHCO}_3$  was purchased from Sigma-Aldrich.

### 2.1.2 C1R cell lines

The C1R cell line is a human B lymphoblastoid cell line which lacks expression of HLA-A and HLA-B antigens.<sup>436</sup> The untransfected C1R cell line and a C1R cell line stably transfected to express CD1d were a gift from the laboratory of Professor Vincenzo Cerundolo (Oxford, UK). Both cell lines were maintained in cIMDM. CD1d expression status was verified by flow cytometry before cell use.

### 2.1.3 Cell counting

Cell numbers were determined by staining an aliquot with 0.4% trypan blue (Invitrogen) and counting unstained (live) cells using a haemocytometer (Weber Scientific, Middlesex, England).

### 2.1.4 Culture conditions

All cells were cultured at 37 °C with 5% CO<sub>2</sub> and 95% humidity in a HERAcell™ tissue culture incubator (Thermo Scientific, North Shore, New Zealand).

## 2.2 Patients and ethical considerations

Study participants were recruited by invitation letter and by poster advertisement (appendix A). All participants were provided with written information regarding the study (appendix B). Exclusion criteria included any other active malignancy or previous chemotherapy for a disease other than CLL, as determined by completion of a participant questionnaire (appendix C), and by review of hospital records where relevant. All donors provided written informed consent (appendix D).

All patients met morphologic and flow cytometric criteria for a diagnosis of CLL<sup>437</sup>. The Rai<sup>126</sup> and Binet<sup>127</sup> clinical stages of patients with CLL were determined by physical examination and by analysis of peripheral blood counts performed on the day of PBMC cryopreservation.

Ethical approval for this study was obtained from the Central Regional Ethics Committee of New Zealand (application reference CEN/08/01/002). This study was conducted in accordance with the University of Otago Code of Ethical Conduct.

## 2.3 Venepuncture and PBMC cryopreservation

Peripheral blood was drawn into heparinized tubes before dilution 1:1 in DPBS. Diluted blood was layered over a sodium diatrizoate and polysaccharide solution (Lymphoprep™; Axis-Shield, Oslo, Norway) before centrifugation at 800 g for 25 minutes at room temperature. The PBMC fraction was aspirated, washed and resuspended in 90% FCS with

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## 2.4. B cell depletion and enrichment

10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and frozen in a controlled-rate freezing container. Samples were stored at -180 °C in gaseous phase liquid nitrogen until use.

Additional blood samples were drawn into EDTA and serum-separating vacuum tubes (BD Vacutainer™, BD, North Ryde, Australia) for determination of absolute differential blood count using a Sysmex XE 2100 analyser (Roche Diagnostics, Auckland, New Zealand), and of serum immunoglobulin G (IgG) levels using an immunoturbidimetric assay (Tina-Quant™ IgG Gen2; Roche Diagnostics), respectively.

## 2.4 B cell depletion and enrichment

B cells were depleted and positively selected using anti-CD19-coated immunomagnetic beads (Dynabeads™ CD19 pan-B; Invitrogen Dynal AS, Oslo, Norway). Immunomagnetic beads were washed as per manufacturer's instructions, and PBMCs were counted and resuspended at  $1 \times 10^6$ /mL to  $2.5 \times 10^7$ /mL in immunomagnetic bead buffer in 5 mL polystyrene tubes. Between 0.5 and 4 beads per target cell were added. The cell suspension was incubated for 30 minutes at 4 °C with tilting and rotation. Sample tubes were placed in a DynaMag™ magnet (Invitrogen) for two minutes, and the supernatant containing unbound cells aspirated (B cell-depleted PBMCs). Between one and four serial rounds of B cell depletion were performed to achieve a residual level of CD5+CD19+ CLL cells of < 5% in B cell-depleted PBMCs, as confirmed by flow cytometric analysis.

For some experiments, positively-selected CD19+ cells were retained and immunomagnetic beads were detached using anti-antigen-binding fragment (Fab) antibodies specific for the bead-bound anti-CD19 antibody (DETACHaBEAD™ CD19; Invitrogen Dynal AS), as per manufacturer's instructions. The purity of CD19+ CD5+ CLL cells within the positively-selected B cell population was  $\geq 95\%$ .

For some experiments, CLL cells were sorted by fluorescence-activated cell sorting (FACS): PBMCs were stained for surface markers as outlined in section 2.6, then resuspended at  $1 \times 10^6$ /mL in sterile azide-free flow cytometry buffer. CLL cells were sorted on a BD FACSVantage DiVa flow cytometer (Becton Dickinson, San Jose, CA) based on co-expression of CD19 and CD5. Purity of CLL cells was verified after FACS sorting, and was  $\geq 98.5\%$ .

## 2.5 Loading and tetramerization of CD1d monomer

Biotinylated human CD1d monomer at 2 mg/mL was obtained from the National Institutes of Health (NIH) Tetramer Core Facility. The concentration was adjusted to 1 mg/mL by addition of an equal volume of phosphate-buffered saline (PBS)/PI, comprising DPBS with 2 mM EDTA and a protease inhibitor cocktail to give a final concentration of 1  $\mu$ M pepstatin A, 2  $\mu$ M leupeptin, 5  $\mu$ M bestatin, 80 nM aprotinin, 100  $\mu$ M 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride and 1.5  $\mu$ M E-64 (Halt Protease Inhibitor Cocktail, Thermo Scientific).

20  $\mu\text{L}$   $\alpha$ -GalCer 500  $\mu\text{g}/\text{mL}$  stock (a gift from Industrial Research Laboratories, Lower Hutt, New Zealand) was sonicated at 37 °C for fifteen minutes (Ultrasonic cleaner, Unisonics, Sydney, Australia), then added carefully to 100  $\mu\text{L}$  of 1 mg/mL CD1d monomer solution. 100  $\mu\text{L}$  of DPBS containing 0.5% Tween-20 (Sigma) was added, and mixed by pipetting. The volume was made up to 1 mL by addition of PBS/PI. The CD1d monomer and  $\alpha$ -GalCer solution was incubated in a 37 °C water bath for three hours. The loaded CD1d monomer was concentrated three times in a Vivaspin 500 10 kDa centrifugal ultrafiltration concentrator (Sigma-Aldrich), then resuspended in 50  $\mu\text{L}$  PBS/PI. Aliquots of unloaded CD1d tetramer were simultaneously prepared using the same method, but without addition of  $\alpha$ -GalCer, for subsequent use as negative staining controls.

Loaded and unloaded CD1d monomer was tetramerized and labelled as follows: 50 $\mu\text{L}$  aliquots of monomer were protected from light and stored on ice during the addition of 300  $\mu\text{L}$  phycoerythrin (PE)-labelled streptavidin (BD Biosciences), added in 30  $\mu\text{L}$  aliquots every fifteen minutes, with thorough mixing. Loaded and unloaded PE-labelled CD1d tetramer aliquots were stored at 4 °C until use.

### 2.6 Immunofluorescent staining of cell surface markers for flow cytometry

Fluorescent-labelled antibodies were obtained from BD Biosciences (Franklin Lakes, NJ), Beckman Coulter (Brea, CA), Biolegend (San Diego, CA), and Invitrogen. The antibody clones, isotypes and fluorophores used are listed in table 2.1.

B cell-depleted or whole PBMCs were washed twice and resuspended at 1 to 10  $\times 10^6/\text{mL}$  in PBS. 200  $\mu\text{L}$  aliquots were dispensed into round-bottomed 96-well plates and centrifuged. Crystallizable fragment (Fc) receptors were blocked by incubation of cells for 15 minutes with 2 mg/mL polyclonal human IgG (Intragam P™, CSL Limited, Broadmeadows, Australia). For surface staining, cells were incubated for 20 minutes at 4 °C with the fluorescent-labelled antibodies or tetramer indicated at optimal concentrations as determined by prior experiments, before two washes in flow cytometry buffer. Cells were stained with an amine-reactive viability dye (LIVE/DEAD Fixable™ Blue; Molecular Probes, Eugene, OR) according to manufacturer's instructions then resuspended in FACS buffer, or were resuspended in flow buffer containing 0.5  $\mu\text{g}/\text{mL}$  4',6-diamidino-2-phenylindole (DAPI; Molecular Probes), to allow exclusion of dead cells.



## 2.6. Immunofluorescent staining of cell surface markers for flow cytometry

**Table 2.1 Fluorescent-labelled antibodies** List of antibodies and antibody cocktails used for flow cytometry. APC = allophycocyanin; FITC = fluorescein isothiocyanate; Lin 1 = lineage cocktail 1 (anti-CD3, -CD14, -CD16, -CD19, -CD20 and -CD56); PE = phycoerythrin; PerCP = peridinin-chlorophyll protein complex. All isotypes are mouse anti-human, unless otherwise stated

Antigen(s)	Clone(s)	Isotype(s)	Fluorophore(s)	Supplier
CD1c	L161	IgG <sub>1</sub> κ	PE	Biolegend
CD1d	CD1d42	IgG <sub>1</sub> κ	PE	BD Biosciences
CD3	UCHT1	IgG <sub>1</sub> κ	APC	BD Biosciences
CD3	SK7	IgG <sub>1</sub> κ	APC-H7, FITC, PE	BD Biosciences
CD3, CD16 + CD56, CD45, CD19	SK7, B73.1 + NCAM16.2, 2D1, SJ25C1	IgG <sub>1</sub> κ, IgG <sub>2b</sub> κ	FITC, PE, PerCP, APC	BD Biosciences
CD4	OKT4	IgG <sub>2b</sub> κ	Pacific Blue	Biolegend
CD4	SK3	IgG <sub>1</sub> κ	APC	Biolegend
CD5	UCHT2	IgG <sub>1</sub> κ	APC	Biolegend
CD5	L17F12	IgG <sub>2a</sub> κ	PE	BD Biosciences
CD8	RPA-T8	IgG <sub>1</sub> κ	Alexa 700	BD Biosciences
CD8	SK1	IgG <sub>1</sub> κ	APC	BD Biosciences
CD11c	Bu15	IgG <sub>1</sub> κ	APC	Biolegend
CD11c	S-HCL-3	IgG <sub>2b</sub> κ	PE	BD Biosciences
CD14	MφP9	IgG <sub>2b</sub> κ	APC, PE	BD Biosciences
CD16	3G8	IgG <sub>1</sub> κ	FITC	Biolegend
CD19	SJ25C1	IgG <sub>1</sub> κ	APC, PE	BD Biosciences
CD19	4G7	IgG <sub>1</sub> κ	FITC	BD Biosciences
CD23	9P25	IgG <sub>1</sub> κ	FITC	Beckman Coulter
CD25	2A3	IgG <sub>1</sub> κ	FITC	BD Biosciences
CD40	5C3	IgG <sub>1</sub> κ	FITC	BD Biosciences
CD54	HA58	IgG <sub>1</sub> κ	PE	BD Biosciences
CD80	L307.4	IgG <sub>1</sub> κ	PE	BD Biosciences
CD86	IT2.2	IgG <sub>2b</sub> κ	PE	Biolegend

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Table 2.1, continued from page 67

Antigen(s)	Clone(s)	Isotype(s)	Fluorophore(s)	Supplier
CD154	TRAP1	IgG <sub>1</sub> κ	APC	BD Biosciences
CD161	DX12	IgG <sub>1</sub> κ	FITC	BD Biosciences
FoxP3	259D/C7	IgG <sub>1</sub> κ	PE	BD Biosciences
Granzyme B	GB11	IgG <sub>1</sub> κ	FITC	BD Biosciences
HLA Class I	B9.12.1	IgG <sub>1</sub> κ	FITC	Beckman Coulter
HLA-DR	L243	IgG <sub>2a</sub> κ	FITC	BD Biosciences
HLA-DR	L243	IgG <sub>2a</sub> κ	PerCP	Biolegend
IFN-γ	B27	IgG <sub>1</sub> κ	Alexa 488	Invitrogen
IL-4	MP4-25D2	Rat IgG <sub>1</sub> κ	APC	Biolegend
iNKT cell	6B11	IgG <sub>1</sub> κ	FITC, PE	BD Biosciences
Lin 1*	SK7, MφP9, 3G8, SJ25C1, L27, NCAM16.2*	IgG <sub>1</sub> κ, IgG <sub>2b</sub> κ	FITC	BD Biosciences
ZAP-70	1E7.2	IgG <sub>1</sub> κ	PE	Biolegend

\* Lineage cocktail 1 (Lin1) contains antibody clones directed against CD3, CD14, CD16, CD19, CD20 and CD56, respectively.

## 2.7. Immunofluorescent staining of intracellular cytokines, FoxP3, granzyme B and ZAP-70 for flow cytometry

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### 2.7 Immunofluorescent staining of intracellular cytokines, FoxP3, granzyme B and ZAP-70 for flow cytometry

PBMCs were resuspended to  $1 \times 10^6$ /mL in cIMDM. For intracellular cytokine and granzyme B staining, cells were stimulated by addition of ionomycin  $1 \mu\text{g}/\text{mL}$  (Merck, Manukau City, New Zealand), phorbol 12-myristate 13-acetate  $10 \text{ ng}/\text{mL}$  (PMA; Sigma-Aldrich) and protein transport was inhibited by addition of brefeldin A  $2 \mu\text{g}/\text{mL}$  (eBioscience, San Diego, CA). Cells were then incubated at  $37^\circ\text{C}$  for four hours. No stimulation step was used for ZAP-70 staining.

Cells were washed, blocked with human IgG, and stained for surface markers, as described in section 2.6. Cells were then fixed, permeabilized, stained for intracellular markers, and washed using a fixation/permeabilization kit (BD Cytotfix/Cytoperm™; BD Biosciences) as per manufacturer's instructions, before flow cytometric analysis. For intracellular FoxP3 staining a specific FoxP3 staining buffer set (eBioscience) was used for the fixation and permeabilisation steps as per manufacturer's instructions.

Stained unactivated PBMCs, and isotype control-stained activated PBMCs were used as negative controls, and the latter used to set gates for positive intracellular cytokine and granzyme B staining. For ZAP-70 staining, CD5+ CD19- T cells were used as an internal positive control to set the gate for ZAP-70 positivity, as previously described.<sup>133</sup> Cases of CLL were considered ZAP-70 positive if  $> 20\%$  of CD19+ CD5+ cells stained positively for ZAP-70.

## 2.8 Flow cytometric analysis

Flow cytometric data were acquired using an LSR II flow cytometer (Becton Dickinson)—the laser and filter configuration used is shown in table 2.2. Automated compensation was performed using the appropriate fluorophore-labelled antibodies bound to anti-mouse immunoglobulin (Ig) coated particles (BD Compbeads™; BD Biosciences). For non-antibody stains, automated compensation was performed using stained and unstained populations of positive control cells. Data were analysed using FlowJo™ software (Tree Star Incorporated, Ashland, OR).

The principal immunofluorescence panels used for the flow cytometry experiments presented in this thesis are listed in table 2.3. Except where otherwise stated, cells were serially gated to exclude doublets (on forward scatter height versus forward scatter area then side scatter height versus side scatter area plots) and to exclude dead cells and debris (DAPI or LIVE/DEAD fixable blue negative), before application of an appropriate forward and side scatter gate.

Lymphocytes were identified using a low forward and side scatter gate (figure 3.5) except in functional assays or where activation markers were assessed, when the forward and

**Table 2.2 Flow cytometer configuration** Configuration of the LSR II flow cytometer used for the experiments in this thesis. APC = allophycocyanin; BP = band pass; CFSE = carboxyfluorescein succinimidyl ester; CTO = CellTracker™ Orange; DAPI = 4',6-diamidino-2-phenylindole; FITC = fluorescein isothiocyanate; LP = long pass; PE = phycoerythrin; PerCP = peridinin-chlorophyll protein complex.

Excitation wavelength (nm) (colour)	Filter wavelengths (nm)	Fluorophore(s) or parameter
355 (ultraviolet)	450/50 BP	DAPI, LIVE/DEAD fixable blue
405 (violet)	450/50 BP	Pacific blue
405 (violet)	560/40 BP	Pacific orange
488 (blue)	515/20 BP & 505 LP	FITC, Alexa 488, CFSE
488 (blue)	685/35 BP & 640 LP	PerCP
488 (blue)	488/10 BP	Side scatter
532 (green)	575/26 BP	PE, CTO
640 (red)	670/14 BP	APC
640 (red)	780/60 BP & 740 LP	APC-H7
640 (red)	710/50 BP & 685 LP	Alexa 700

side scatter gates were extended to include activated (blasting) cells. Within the lymphocyte gate, T cells were identified as CD19-CD3+, NK cells were identified as CD19-CD3- and CD16/CD56+, NK-like T cells were identified as CD19-CD3+ and CD16/CD56+, B cells were identified as CD19+, CLL cells were identified as CD19+CD5+, and 'normal' B cells were identified as CD19+CD5-.

To identify dendritic cells an intermediate forward and side scatter gate was applied (figure 4.2a). Within this gate, myeloid DCs were identified as Lin1-, HLA-DR+ and CD11c high. Plasmacytoid DCs were identified as Lin1-, HLA-DR+ and CD123 high.

Monocytes were identified using a high forward and side scatter gate (figure 4.5a). Within this gate, CD14hi monocytes were identified as CD14 high and CD16-. CD16+ monocytes were identified as CD14 low and CD16+.

Except where otherwise indicated, relative numbers of PBMC subsets were expressed as a proportion of live PBMCs, determined by flow cytometry of cryopreserved samples.

For some experiments, absolute numbers of PBMC subsets were estimated from Coulter full blood counts of whole blood performed on the same day as PBMC cryopreservation, using the following formula: absolute number of circulating PBMCs = absolute lymphocyte count + absolute monocyte count.

For quantitative flow cytometric analyses, positively stained samples were analysed alongside identically-treated fluorescence minus one (FMO) controls, or controls stained with an isotype-matched control antibody. Net median fluorescence intensity was calculated

**Table 2.3 Immunofluorescent antibody panels** Principal immunofluorescence panels used for the multicolour flow cytometry experiments presented in this thesis.

Panel purpose	Fluorophores and antigens
CLL and B cell identification and surface phenotype	DAPI or LIVE/DEAD fixable blue, FITC-CD19, APC-CD5, APC-H7-CD3
B cell activation status	DAPI or LIVE/DEAD fixable blue, FITC-CD40 or -CD25 or -HLA-DR, PE-CD80 or -CD86 or -CD54, APC-CD19, APC-H7-CD3
CLL cell ZAP-70 expression	FITC-CD19, PE-ZAP-70, APC-CD5
iNKT and T cell enumeration and surface phenotype	DAPI or LIVE/DEAD fixable blue, Pacific blue-CD4, FITC-CD25 or -CD161, PE-anti-iNKT cell (clone 6B11) or PE-CD1d tetramer, APC-CD19, Alexa 700-CD8, APC-H7-CD3
iNKT and T cell intracellular cytokines	LIVE/DEAD fixable blue, Pacific blue-CD4, Alexa 488-IFN- $\gamma$ , PE-anti-iNKT cell (clone 6B11) or PE-CD1d tetramer, APC-IL-4, Alexa 700-CD8, APC-H7-CD3
iNKT and T cell proliferation	DAPI, Pacific blue-CD4, CFSE, PE-anti-iNKT cell (clone 6B11) or PE-CD1d tetramer, APC-CD19, Alexa 700-CD8, APC-H7-CD3
Treg identification	Pacific blue-CD4, FITC-CD25, PE-FoxP3, APC-H7-CD3
NK and NK-like T cell identification	DAPI or LIVE/DEAD fixable blue, FITC-CD161, PE-CD3, APC-CD19
Myeloid dendritic cell enumeration and phenotype	DAPI or LIVE/DEAD fixable blue, FITC-Lin1, PE-CD1d or CD1c or CD86 or CD80, PerCP-HLA-DR, APC-CD11c
Monocyte enumeration and phenotype	DAPI or LIVE/DEAD fixable blue, FITC-CD16, PE-CD1d or CD86 or CD80, APC-CD14

by subtracting the median fluorescence intensity of the FMO or isotype-matched control stained sample from that of the positively-stained sample.

### 2.9 IFN- $\gamma$ ELISpot assay

IFN- $\gamma$  ELISpot was performed using a human IFN- $\gamma$  ELISpot kit (BD Biosciences) according to manufacturer's instructions.  $5 \times 10^5$  B cell-depleted PBMCs were washed, resuspended in cIMDM and dispensed into pre-coated ELISpot plates before addition of  $\alpha$ -GalCer 200 ng/mL, PBS with 0.5% Tween-20 vehicle as negative control, or phytohaemagglutinin-P 1  $\mu$ g/mL (PHA-P; Sigma-Aldrich) as positive control. For some experiments, an unlabelled purified anti-CD1d monoclonal antibody (clone CD1d42; BD Biosciences) was added at 0.5  $\mu$ g/mL to confirm the CD1d dependence of  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpots. Plates were cultured for 18 hours at 37 °C.

Following culture, the cell suspension was aspirated, and the wells washed twice with deionised water, then thrice with 200  $\mu$ L/well of the manufacturer's supplied wash buffer. 100  $\mu$ L/well detection antibody was added, and the plates incubated for two hours at 20 °C. Detection antibody was discarded, and the wells washed thrice with 200  $\mu$ L/well wash buffer before addition of 100  $\mu$ L/well streptavidin-horseradishperoxidase (HRP) solution and a further one hour incubation at 20 °C. Streptavidin-HRP was discarded, and the wells washed four times with 200  $\mu$ L/well wash buffer, then twice with 200  $\mu$ L/well PBS. 100  $\mu$ L/well 3-amino-9-ethylcarbazole (AEC) substrate was added, and the plates monitored visually for spot development. After 20 to 40 minutes, the reaction was halted by washing wells twice with deionised water, and the plate dried overnight.

ELISpots were enumerated using an automated ELISpot reader (Autoimmun Diagnostika, Strassberg, Germany). Spot-forming units (SFUs) in response to vehicle were subtracted from SFUs in response to  $\alpha$ -GalCer to calculate  $\alpha$ -GalCer-induced ELISpots.

### 2.10 iNKT cell proliferation assays

$2 \times 10^5$  to  $2 \times 10^6$  PBMCs were cultured at 37 °C in cIMDM with either 100 ng/mL  $\alpha$ -GalCer or PBS/0.5% Tween-20 vehicle. Recombinant human IL-2 100 U/mL (Chiron Corporation, Emeryville, CA) was added at 24 hours. For some experiments, recombinant human IL-7, IL-15 or IL-21 (all from eBioscience, San Diego, CA) were used instead of IL-2.

Cells were harvested after seven days. The absolute number of live cells was enumerated by Trypan blue staining (as described in section 2.1.3), and the proportion of iNKT cells was determined by flow cytometry.

To assess the effect of CLL cells on iNKT cell proliferation, for some experiments positively-selected autologous CLL cells were added back to the B-cell depleted PBMCs before the addition of  $\alpha$ -GalCer and IL-2, as above.

### 2.11 Generation and maintenance of iNKT cell lines

Following initial proliferation of iNKT cells as above, iNKT cells were sorted by FACS (as described in section 2.4) based on co-expression of CD3 and the V $\alpha$ 24J $\alpha$ 18 TCR- $\alpha$  chain, detected with the 6B11 antibody clone.

Sorted iNKT cells ( $4 \times 10^3$  to  $2 \times 10^5$ ) were dispensed into 24-well plates.  $2 \times 10^5$  to  $1 \times 10^6$  CD1d-transfected C1R cells (C1R-CD1d) were cultured in the presence of 200 ng/mL  $\alpha$ -GalCer for 24 hours,  $\gamma$ -irradiated to 50 Gy (Gammacell 3000 Elan irradiator, Best Theratronics, Ottawa, Canada), and washed three times, before addition to sorted iNKT cells for restimulation. 100 U/mL recombinant human IL-2 was added 24 hours after the C1R-CD1d cells.

Resultant polyclonal iNKT cell lines were split once the concentration reached  $\geq 2 \times 10^6$  cells per well. Cell lines were restimulated with  $\alpha$ -GalCer-pulsed irradiated C1R-CD1d cells every two to three weeks, and maintained in media containing 50 U/mL IL-2. For some cell lines, FACS sorting was repeated to maintain the purity of iNKT cells  $\geq 95\%$ .

### 2.12 Cytokine bead array analysis

$2 \times 10^4$  C1R-CD1d cells were pulsed with 200 ng/mL  $\alpha$ -GalCer or vehicle for two hours,  $\gamma$ -irradiated to 50 Gy, then added to  $4 \times 10^4$  cultured iNKT cells. After four hours, supernatant was harvested for cytokine analysis. In separate experiments,  $2 \times 10^6$  whole or B cell-depleted PBMCs were cultured in the presence of  $\alpha$ -GalCer or vehicle. IL-2 100 U/mL was added at 17 to 24 hours. Supernatant was taken at 6 to 72 hours. Supernatants were cryopreserved at  $-80^\circ\text{C}$  until analysis.

Supernatant cytokine concentrations were determined by cytokine bead array (Milliplex™; Millipore Corporation, Billerica, MA), according to bead manufacturer's instructions. Briefly, 25  $\mu\text{L}$  of each culture supernatant and cytokine standard was dispensed into pre-wetted filter plates in duplicate, and diluted with assay buffer or culture medium, respectively. Capture antibody-conjugated microbeads were sonicated, vortexed and mixed, and added to each well. The plate was sealed and incubated with agitation for one hour at  $20^\circ\text{C}$ . Fluid was removed by vacuum filtration, and the wells washed twice with wash buffer. Detection antibodies were added, and the plate incubated for 30 minutes at  $20^\circ\text{C}$  with agitation. PE-labelled streptavidin was added to each well before a further 30 minute incubation at  $20^\circ\text{C}$  with agitation. Fluid was removed by vacuum filtration, and each well washed twice, before the contents were resuspended in 100  $\mu\text{L}$  sheath fluid (Bio-Rad Laboratories, Hercules, CA) with agitation.

Plates were analysed on a Bio-Plex™ analyser (Bio-Rad Laboratories). A cytokine standard curve was generated using a spline-fitting curve model, and was used to determine cytokine concentrations in the experimental samples. The lower limit of detection for each cytokine was 3.2 pg/mL, determined by the lowest dilution of the cytokine standard used.

## 2.13 Cytotoxicity assays

### 2.13.1 Lactate dehydrogenase release assay

$2.5 \times 10^4$  untransfected C1R or C1R-CD1d target cells were cultured with 200 ng/mL  $\alpha$ -GalCer or PBS/Tween vehicle for one hour in phenol-red free RPMI-1640 with 5% human AB serum before addition of iNKT effector cells at indicated effector:target ratios, in triplicate. Effector only, target only and background (medium only) controls were included, and to determine maximal lactate dehydrogenase (LDH) release, additional aliquots of target cells were incubated with 1/10 LDH assay lysis solution (Sigma-Aldrich).

After incubation for four hours at 37 °C, supernatants were harvested and LDH content determined using an LDH assay kit (Sigma-Aldrich), as per manufacturer's instructions. Briefly, 50  $\mu$ L of each supernatant was dispensed into flat-bottomed 96-well plates, and 100  $\mu$ L of LDH assay mixture (comprising LDH substrate, dye and co-factor solutions) added. The plates were incubated for 30 minutes at 20 °C in the dark, after which 15  $\mu$ L of 1N HCl was added to each well to terminate the reaction.

LDH activity was measured by spectrophotometry using a Versamax™ microplate reader (Molecular Devices, Surrey Hills, VIC, Australia). Background absorption at 690 nm was subtracted from absorption at 490 nm. Specific lysis was calculated according to the formula (experimental LDH release – spontaneous effector LDH release – spontaneous target LDH release)  $\div$  (maximal target LDH release – spontaneous target LDH release), as previously described.<sup>438</sup>

### 2.13.2 VITAL assay

The *in vivo/in vitro* technique for assessing lysis (VITAL) assay was performed on *in vitro* cultured PBMCs as previously described.<sup>439</sup> Briefly, C1R-CD1d target cells were cultured for two hours with 100 ng/mL  $\alpha$ -GalCer or with vehicle, then resuspended at  $2 \times 10^6$ /mL in PBS.

The  $\alpha$ -GalCer-pulsed C1R-CD1d target cells were labelled with cell tracker orange (CTO; Molecular Probes) by addition of 10  $\mu$ M CTO followed by incubation at 37 °C for fifteen minutes. The cells were then centrifuged and resuspended in warmed cIMDM, incubated at 37 °C for a further twenty minutes, and washed twice in PBS.

Unpulsed C1R-CD1d target cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) as follows: CFSE was added to the cell suspension to a final concentration of 0.2  $\mu$ M. The cell suspension was mixed thoroughly, then incubated for eight minutes at 20 °C, mixing twice during the incubation. An equal volume of FCS was added to quench the reaction. Cells were washed twice, then resuspended in cIMDM.

Each population of labelled target cells was resuspended at  $2 \times 10^5$ /mL in cIMDM. 25  $\mu$ L of this suspension was dispensed to wells of a 96-well round-bottomed plate, in duplicate ( $5 \times 10^3$  of each target cell population/well). Additional wells containing each target population alone were used for compensation controls. Whole healthy donor PBMCs were added as effectors, at the effector:target ratios indicated.



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## 2.14. IFN- $\gamma$ , $\alpha$ -GalCer and resiquimod effects on CLL cell surface markers

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Following a six hour incubation at 37 °C, cells were harvested, washed, resuspended in buffer containing propidium iodide (PI; BD Biosciences) for dead cell exclusion, and analysed by flow cytometry to determine the relative proportions of CTO-labelled  $\alpha$ -GalCer-pulsed and CFSE-labelled unpulsed target cells.

## 2.14 IFN- $\gamma$ , $\alpha$ -GalCer and resiquimod effects on CLL cell surface markers

Leukaemic CLL cells were immunomagnetically selected, and immunomagnetic beads removed, as detailed in section 2.4.  $1 \times 10^6$  CD19+ cells ( $\geq 95\%$  CD19 and CD5 co-positive) were cultured in cIMDM for 24 to 48 hours in the presence of vehicle alone or with 1000 U/mL recombinant human IFN- $\gamma$  (Peprotech, Rocky Hill, NJ), 100 ng/mL  $\alpha$ -GalCer, or resiquimod at the indicated concentrations (Invivogen, San Diego, CA). Cells were harvested and stained for MHC Class I, Class II, CD1d and CD86 expression in parallel with FMO or isotype controls, as detailed in section 2.6.

## 2.15 Vaccine recall responses

For the assessment of vaccine recall responses, the following vaccines were used:

- VAXIGRIP™ (Sanofi Pasteur, Lyon, France), a purified, inactivated adjuvant-free vaccine containing influenza virus strains A/Brisbane/59/2007IVR-148, A/Uruguay/716/2007 NYMC X-175C and B/Florida/4/2006
- ADT Booster™ (CSL Biotherapies, Parkville, Australia), containing diphtheria toxoid 4 IU/mL, tetanus toxoid 40 IU/mL, and aluminium hydroxide adjuvant.

### 2.15.1 Thymidine incorporation

Thawed PBMCs from vaccinated donors were cultured at  $2 \times 10^5$ /well in 96-well plates in the presence of various concentrations of influenza or tetanus toxoid vaccines in cIMDM for five days, with the addition of 100 ng/mL  $\alpha$ -GalCer or vehicle, in triplicate.

Twelve hours before the end of the culture period,  $^3\text{H}$  thymidine (Amersham Biosciences, Little Chalfont, United Kingdom) was added at  $1 \mu\text{Ci}$  [0.037 MBq]/well. At the end of the culture period, plate contents were aspirated through glass fibre filter mats (PerkinElmer, Waltham, MA) using an automated plate harvester (Tomtec, Hamden, CT). Filter mats were dried, and placed into plastic sample bags before addition of scintillation liquid cocktail (Beta Scint™; Wallac, Turku, Finland).  $^3\text{H}$  thymidine incorporation was measured using an automated beta scintillation counter (1450 Microbeta Plus; Wallac).

### 2.15.2 CFSE dilution

For some experiments, CFSE dilution was used to assess the proliferation of T cell subsets. Thawed PBMCs were labelled with 0.2  $\mu\text{M}$  CFSE, as described in section 2.13.2.  $1 \times 10^6$ /well CFSE-labelled PBMCs were dispensed into 24-well plates before addition of vaccine and/or  $\alpha$ -GalCer. Cells were cultured for five days at 37 °C, then harvested and stained for surface markers. Flow cytometry was used to determine CFSE dilution within T cell subsets.

## 2.16 *In vitro* alloresponse to CLL cells

CLL cells were purified by flow cytometric cell sorting or positive immunomagnetic selection and incubated for 18 hours with vehicle, 200 ng/mL  $\alpha$ -GalCer, 1  $\mu\text{g}/\text{mL}$  resiquimod (Invivogen, San Diego, CA), or both, then  $\gamma$ -irradiated (50 Gy) and washed four times. Treated, irradiated CLL cells were added at  $4 \times 10^4$ /well to  $2 \times 10^6$ /well healthy donor PBMCs in 96-well plates, and cultured in cIMDM for five days.  $^3\text{H}$  thymidine was added during the final twelve hours of culture, and thymidine incorporation measured by scintillation counter as described in section 2.15.1.

For some experiments, responding PBMCs were labelled with 0.2  $\mu\text{M}$  CFSE, as described in section 2.13.2, before addition of  $4 \times 10^4$ /well irradiated CLL cells as stimulators to  $2 \times 10^5$  responders, in 96-well plates. After incubation at 37 °C for five days, cultures were harvested, stained for surface markers and analysed by flow cytometry to determine the number of divided (CFSE-low) cells within the iNKT and CD4+ and CD8+ T cell subsets.

## 2.17 Autologous responses to CLL cells

CLL cells were purified by FACS or positive immunomagnetic selection as described in section 2.4, then incubated for 18 hours with vehicle or 200 ng/mL  $\alpha$ -GalCer, before  $\gamma$ -irradiation (50 Gy), four washes, and resuspension in cIMDM. For some experiments, additional aliquots of unpulsed or  $\alpha$ -GalCer-pulsed CLL cells were cryopreserved at -180 °C in 90% FCS, 10% DMSO for future stimulations.

Between  $1 \times 10^5$  and  $1 \times 10^6$  CLL cells (the same number of treated and untreated cells were used for each donor) were added to  $1 \times 10^6$  autologous B cell-depleted PBMCs in cIMDM in 48-well plates. 1 ng/mL IL-7 (eBioscience) was added at 24 hours. At one week intervals, further aliquots of CLL cells were thawed, washed, resuspended in cIMDM, and added to the responding cell cultures, with a further 1 ng/mL IL-7 added 24 hours later, to a total of three stimulations. One week after the third stimulation, cultures were harvested, absolute live cell counts determined by trypan blue exclusion, and flow cytometry performed to determine iNKT cell numbers.

For some experiments,  $1 \times 10^6$  responding PBMCs were labelled with 0.2  $\mu\text{M}$  CFSE and resuspended in cIMDM, before addition of  $5 \times 10^5$  autologous untreated or  $\alpha$ -GalCer-

treated CLL cells in the absence of exogenous cytokines. Proliferation of PBMC subsets was assessed after five days of culture by flow cytometry.

### 2.18 Statistical analyses

Data were analysed using Prism™ 5.0 (GraphPad Software, La Jolla, CA). Non-parametric tests were used for all analyses except where otherwise stated: The Mann Whitney test was used for unpaired data, the Wilcoxon matched pairs test for paired data, and Kruskal Wallis analysis of variance (ANOVA) with Dunn's post-test for multiple comparisons. Bars and error bars represent medians and interquartile ranges except where otherwise stated. Categorical data were compared using Fisher's exact test. Survival curves were compared using the log-rank (Mantel-Cox) test. Linear and nonlinear regressions were performed using the least squares fitting method, without weighting. For some analyses, Pearson's correlation coefficient was calculated. For all analyses, two-tailed P values were calculated, and a P value < 0.05 was considered significant.



## Chapter 3

# Frequency and immunophenotype of iNKT cells in patients with CLL

### 3.1 Introduction

As discussed in section 1.3.3, preclinical studies suggest that iNKT cells have a number of potential roles in the immunotherapy of cancer, including:

- Production of cytokines such as IFN- $\gamma$  with the potential to enhance NK cytotoxicity or modulate the number and function of immunosuppressive cells.<sup>402</sup>
- Activation of DCs via CD40L signals to enhance peptide-specific CD4+ and CD8+ T cell responses against a co-presented tumour antigen.<sup>258</sup>
- Direct cytotoxicity against CD1d positive tumour cells in the presence of strong ligands (such as  $\alpha$ -GalCer) and/or activating NK receptor signals.<sup>310</sup>

The number of circulating iNKT cells may dictate responses to iNKT cell-directed immunotherapy: In a phase I clinical study, cytokine responses to systemically administered  $\alpha$ -GalCer were stronger in patients with higher numbers of circulating iNKT cells.<sup>303</sup> However, reduced numbers and impaired function of circulating iNKT cells have been reported in patients with a range of malignancies, including advanced solid cancers, prostate cancer and multiple myeloma.<sup>210,303,304,307</sup>

Subsets of iNKT cells with differing function have been described, as detailed in section 1.2.2.3. In humans, CD4+ iNKT cells produce Th2-type cytokines, whereas their CD4- counterparts do not.<sup>218,219</sup> This may be of functional significance, as in mice, only the CD4- iNKT cell subset was capable of tumour rejection in a sarcoma model.<sup>222</sup>

As discussed in section 1.1.3.5, numerous abnormalities of innate and adaptive immunity have been described in patients with untreated CLL. These include a significant increase in the number of circulating T cells with an inversion of the CD4/CD8 T cell ratio,<sup>83,84</sup> the presence of oligoclonal T cell expansions in both CD4+ and CD8+ T cell subsets,<sup>87,88</sup> and impaired T cell proliferative responses to common mitogens.<sup>440</sup> The treatment of CLL also affects T cell numbers: the purine analogue fludarabine, which is widely used as a

## Chapter 3. Frequency and immunophenotype of iNKT cells in patients with CLL

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component of first-line chemotherapy, induces a profound and prolonged depletion of T cells, particularly of the CD4+ subset.<sup>175</sup>

The numbers of iNKT cells have not previously been reported in patients with CLL. Therefore, as a first step to exploring the feasibility of iNKT cell-mediated immunotherapy of CLL, I investigated the number and immunophenotype of iNKT cells in patients with untreated and fludarabine-treated CLL.

### 3.2 Aims

I hypothesised that, as in other malignancies, the frequency of circulating iNKT cells would be reduced in patients with CLL, and that iNKT cells would display an abnormal immunophenotype in patients, compared to healthy controls.

Specifically, these experiments aimed to:

- Enumerate circulating iNKT cells from the peripheral blood of patients with untreated and fludarabine-treated CLL and of healthy age-matched controls.
- Determine the immunophenotype of circulating iNKT cells in patients and controls, focussing on the expression of CD4, CD8 and activation markers.

### 3.3 Results

#### 3.3.1 Patient and control characteristics

Patients with untreated (n = 30) and treated (n = 10) CLL were recruited. Because the frequency of circulating iNKT cells declines with age,<sup>210,223,441</sup> healthy age-matched controls (n = 31) were also recruited. As circulating iNKT cell numbers are known to be reduced in patients with solid cancers,<sup>303,304</sup> a history of any active malignancy other than CLL was used as an exclusion criterion in both patient and control groups.

The number, sex and age distribution of controls and untreated and treated patients is given in table 3.1. The age distribution of the three cohorts was closely matched (figure 3.1, table 3.1). The trend towards a male preponderance among patients who had received treatment for CLL (difference non-significant, Fisher's exact test) is consistent with the literature.<sup>8</sup>

Clinical stage was determined on the day of PBMC cryopreservation by clinical examination and peripheral blood count. The majority of patients included in this study were at an early clinical stage: 17% of untreated and 30% of treated patients had features of more advanced Binet stage B or C disease.

Treated patients had previously received the following lines of chemotherapy: chlorambucil (40%); fludarabine monotherapy (40%); fludarabine with cyclophosphamide (60%); fludarabine, cyclophosphamide and rituximab (20%); cyclophosphamide, doxorubicin, vincristine and prednisone (10%). The median time since the last cycle of chemotherapy

was 22 months (range 3 – 87 months). All but one of the chemotherapy-treated patients had received at least one cycle of a fludarabine-containing regimen.

The distributions of haemoglobin concentration, platelet and lymphocyte counts, taken on the day of phlebotomy for the study, are given in figure 3.1. As would be expected, absolute lymphocyte count was significantly higher in untreated patients than in controls or patients who had received chemotherapy. Platelet count was significantly lower among chemotherapy-treated patients than in healthy controls or in untreated patients, likely to represent a consequence of chemotherapy-induced myelosuppression.

**Figure 3.1 Age and peripheral blood counts of study participants** Absolute peripheral blood counts and haemoglobin were determined by Coulter counter analysis of whole EDTA-anticoagulated blood taken on the same day as PBMC isolation. (a) Age, (b) haemoglobin, (c) lymphocyte count, (d) platelet count distribution of study participants (\*  $p < 0.05$ ; \*\*\*  $p < 0.0001$ ; Kruskal Wallis test with Dunn's multiple comparison).

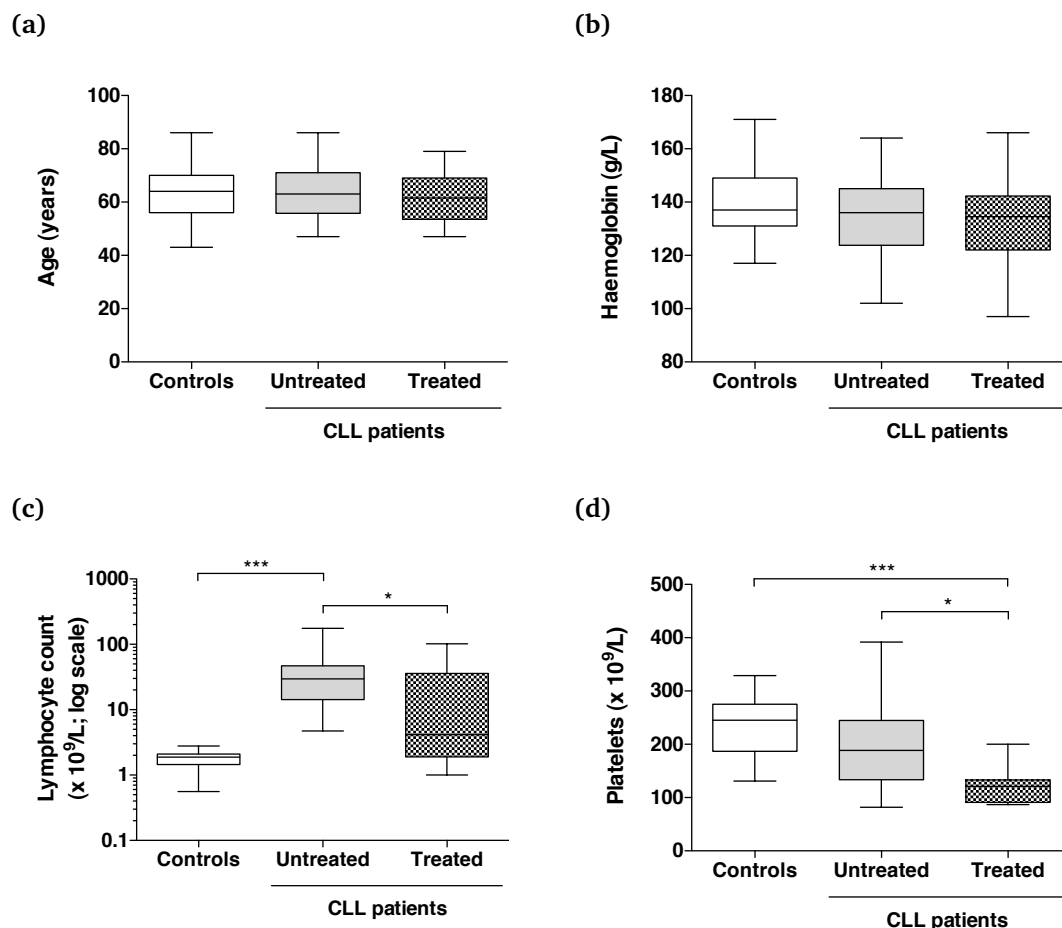


Table 3.1 Control and patient characteristics.

Characteristic	Controls	Untreated CLL	Treated CLL
n	31	30	10
Male (%)	40	47	70
Median age (range), years	64 (43 – 86)	63 (47 – 86)	62 (47 - 79)
Binet stage B or C (%)	–	17	30
Median lymphocyte count (range), x 10 <sup>9</sup> /L	1.9 (0.6 – 2.8)	29.6 (4.7 – 175.1) *	4.2 (1.0 – 102.3)
Median haemoglobin (range), g/L	137 (117 – 171)	136 (102 – 164)	134.5 (97 – 166)
Median platelet count (range), x10 <sup>9</sup> /L	245 (131 – 329)	188.5 (82 – 392)	121 (87 – 200)
Median time since last chemotherapy (range), days	–	–	713 (103 – 2649)

\* One patient in the untreated CLL cohort with a CLL score of 5 had a lymphocyte count < 5 x10<sup>9</sup>/L on the day of phlebotomy. This patient's lymphocyte count was persistently > 5 x10<sup>9</sup>/L before donating blood for this study, and has risen to > 10 x10<sup>9</sup>/L again since taking part, so this patient has been included in the CLL cohort.



Table 3.2 Immunomagnetic depletion of leukemic cells from CLL PBMC samples

Lymphocyte count ( $\times 10^9/L$ )	CLL cells (% of lymphocytes)		
	Pre-depletion	Post-depletion	
		Single round	Three rounds
16.9	59.9	1.6	0.4
19.0	86.3	6.3	1.7
26.9	82.9	13.7	2.8
38.0	94.0	10.8	0.3
39.9	84.6	3.1	0.3
61.9	94.6	26.0	0.8
84.0	92.5	5.1	1.3
129.5	95.5	31.2	0.3
175.1	95.9	13.4	0.7

### 3.3.2 Immunomagnetic depletion of B cells

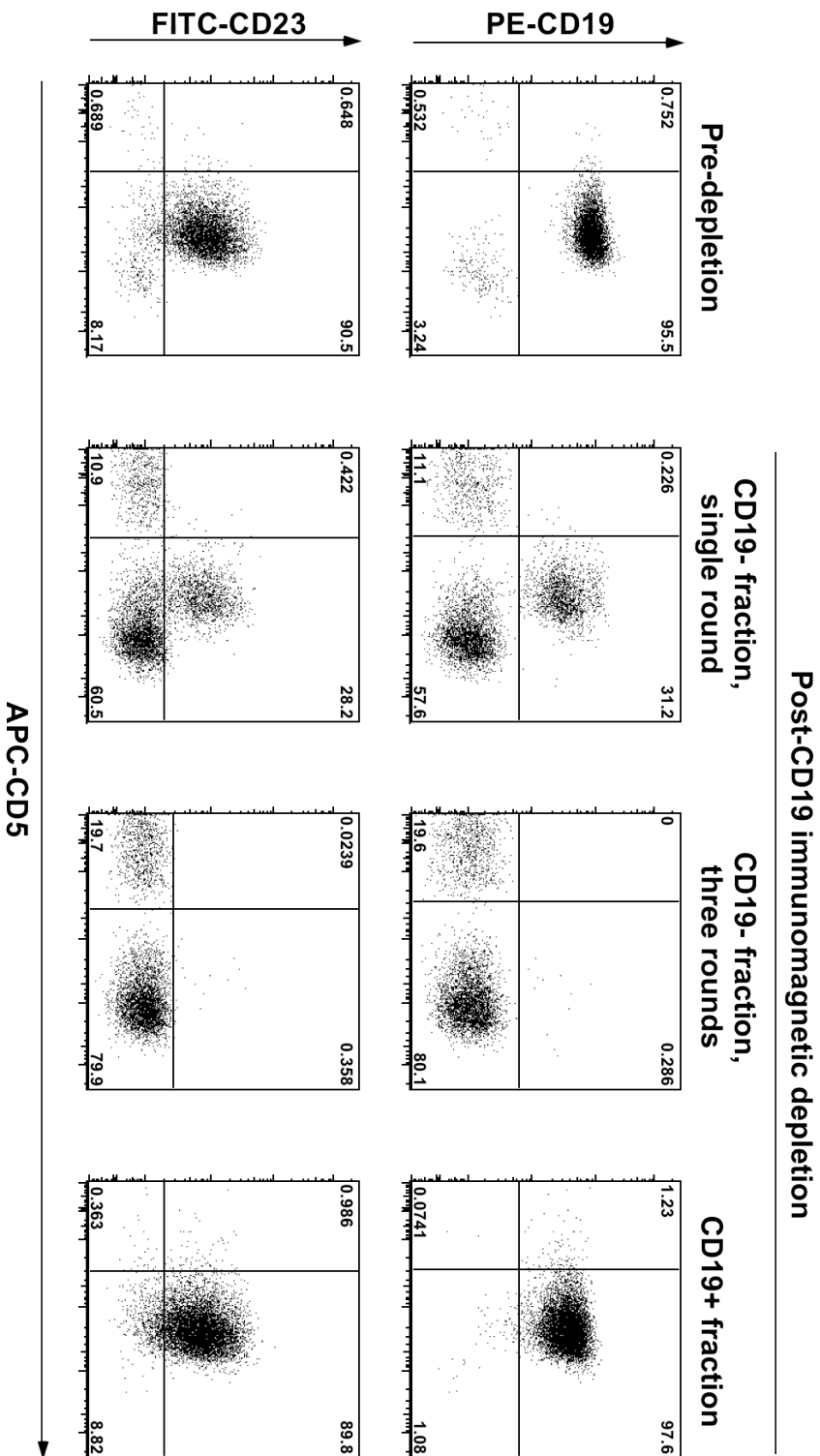
Invariant natural killer T cells constitute a small proportion of circulating T cells in humans, particularly in older individuals,<sup>210,442</sup> posing challenges to their analysis.

Flow cytometry allows the analysis of multiple parameters on individual cells at high speed, and was therefore selected as the most appropriate technique to enumerate and phenotyping of iNKT cells. However, the overwhelming proportion of malignant B cells (characterised by the immunophenotype CD5+CD19+ or CD5+CD23+) observed in the peripheral blood of some patients with CLL (table 3.2; figure 3.2) complicated flow cytometric analysis by necessitating the acquisition of extremely large numbers of events to detect any iNKT cell events.

Detailed analysis of this rare T cell subset therefore demanded depletion of malignant B cells from the stored PBMC samples. I chose to deplete B cells using anti-CD19 immunomagnetic beads. The reasons for using a negative isolation technique, rather than a positive isolation (selecting T cells), was that the negative isolation method leaves T and iNKT cells untouched and unactivated for subsequent analysis, and also allows characterisation of other PBMC populations such as DCs, monocytes and NK cells.

In some patients with CLL, particularly those with a very high peripheral blood lymphocyte count, a single round of CD19 depletion was insufficient to fully remove residual CLL cells. Three sequential rounds of immunomagnetic depletion successfully depleted CLL cells to <5% of residual lymphocytes in all cases (table 3.2; figure 3.2).

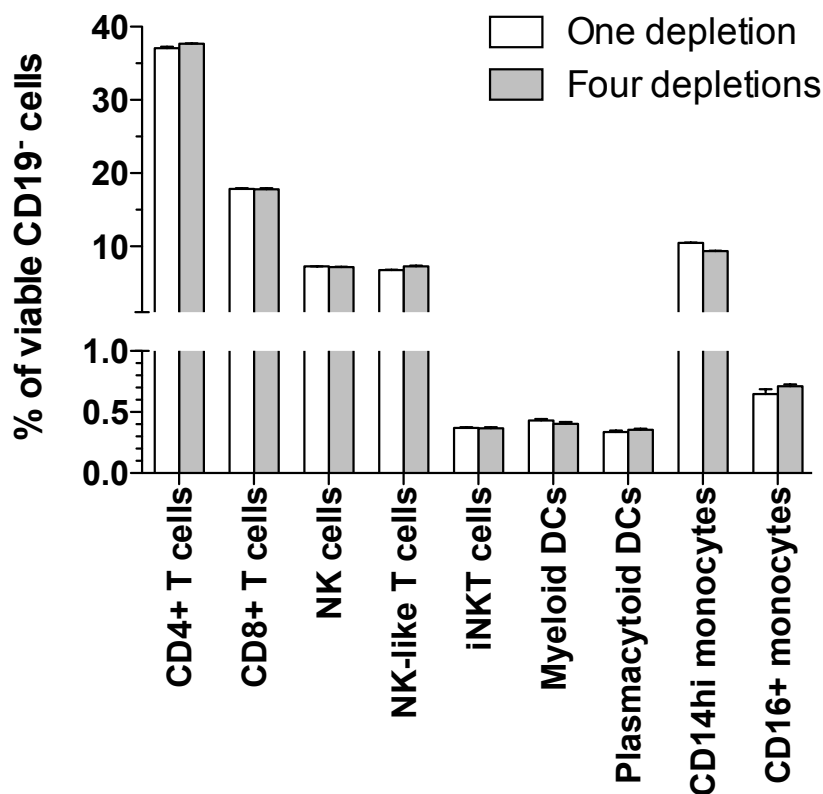
**Figure 3.2 Immunomagnetic depletion of CLL cells from patient PBMCs.** The number of CLL cells in patient PBMCs was determined by flow cytometry before and after one or three serial rounds of CD19 immunomagnetic B cell depletion. CLL cells are identified by the immunophenotype CD5+CD19+ or CD5+CD23+



Each round of immunomagnetic depletion involves a 30-minute incubation at 4 °C with tilting and rotation. To exclude the possibility that this led to selective depletion or activation of PBMC subsets, I compared the numbers and activation status of PBMC populations after one and four rounds of immunomagnetic depletion.

I observed no major differences in the proportions of CD4+, CD8+, NK, NK-like T and iNKT cells, myeloid and plasmacytoid DCs, and CD14 high and CD16+ monocytes among viable CD19- cells (figure 3.3).

**Figure 3.3 Effect of serial CD19 immunomagnetic depletion on major and minor PBMC subsets.** Healthy donor PBMCs were analysed by flow cytometry to determine the proportions of CD4+ and CD8+ T cells, NK cells, NK-like T cells, iNKT cells, myeloid and pDCs and monocytes, after one or four sequential rounds of CD19 immunomagnetic depletion, as a percentage of viable CD19- cells. Performed in triplicate; bars and error bars represent mean and standard error of the mean of replicates, respectively.



Expression of the activation marker CD86 was determined on viable monocytes (gated as high forward and side scatter, CD14+ events) and on viable mDCs (gated as described in section 2.8). Serial depletion rounds did not result in marked monocyte or dendritic cell activation (figure 3.4a).

## Chapter 3. Frequency and immunophenotype of iNKT cells in patients with CLL

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Expression of the activation marker CD69 was determined on viable T cells (gated as CD3+CD19- events with low to high forward and side scatter), and did not change between one and four serial rounds of immunomagnetic B cell depletion (figure 3.4b).

Together, these experiments indicated that B cells could be effectively removed from patient and control samples using serial rounds of CD19 immunomagnetic depletion without major numerical or immunophenotypic changes in the remaining PBMC populations.

### 3.3.3 Flow cytometric identification of invariant natural killer T cells

A number of reagents have been employed for the flow cytometric enumeration of iNKT cells, including antibodies against the V $\alpha$ 24 and V $\beta$ 11 chains, CD1d tetramers loaded with  $\alpha$ -GalCer, and the 6B11 antibody clone, which is directed against the complementarity determining region (CDR) 3 of the V $\alpha$ 24J $\alpha$ 18 TCR- $\alpha$  chain expressed by human iNKT cells.<sup>218,443</sup> As not all V $\alpha$ 24 and V $\beta$ 11 co-expressing T cells are either invariant or CD1d-reactive,<sup>218</sup> I used the two more specific reagents to identify iNKT cells: the 6B11 antibody clone and an  $\alpha$ -GalCer-loaded CD1d tetramer.

The low frequency of circulating iNKT cells in humans necessitated a stringent gating strategy including exclusion of doublet events using forward scatter and side scatter height versus area plots, exclusion of dead cells using a viability dye, and exclusion of B cells using an anti-CD19 antibody, illustrated in figure 3.5. To enhance iNKT cell discrimination, the brightest available fluorophore, PE, was used to label the  $\alpha$ -GalCer-loaded CD1d tetramer and the 6B11 antibody clone. Together, these measures resulted in a false positive event rate, as determined by staining with unloaded CD1d tetramer and with a PE-labelled isotype control antibody, of 0.001% of T cells (figure 3.6).

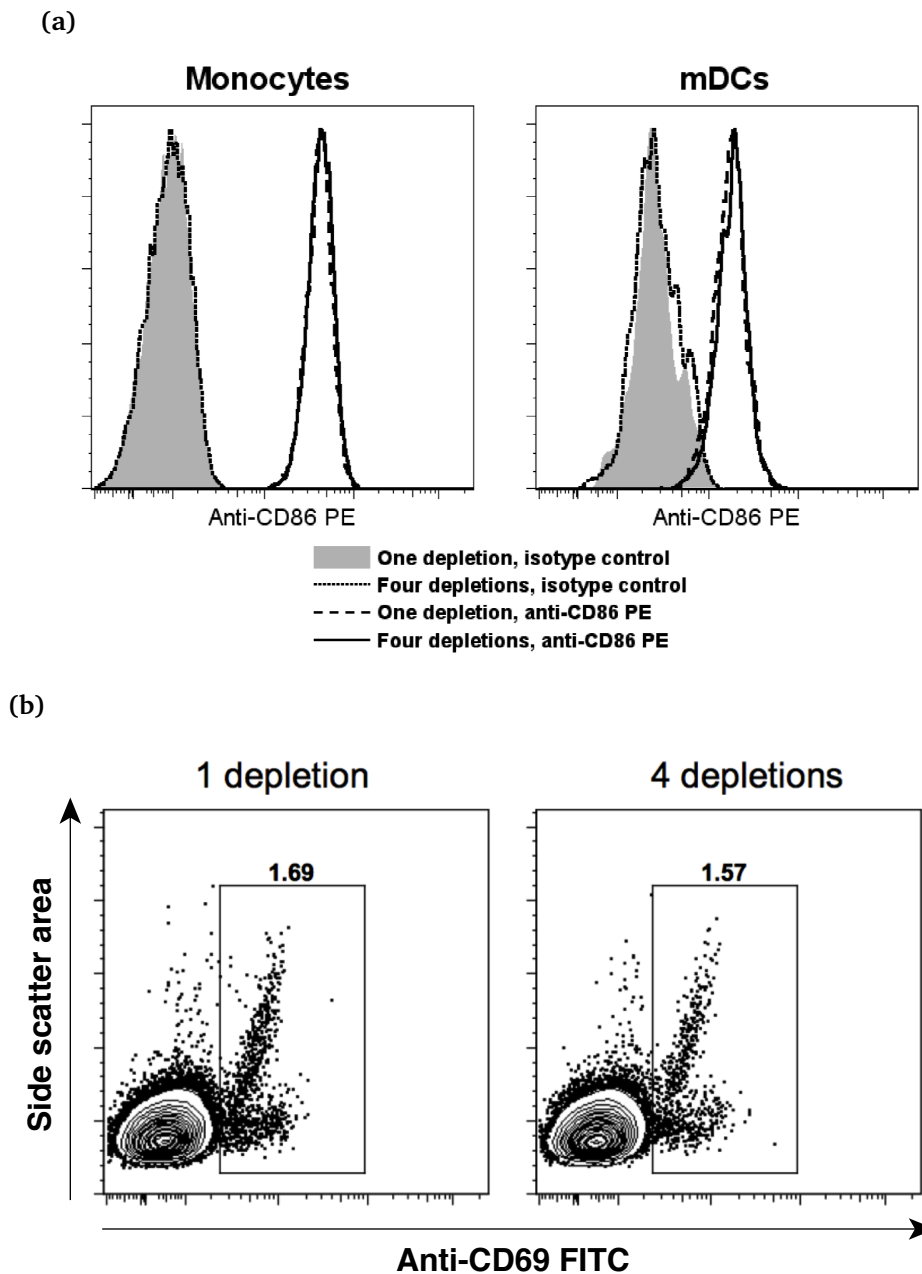
Comparing the 6B11 antibody clone with an  $\alpha$ -GalCer-loaded CD1d tetramer, I found that both could reliably identify iNKT cells, and that the numbers assessed using the two techniques correlated closely (figure 3.6), consistent with published observations.<sup>218,286</sup>

### 3.3.4 Enumeration of circulating iNKT cells in patients and controls

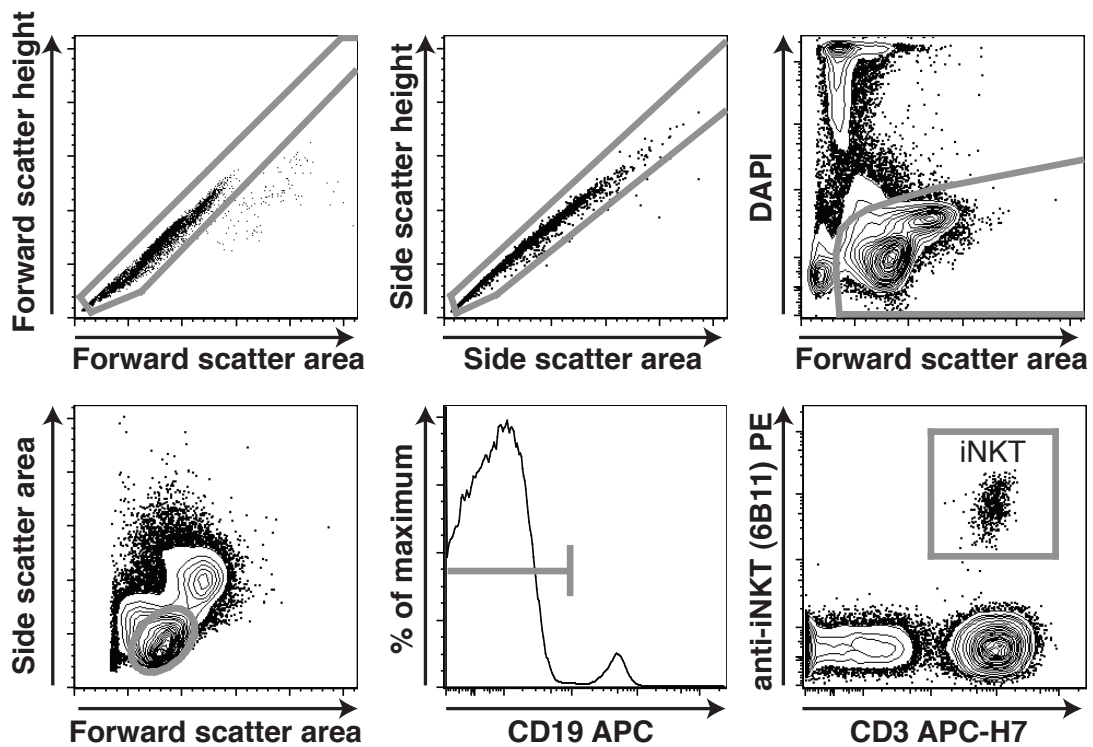
For the enumeration of iNKT cells, the 6B11 antibody was selected because of lower background staining than that observed using the CD1d tetramer (figure 3.6). A median of 154914 T cells (range 48949 – 323685) were analysed per donor. False positive iNKT cell events, as determined using isotype control antibody, occurred at rate of 0.00003% of T cell events. This was lower than the lower limit of detection as determined by the median number of T cell events analysed, at 0.0006%. Thus, the median lower limit of iNKT cell detection was approximately 1 in 10<sup>5</sup> T cell events, or 0.001%.

Among healthy controls (n = 31), median iNKT cell frequency was 0.022% of T cells (range 0.004 – 0.302%). An age-related decline in iNKT cells was seen (95% confidence interval for slope -0.060 to -0.012; non-linear regression). The frequency of iNKT cells did

**Figure 3.4 Activation status of monocytes, mDCs and T cells after serial immunomagnetic B cell depletion.** Healthy donor PBMCs were analysed by flow cytometry after one or four sequential rounds of CD19 immunomagnetic depletion. (a) expression of the activation marker CD86 on monocytes and on myeloid DCs. (b) T cell activation, as determined by expression of CD69 and increase in side scatter.

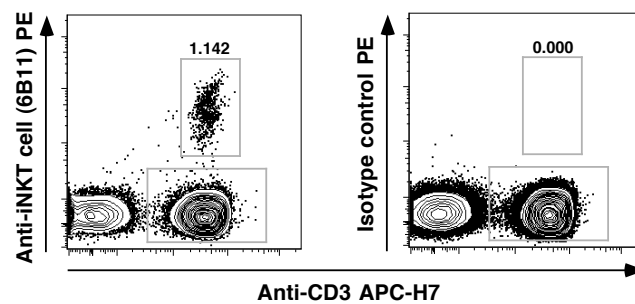


**Figure 3.5 Flow cytometric gating to identify iNKT cells** PBMCs from a healthy donor with a high proportion of iNKT cells stained and analysed by flow cytometry. Light gray lines illustrate gates. Gates were sequentially applied in the following order: forward scatter singlets; side scatter singlets; live cells; lymphocytes (by forward and side scatter); CD19 negative cells; CD3+6B11+ cells (iNKT cells).

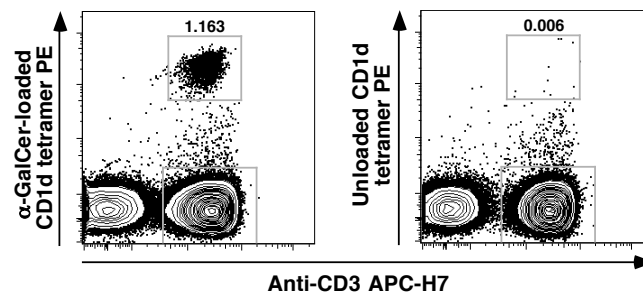


**Figure 3.6 Comparison of CD1d tetramer and 6B11 antibody clone for iNKT cell identification** (a) Identification of iNKT cells with the 6B11 antibody clone, (b) identification of iNKT cells with  $\alpha$ -GalCer-loaded CD1d tetramer in the same donor. Numbers represent percentage of live CD19- lymphocytes within the iNKT cell gate. (c) Comparison of iNKT cell numbers as assessed by 6B11 antibody or  $\alpha$ -GalCer-loaded CD1d tetramer in healthy donors ( $n = 18$ ;  $r^2 = 0.97$ , linear regression)

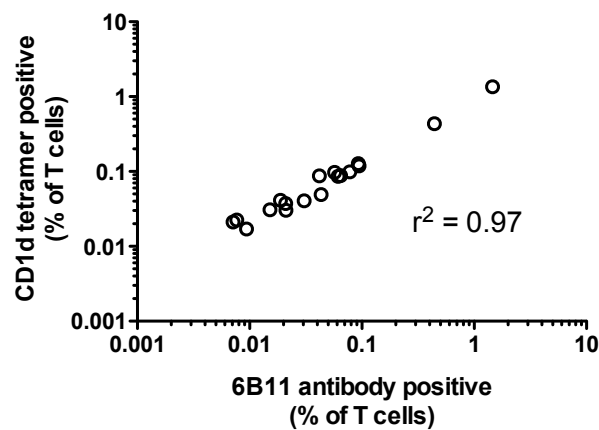
(a)



(b)



(c)



### Chapter 3. Frequency and immunophenotype of iNKT cells in patients with CLL

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not differ significantly between males and females (median 0.019% of T cells in males, 0.026% in females;  $p = 0.35$ ).

Compared to healthy controls, the frequency of iNKT cells in patients, expressed as a percentage of circulating T cells, was significantly reduced (median 0.02% in controls, 0.01% in patients; figure 3.7a).

To calculate absolute numbers of circulating iNKT cells and other T cell subsets, the absolute lymphocyte count, as determined by analysis of whole blood taken on the day of PBMC cryopreservation using a commercial blood count analyser, was used. The number of T cells as a proportion of all lymphocytes, had been determined by flow cytometric analysis of lymphocyte CD3 expression of cryopreserved PBMCs, before B cell depletion steps. Together, this provided a measure of the absolute number of circulating T cells, which was used to calculate absolute numbers of iNKT cells and of other T cell subsets.

Considering absolute numbers of circulating iNKT cells, the difference between patients and controls was no longer apparent (figure 3.7b). This was due to an absolute T cell lymphocytosis observed among patients with CLL (median  $1.0 \times 10^6$ /L T cells in controls,  $2.5 \times 10^6$ /L in patients;  $p < 0.0001$ ), a phenomenon that has been described previously.<sup>83,84</sup>

Stratification of results by disease status did not reveal any relationship between iNKT cell frequency and the clinical stage, lymphocyte count, ZAP-70 status or history of prior fludarabine chemotherapy (figure 3.8). As discussed in section 1.1.5.7, fludarabine has a profound and prolonged effect on T cell populations.<sup>175</sup> Therefore, for this, and for future comparisons of chemotherapy-treated versus untreated patients, the single patient who had received chlorambucil chemotherapy only (and who had never received fludarabine) was not included. Similar results were obtained throughout when the chlorambucil-treated patient was included.

Together, these data suggest that absolute iNKT cell numbers are preserved in CLL, but are reduced as a proportion of T cells, because of an absolute increase in the number of T cells which do not bear the invariant Va24Ja18 receptor.

#### 3.3.5 Analysis of T cell subsets

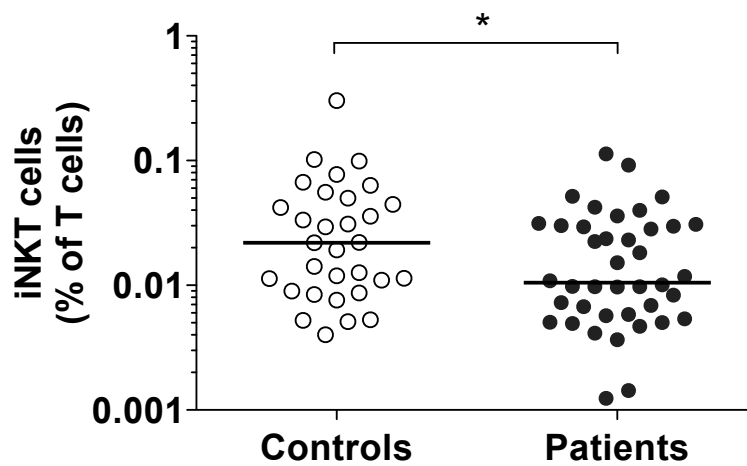
In order to place the results of iNKT cell numbers into context, I analysed the numbers of other major T cell subsets: CD4+, CD8+, CD4-CD8-, NK-like T and T<sub>reg</sub> cells. These are presented as percentages of all T cells in figure 3.9. The results indicate a relative reduction in CD4+ T cells and increase in CD8+ T cells in patients, a finding that was more marked among those who had received fludarabine. Reductions in the CD4/CD8 ratio are well described in CLL.<sup>83,84</sup>

In terms of absolute numbers, increases in all T cell subsets were evident in untreated patients (figure 3.10). In patients who had received fludarabine, a trend towards reduced numbers of CD4+ T cells was observed. These findings are consistent with published data utilising counting beads to accurately determine absolute counts in smaller group of patients.<sup>444</sup>

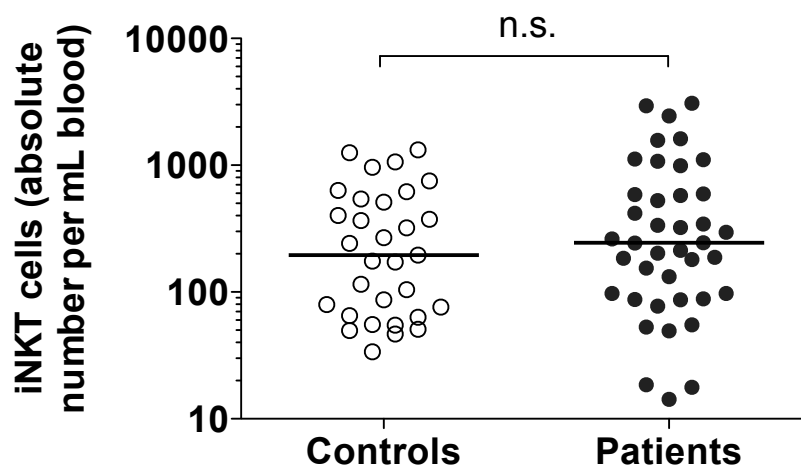


**Figure 3.7 Relative and absolute iNKT cell numbers in patients and controls.** Invariant natural killer T cells were enumerated by flow cytometry of B-cell depleted PBMCs from 40 patients with CLL and 31 healthy age-matched controls using the 6B11 antibody clone, and expressed as a proportion of all T cells (a), or as an absolute number (b). (Bars represent medians; \*  $p < 0.05$ ; n.s. = not significant, Mann Whitney test)

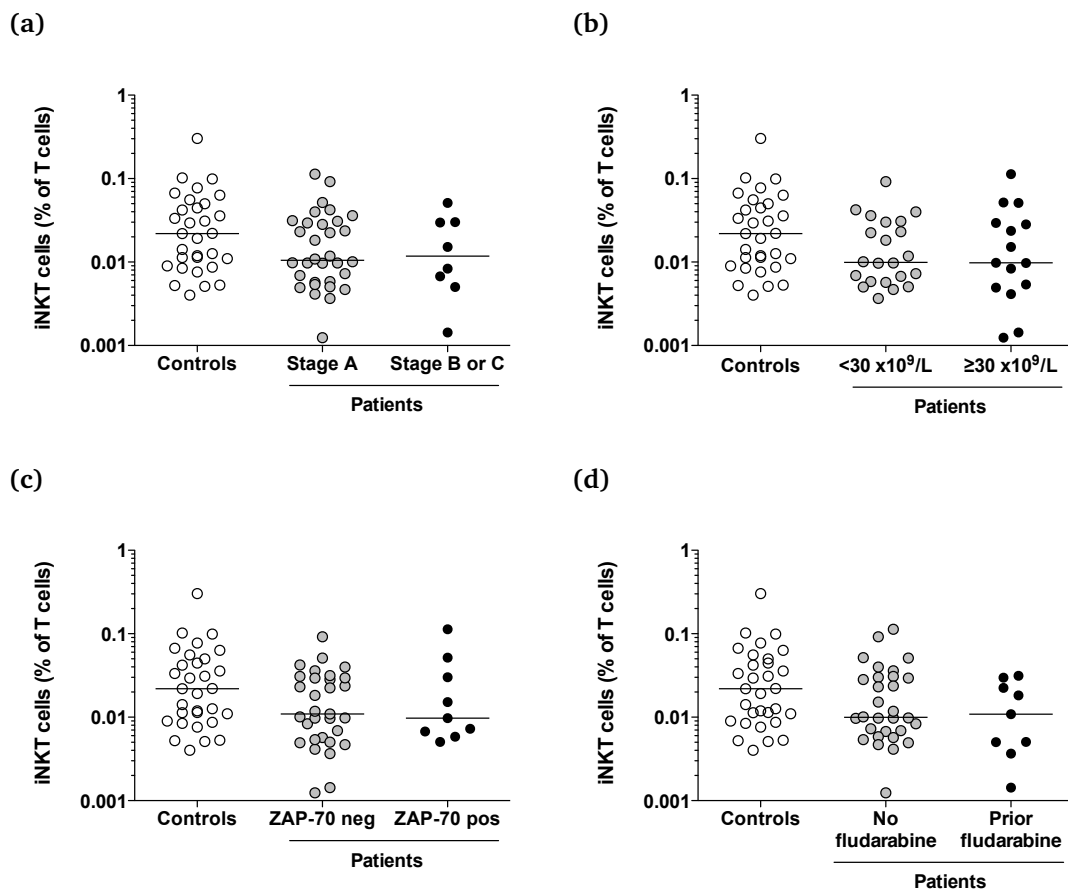
(a)



(b)



**Figure 3.8** NKT cell frequency stratified by stage, lymphocyte count, ZAP-70 status, and prior treatment. iNKT cell frequency, expressed as a proportion of T cells, were determined in PBMCs from 30 patients with CLL and 31 healthy age-matched controls. Patient results are stratified according to (a) Binet clinical stage, (b) absolute lymphocyte count, (c) ZAP-70 expression status, and (d) history of prior fludarabine chemotherapy. (Bars represent medians)



The data I present do not demonstrate a relative increase in  $T_{reg}$  numbers among patients with CLL (figure 3.9), although a trend towards an absolute increase in  $T_{reg}$  numbers was seen (figure 3.10). This is at odds with some observations,<sup>98</sup> but a recent systematic analysis of T cell subset numbers in patients with B cell malignancies also reported normal  $T_{reg}$  numbers in patients with CLL.<sup>444</sup>

**Figure 3.9 Relative proportions of T cell subsets in controls and patients with CLL.** Flow cytometry of T cell subsets was performed, gating on CD4+, CD8+, double negative (DN; CD4-CD8-), NK-like T (CD161+CD3+) and  $T_{reg}$  (CD4+FoxP3+) subsets of CD3+ T cells. T cell subsets are expressed as a percentage of all T cells. \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; Kruskal Wallis test with Dunn's post tests.

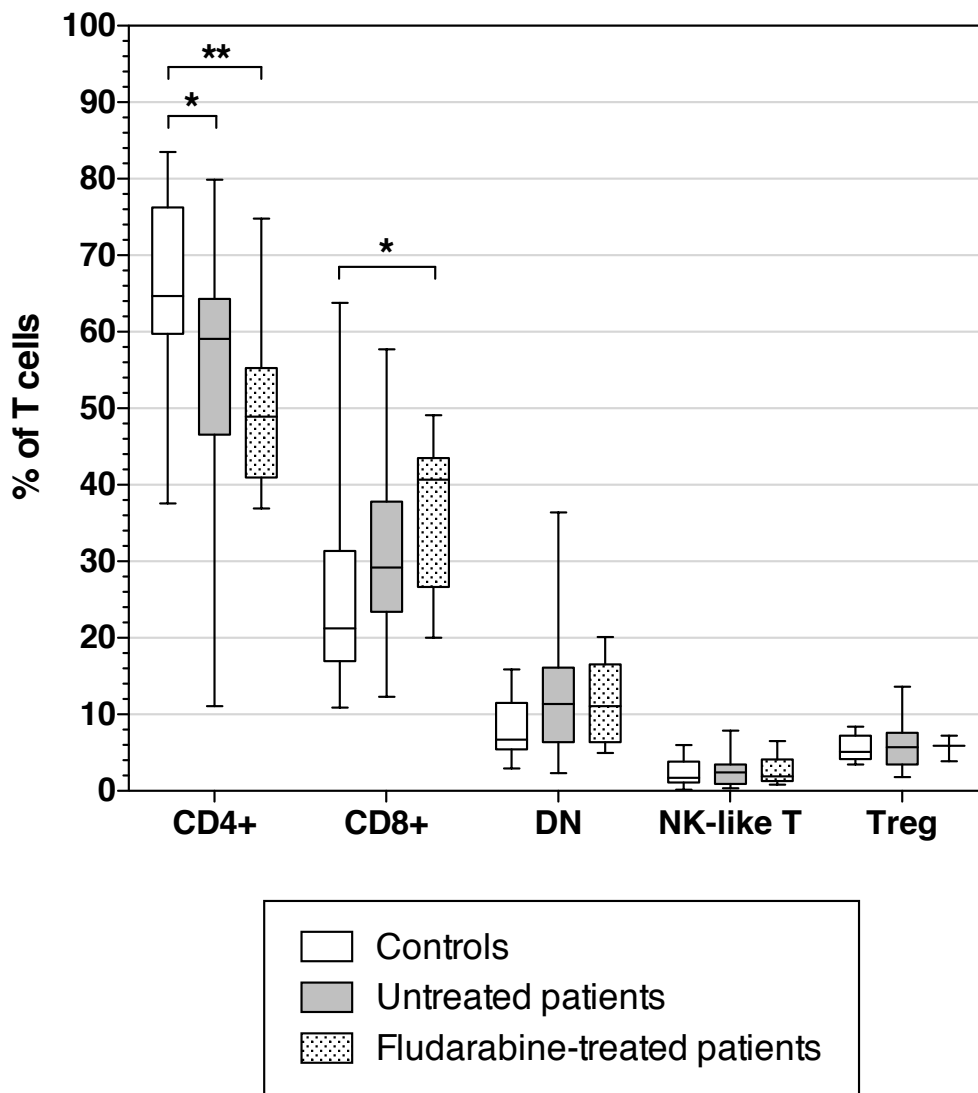
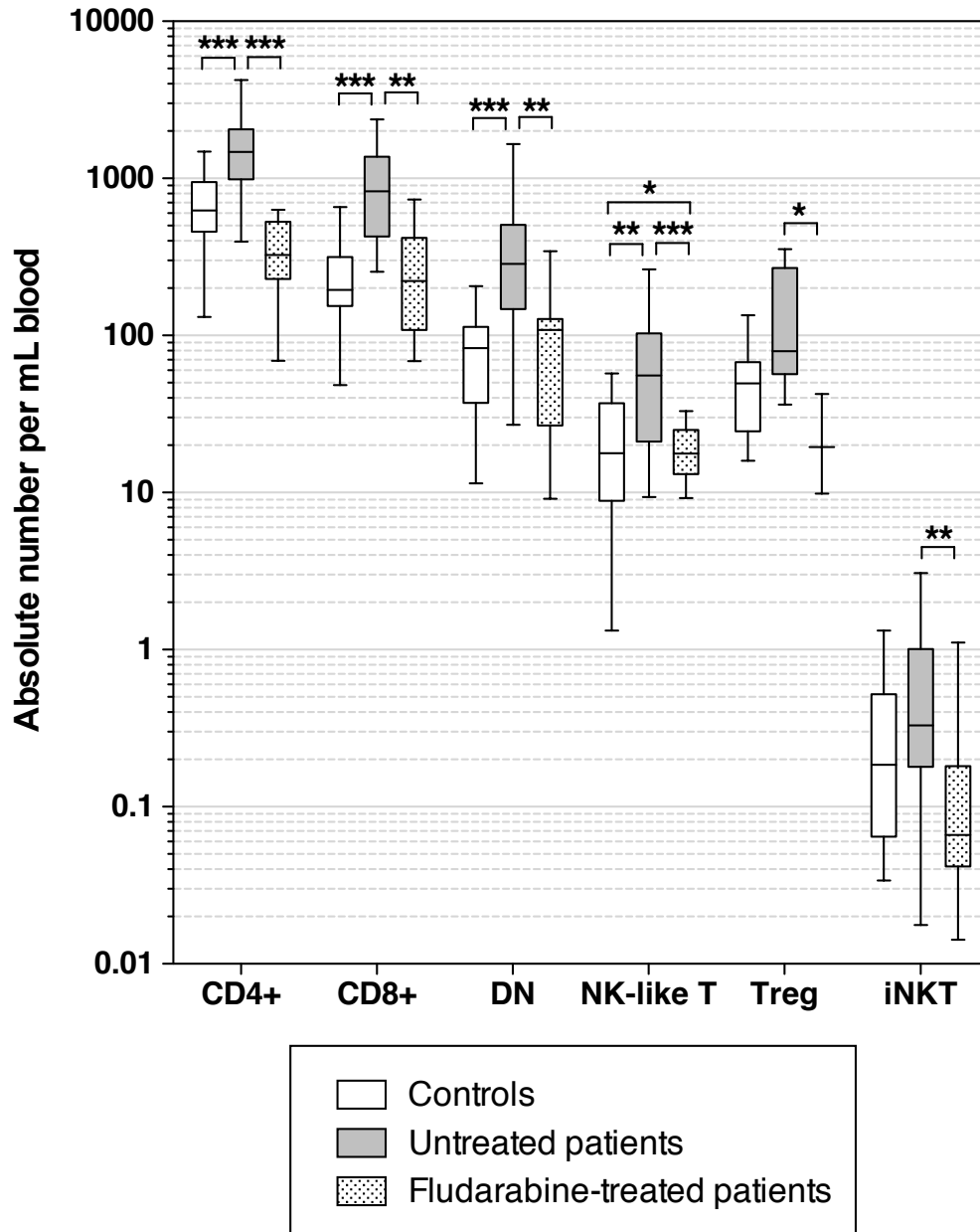


Figure 3.10 Absolute numbers of T cell subsets in controls and patients with CLL. Flow cytometry of T cell subsets was performed, gating on CD4+, CD8+, double negative (DN; CD4-CD8-), NK-like T (CD161+CD3+) and T<sub>reg</sub> (CD4+FoxP3+) subsets of CD3+ T cells. T cell subsets are expressed as absolute numbers. \*\*\* p < 0.001; \*\* p < 0.01; \* p < 0.05; Kruskal Wallis test with Dunn's post tests.



### 3.3.6 Immunophenotype of iNKT cells

Subsets of human iNKT cells with differing function have been identified. The CD4<sup>+</sup> subset is associated with production of Th1 cytokines such as IFN- $\gamma$ , whereas the CD4<sup>+</sup> subset also produces T helper 2 (Th2) cytokines such as IL-4 and IL-13.<sup>218,220</sup> The NK marker CD161 is expressed on the majority of human iNKT cells, and may provide a co-stimulatory function.<sup>227</sup> Activation of T cells, including iNKT cells, results in upregulation of CD25. In one study, upregulation of CD25 on iNKT cells was documented in patients with tuberculosis.<sup>285</sup>

I used multiparameter flow cytometry to investigate iNKT cell expression of CD4, CD8, CD161 and the activation marker CD25 in patients with CLL. The immunophenotype of circulating iNKT cells was similar in patients and controls (figure 3.11). Although I observed a trend towards a higher proportion of CD4<sup>+</sup> iNKT cells in patients (figure 3.11), this was not statistically significant. A statistically significant inverse correlation between the proportion of iNKT cells which were CD4<sup>+</sup> and the number of iNKT cells (as a proportion of T cells) was observed (figure 3.12), a finding which has previously been reported.<sup>218</sup>

### 3.3.7 iNKT cell numbers do not correlate with clinical outcome

Clinical outcome was assessed by review of patient records for the 29 out of 30 patients with untreated CLL for whom follow-up data were available. Events defining progression-free survival were death from any cause or progression of CLL requiring treatment (including palliative treatments or treatment for disease-related autoimmune cytopenias).

During a median follow-up of 38.7 months following phlebotomy (range 12.4 – 48.8 months), eight out of twenty-nine patients progressed: four patients received chlorambucil-based chemotherapy, one received FCR chemotherapy, one was treated with corticosteroids for autoimmune haemolytic anaemia, one patient had radiotherapy to a symptomatic lymph node and one patient died of metastatic lung cancer.

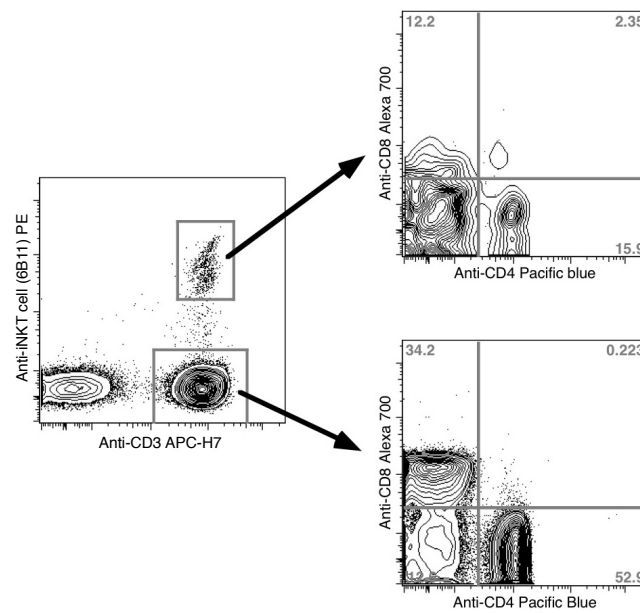
The median iNKT cell frequency was 0.009% of T cells (range 0.004 – 0.051) among patients who progressed or died and 0.011% of T cells (range 0.001 – 0.113) among patients who remained alive and treatment-free (difference not significant, Mann Whitney test).

The median absolute iNKT cell number was 253/mL blood (range 97 – 2452) among patients who progressed or died and 335/mL (range 18 – 2940) among patients who remained alive and treatment-free during follow-up (difference not significant, Mann Whitney test).

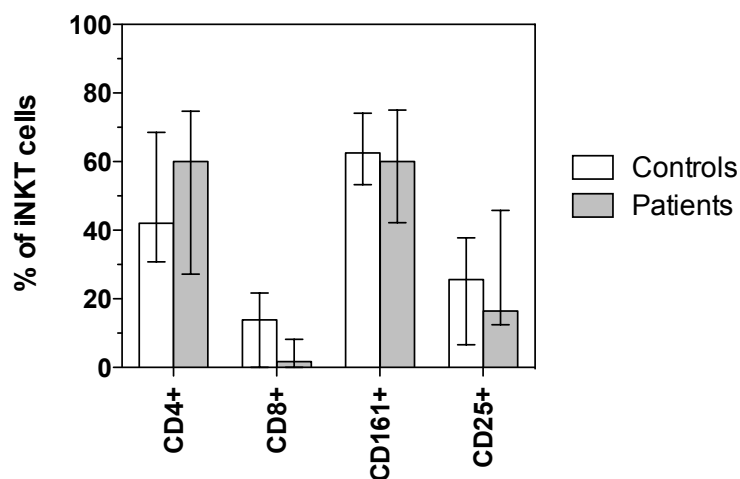
Survival curve analysis showed no difference in progression-free survival between those with a high and those with a low iNKT cell frequency (figure 3.13a) or between those with a high and those with a low absolute number of circulating iNKT cells (figure 3.13b).

**Figure 3.11 Immunophenotype of circulating iNKT cells** (a) Representative flow cytometry plots showing CD4 and CD8 status of T and iNKT cells. (b) Patient (n = 38) and control (n = 28) PBMCs were stained, and expression of CD4, CD8, CD161 and CD25 determined by flow cytometry, gated on iNKT cells (identified using 6B11 antibody clone). Bars represent medians, error bars represent interquartile ranges. Difference not significant for any comparison, Mann Whitney test. (c) CD4 status of iNKT cells presented according to fludarabine treatment status. Difference not significant for any comparison, one-way ANOVA.

(a)

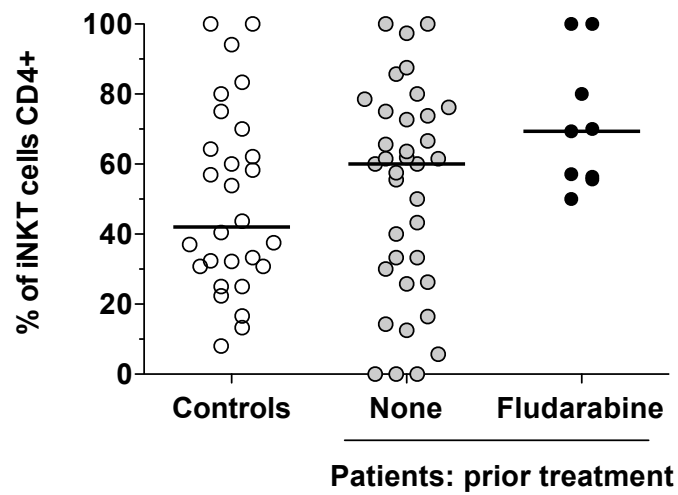


(b)

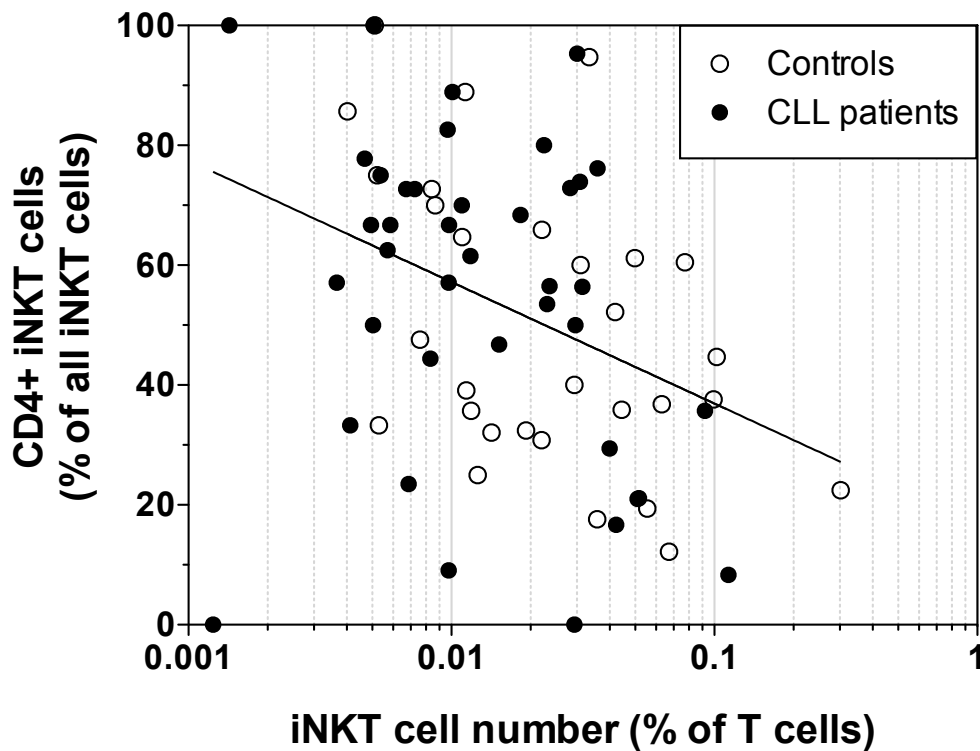


**Figure 3.11 (continued) Immunophenotype of circulating iNKT cells (c) CD4 status of iNKT cells presented according to fludarabine treatment status. Difference not significant for any comparison, one-way ANOVA.**

(c)



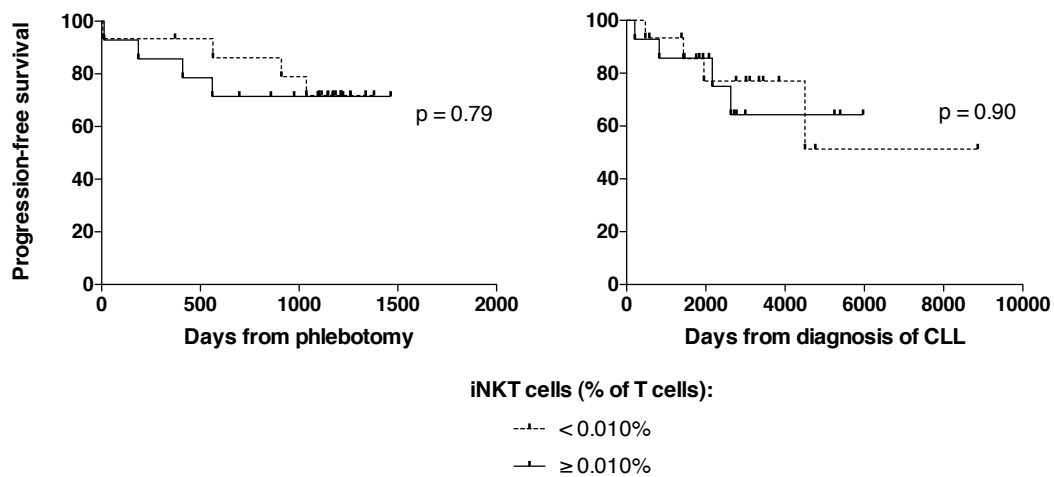
**Figure 3.12 Correlation between iNKT cell numbers and iNKT cell CD4 status** Patient (n = 40) and control (n = 30) PBMCs were analysed by flow cytometry for numbers of iNKT cells (identified as 6B11+CD3+), and for iNKT cell CD4 expression. The percentage of iNKT cells which are CD4+ is plotted against the iNKT cell number (as a percentage of all T cells).  $r^2 = 0.136$ ,  $p < 0.01$  of zero slope, nonlinear regression, semilog fit.



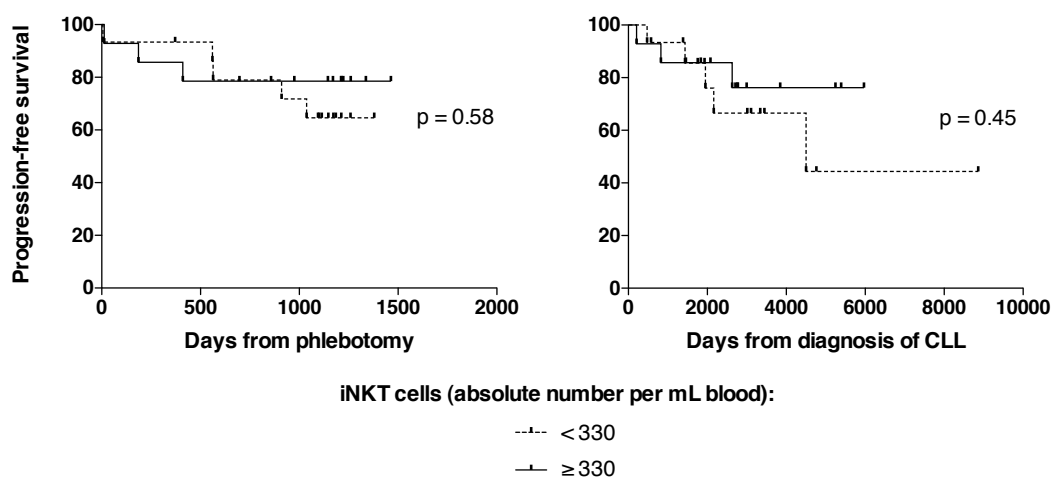


**Figure 3.13 Progression-free survival does not correlate with iNKT cell numbers** Progression-free survival was determined for the 29 patients with untreated CLL for whom follow-up data were available. (a) Progression-free survival did not differ between those with a high iNKT cell frequency ( $\geq 0.01\%$  of T cells, solid line,  $n = 14$ ) and those with a low iNKT cell frequency ( $< 0.01\%$  of T cells, dashed line,  $n = 15$ ), whether determined from the date of phlebotomy for iNKT cell frequency assessment or from the date of diagnosis. (b) Progression-free survival did not differ between those with a high absolute iNKT cell number ( $\geq 330$  per mL blood, solid line,  $n = 14$ ) and those with a low absolute iNKT cell number ( $< 330$  per mL blood, dashed line,  $n = 15$ ), whether determined from the date of phlebotomy for iNKT cell frequency assessment or from the date of diagnosis. P values represent comparison of survival curves using log-rank (Mantel-Cox) test.

(a)



(b)



### 3.4 Summary

In this chapter, I have determined the numbers and immunophenotype of circulating iNKT cells from patients with CLL.

I recruited a cohort of patients with untreated and treated CLL, and a group of healthy controls who were closely age-matched. The sensitivity of flow cytometric detection of iNKT cells was optimised by depletion of contaminating leukaemic B cells, the selection of a highly specific fluorophore (the 6B11 antibody clone), and the use of a stringent gating strategy including exclusion of dead cells, B cells and doublet events.

My results indicate that although reduced as a proportion of T cells, absolute numbers of iNKT cells are preserved in patients with untreated CLL, with a decline among those who had received fludarabine chemotherapy. The combination of a reduced frequency but normal absolute number of iNKT cells may be explained by a failure of iNKT cells to fully participate in the expansion of other T cell subsets observed in patients with untreated CLL. In contrast, reduced absolute numbers of all T cell subsets, including iNKT cells, were seen in those who had received fludarabine-based chemotherapies.

The immunophenotype of circulating iNKT cells is similar in patients to that in controls: no significant differences in CD4, CD8, CD161 and CD25 expression were observed, although there was a trend towards a higher proportion of CD4+ iNKT cells in patients. This trend is keeping with a general inverse correlation between the frequency of iNKT cells overall and the proportion of iNKT cells which were CD4+.

Finally, I sought, but did not find, a correlation between iNKT cell frequency and number and progression free survival among patients with untreated CLL. This suggests that a deficiency of circulating iNKT cells does not directly contribute to CLL disease progression.

In chapter 4, I shall evaluate expression of CD1d, the molecule which presents glycolipid antigens to iNKT cells, on PBMCs from patients with CLL and controls.

## Chapter 4

# CD1d expression on PBMC subsets

### 4.1 Introduction

In chapter 3, I established that absolute numbers of circulating iNKT cells were normal in untreated patients with CLL, and were reduced but detectable in patients who had previously received fludarabine-containing therapies. I found no major differences in the immunophenotype of circulating iNKT cells between patients and controls.

The activation of iNKT cells through their invariant TCR requires the presentation of glycolipid antigens on the MHC Class I-like molecule, CD1d. Many cells of haematopoietic origin express CD1d, including B cells, monocytes and mDCs.<sup>445</sup>

Numeric, phenotypic and functional defects of APCs have been described in patients with CLL, including reduced frequency of circulating DCs,<sup>78</sup> reduced MHC Class I expression on circulating DCs,<sup>80</sup> and reduced allostimulatory of moDCs generated from patients with active disease.<sup>82</sup> The expression of CD1d on circulating APC subsets has not been reported in CLL.

The lysis of tumour cells by iNKT cells requires target cell CD1d expression.<sup>309,310</sup> Expression of CD1d on tumour cells also augmented the efficacy of vaccination with  $\alpha$ -GalCer-pulsed tumour cells in preclinical studies.<sup>422,423</sup> A single published report indicates that CLL cells do express CD1d and, and that they can be lysed by allogeneic iNKT cell lines.<sup>417</sup>

I used flow cytometry to examine the numbers of circulating APC subsets and CD1d expression on APCs and CLL cells, on PBMC samples from patients with CLL and from healthy controls.

### 4.2 Aims

The specific aims of the experiments outlined in this chapter were:

- to enumerate circulating APCs from patients with CLL
- to determine CD1d expression levels on circulating APCs
- to determine CD1d expression levels on CLL cells.

### 4.3 Results

#### 4.3.1 Relative CD1d expression levels on PBMC subsets

Activation of iNKT cells by  $\alpha$ -GalCer requires its presentation on the MHC Class I-like molecule, CD1d. Within PBMCs, the highest levels of CD1d expression were found on mDCs and on monocytes. Leukemic B cells expressed CD1d at a lower level, similar to that of normal B cells (figure 4.1).

Higher levels of CD1d have been reported on CLL cells in cases with unmutated IgVH genes.<sup>417</sup> I do not have data on the IgVH status of the patients in this study. Of the samples exhibiting ZAP-70 positivity (which correlates with unmutated IgVH status), there was no trend towards higher CD1d expression.

In patients with myeloma, another malignancy of mature B cells, CD1d expression on the malignant B cells declines with progression of the disease.<sup>446</sup> I therefore compared CD1d expression levels on CLL cells between patients with early (Binet stage A) and clinically advanced (Binet stages B or C) disease (figure 4.1c). I could find no evidence of reduced CD1d expression on CLL cells in patients with advanced as opposed to early stage disease (figure 4.1).

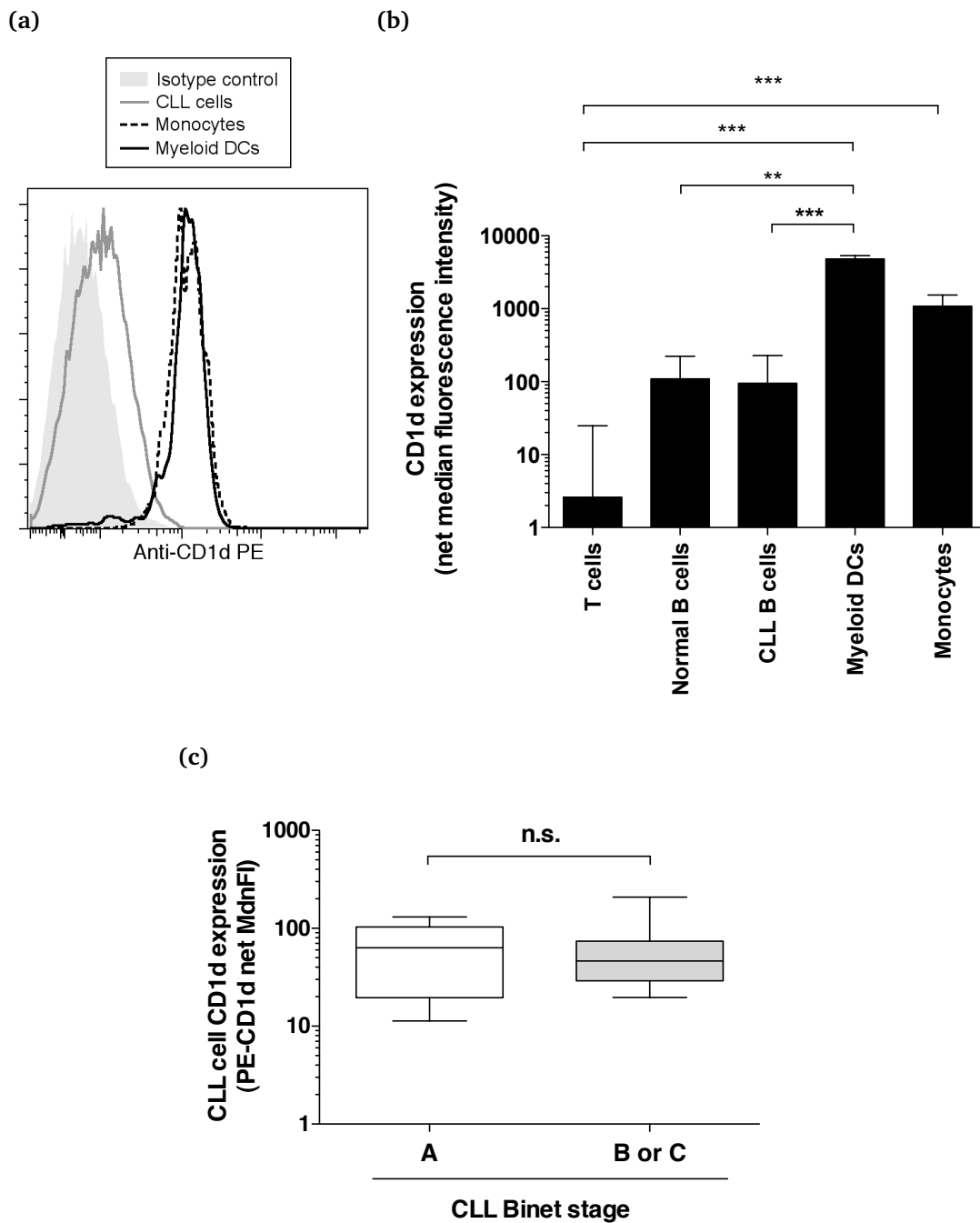
#### 4.3.2 Myeloid dendritic cell numbers and CD1d expression

Numeric, immunophenotypic and functional defects of circulating mDC have been reported in CLL.<sup>78,80</sup> Therefore, I investigated the numbers of, and CD1d expression on, mDC from patients with CLL in more detail.

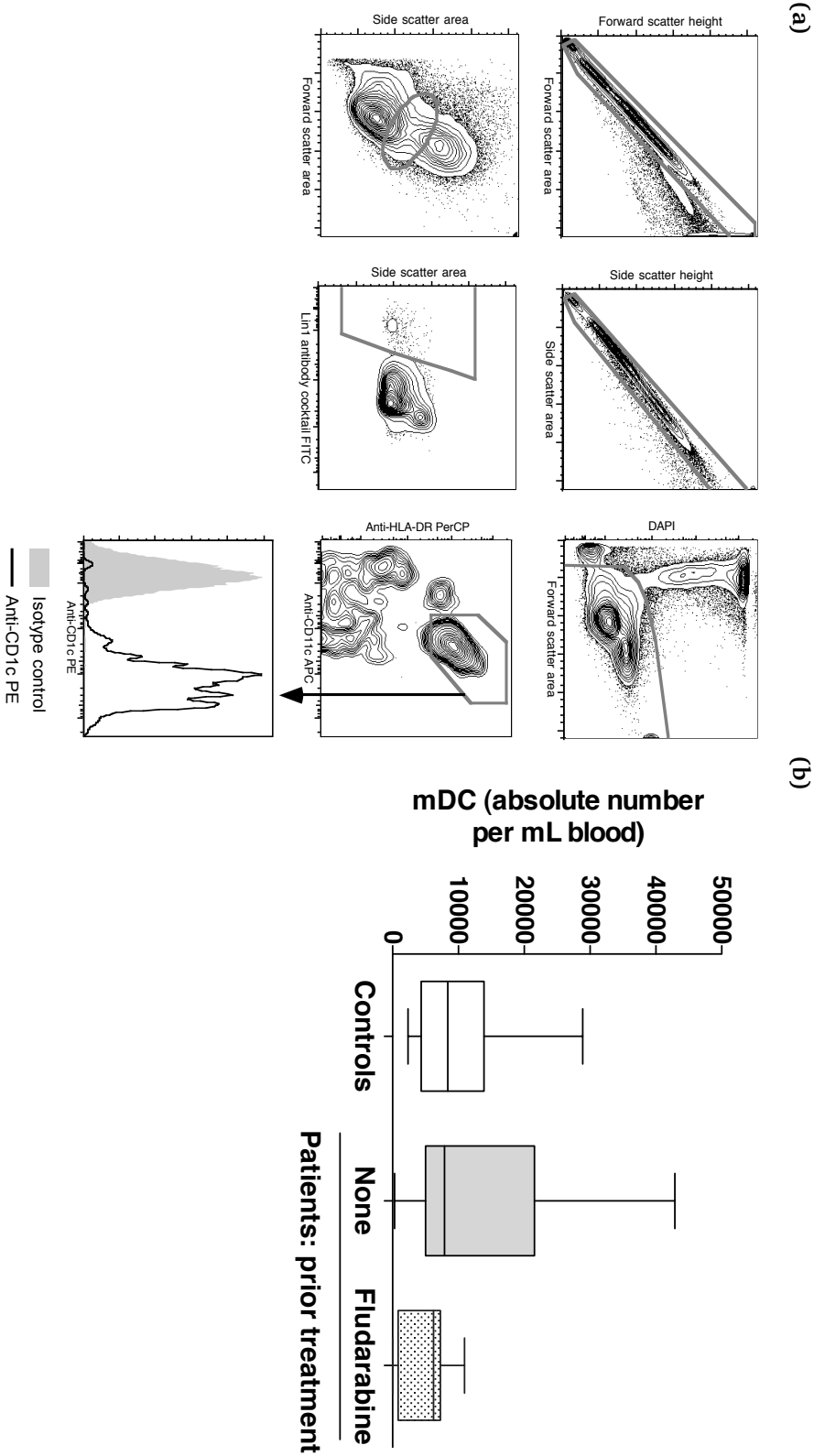
Flow cytometric gating of mDCs was performed by selecting a forward and side scatter gate intermediate between lymphocytes and monocytes, excluding cells expressing lineage markers using a Lin-1 antibody cocktail, then selecting cells which expressed high levels of HLA-DR and CD11c. Cells identified in this manner were overwhelmingly positive for CD1c, also known as Blood Dendritic Cell Antigen 1 (BDCA-1), and considered a specific marker for mDCs (median 98.5% CD1c positive, range 94.4% – 100%, n = 16; figure 4.2a).<sup>447</sup>

As expected, mDC numbers were reduced as a proportion of PBMCs in most patients due to large numbers of circulating leukaemic cells. In contrast, the absolute numbers of circulating mDCs were similar to controls in patients with untreated CLL, with a non-significant trend towards reduction among patients who had received a fludarabine-based chemotherapy (median  $8.4 \times 10^3$ /mL in controls,  $7.9 \times 10^3$ /mL in untreated patients,  $6.2 \times 10^3$ /mL in fludarabine-treated patients, figure 4.2b).

**Figure 4.1 Expression of CD1d on PBMC from patients with CLL.** CD1d expression on PBMCs from patients with CLL was determined by flow cytometry, gating on T cells (CD19<sup>-</sup>, CD5<sup>+</sup> lymphocytes), normal B cells (CD19<sup>+</sup>, CD5<sup>-</sup> lymphocytes), CLL cells (CD19<sup>+</sup>, CD5<sup>+</sup> lymphocytes), myeloid DCs (intermediate forward and side scatter, Lin1<sup>-</sup>, HLA-DR high, CD11c high) and monocytes (high forward and side scatter, CD14<sup>+</sup>). (a) CD1d expression on PBMC subsets from an individual patient with CLL. (b) CD1d expression levels in patients with untreated CLL (n = 10). Bars represent median, error bars represent range, \*\*\* p < 0.001, \*\* p < 0.01, Friedman test with Dunn's multiple comparison. (c) CD1d expression on CLL cells in patients with early (Binet stage A; n = 10) or clinically advanced (Binet stages B or C; n = 9) disease. Bars represent medians; n.s. = not significant, Mann Whitney test.



**Figure 4.2 Numbers of mDC in patients and controls.** (a) Flow cytometric identification of circulating mDC, showing sequential gating strategy. (b) Absolute numbers of circulating mDC in healthy controls (n = 30), untreated CLL (n = 30) and fludarabine-treated CLL patients (n = 9). Difference not significant for any comparison; Kruskal-Wallis test with Dunn's post-tests.



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Assessed by flow cytometry using an anti-CD1d antibody, the surface expression of CD1d on mDC was the same in patients with CLL as in healthy donors (figures 4.3a and 4.3b).

Hypogammaglobulinemia is a common feature of CLL. MoDCs generated in the absence of human IgG have reduced CD1d expression, a finding ascribed to IgG binding to moDC via FcγRIIa.<sup>448</sup> I hypothesized that mDC CD1d expression may correlate positively with serum IgG level. However, I found no relationship between CD1d expression and serum IgG levels in patients or in healthy donors ( $r^2 = 0.00$ ), with normal mDC CD1d expression even in patients with marked hypogammaglobulinaemia (figure 4.3c).

### 4.3.3 Monocyte numbers and CD1d expression

As already noted, monocytes express CD1d at a level intermediate between that of B cells and mDCs (figure 4.1b). As monocytes constitute a high proportion of PBMCs, they may be important for the presentation of  $\alpha$ -GalCer to iNKT cells. Abnormalities of the monocyte population have been described in patients with CLL, including a monocytosis and increased MHC class II expression.<sup>449</sup> I therefore investigated monocyte numbers and CD1d expression in more detail.

Two principal monocytes subpopulations have been identified in human PBMCs: a large population of monocytes with high expression of CD14 and low CD16 expression (CD14hi monocytes), and a smaller population of monocytes that co-express CD16 and CD14 (CD16+ monocytes). CD16+ monocytes are also known as 'inflammatory monocytes', as they possess phenotypic features of tissue macrophages,<sup>450</sup> are potent producers of TNF- $\alpha$  in vitro,<sup>451</sup> and are increased in number in proinflammatory states such as sepsis.<sup>452</sup> These proinflammatory characteristics do not appear to translate to tumour protection, however, as CD16+ monocytes are reported to be increased in numbers in patients with breast cancer and cholangiocarcinoma, and to carry negative prognostic value.<sup>453,454</sup> In vitro, CD16+ monocytes differentiate into moDCs with Th2-skewing properties.<sup>455,456</sup>

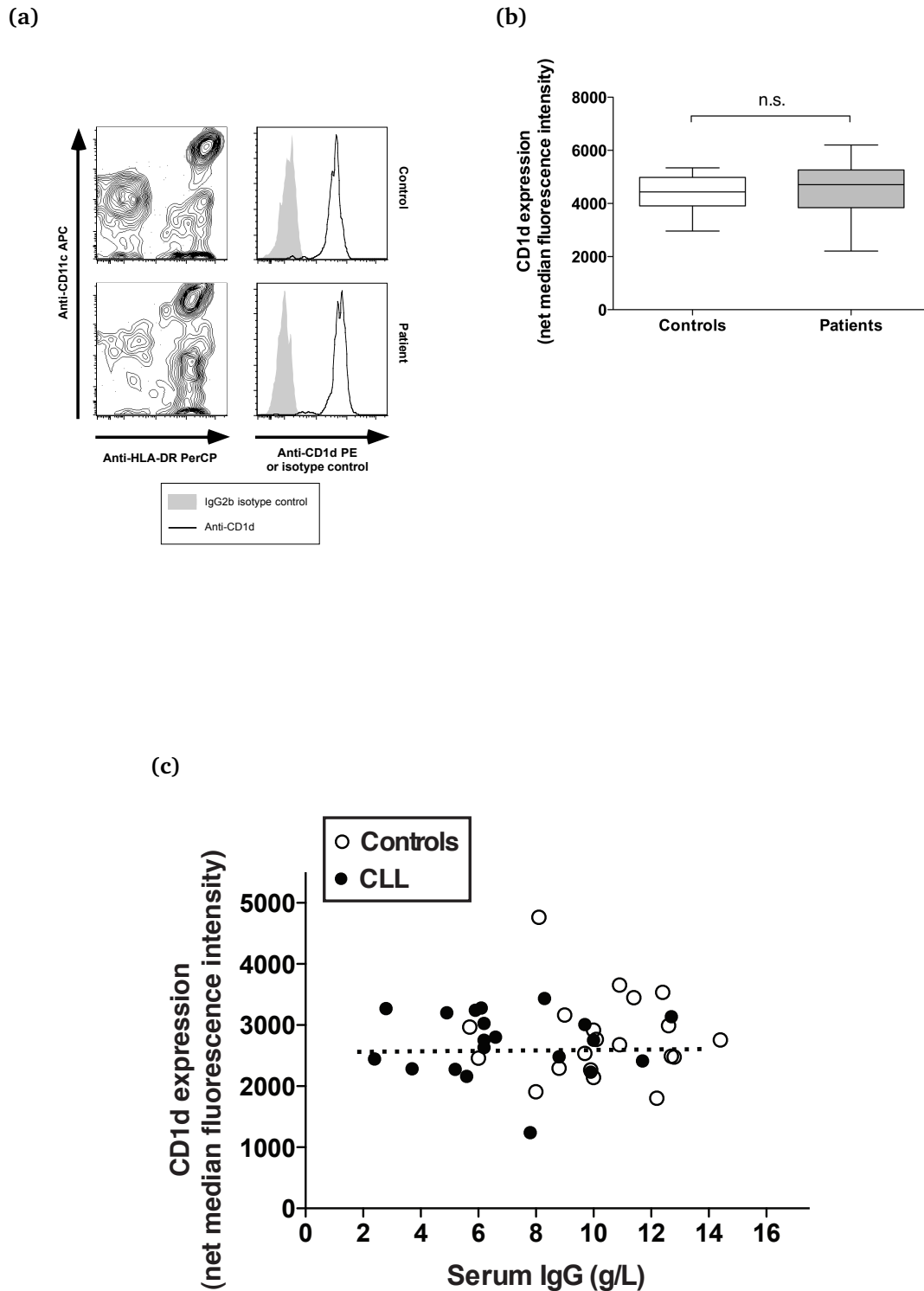
I investigated the numbers of these two monocyte subsets in patients with CLL and controls, and examined CD1d expression levels on each subset.

The absolute number of CD14hi monocytes was similar in patients and controls. A trend towards a higher frequency of CD16+ monocytes in fludarabine-treated patients with CLL was not statistically significant (median 5.9% of monocytes CD16+ in controls, 6.7% in untreated patients, 11.1% in fludarabine-treated patients,  $p = 0.06$ , Kruskal-Wallis test; figure 4.4).

Flow cytometric analysis indicated that CD14hi monocytes consistently expressed CD1d at a significantly higher level than their CD16+ counterparts in both patients and controls ( $p < 0.0001$ , Wilcoxon matched pairs test; figure 4.5a and 4.5b). Comparing patients with controls, expression of CD1d on the two monocyte subsets was normal in patients with CLL (figure 4.5c).

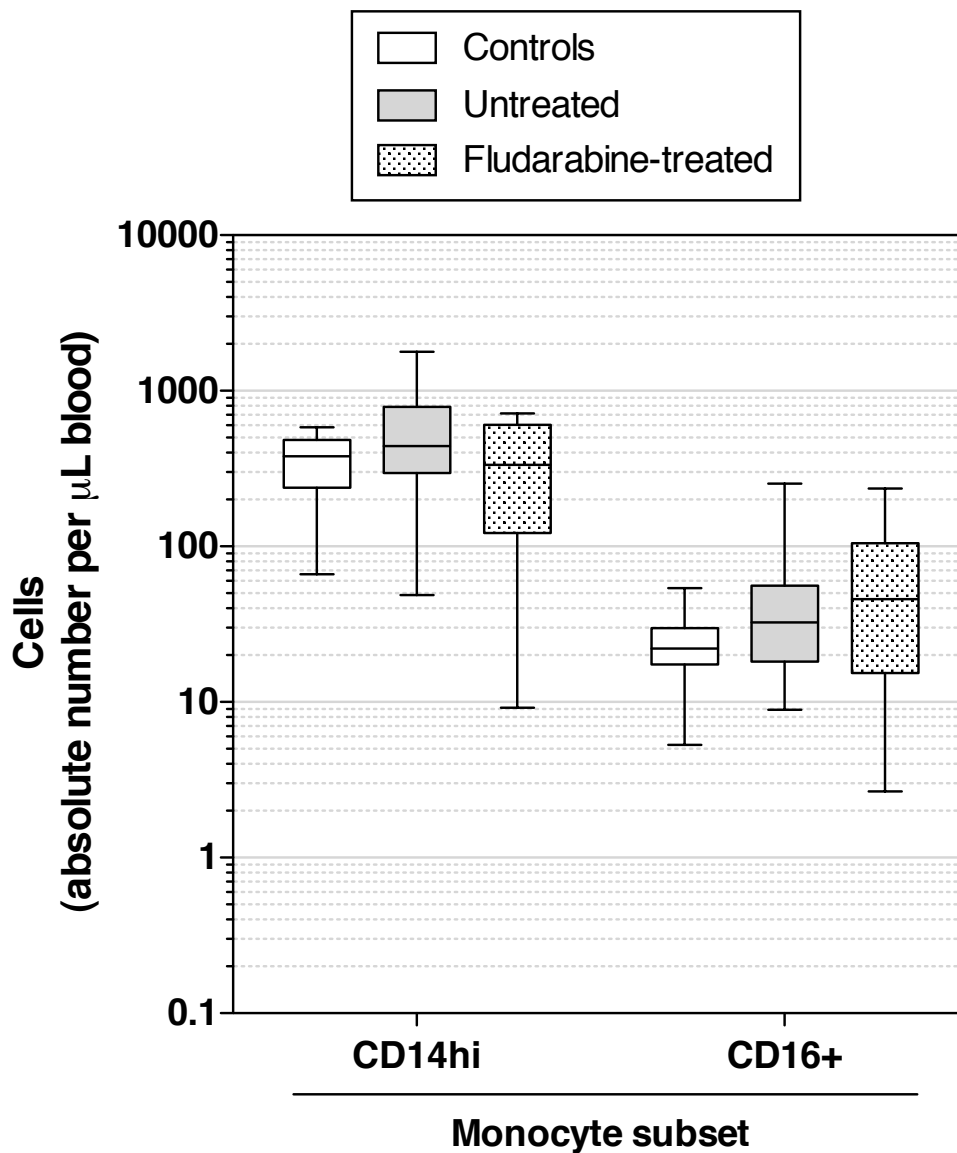
## Chapter 4. CD1d expression on PBMC subsets

**Figure 4.3** Expression of CD1d on patient and control mDC. (a) Representative flow cytometry plots demonstrating expression of CD1d by patient and control mDC. (b) CD1d expression on patient and control mDCs; box represents median +/- interquartile range; whiskers represent range. (c) Myeloid DC CD1d expression in controls (n = 20) and patients with CLL (n = 20), expressed according to serum IgG level. Dotted line represents linear regression ( $r^2 = 0.00$ ).

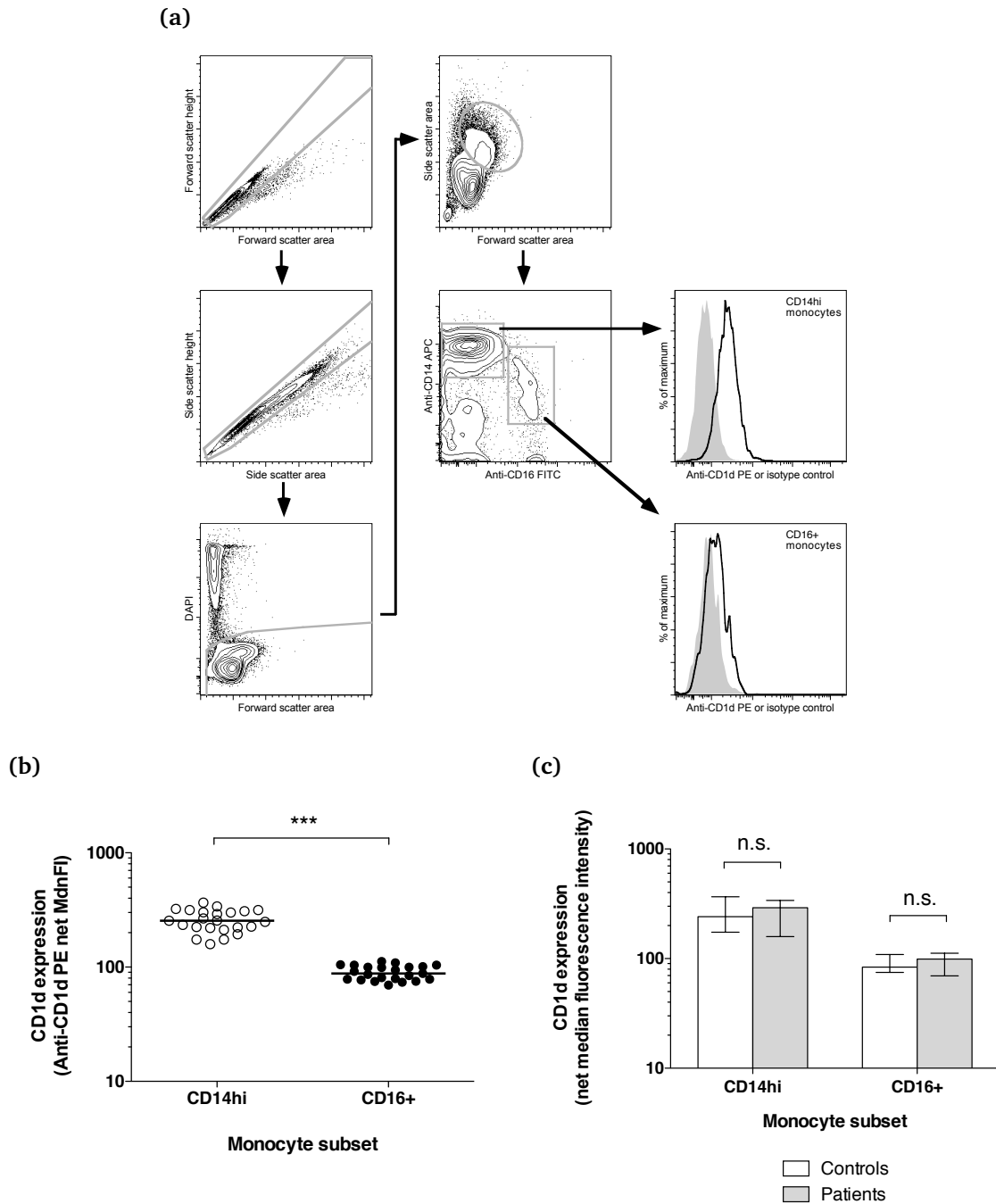




**Figure 4.4 Absolute numbers of monocyte subsets.** The CD14<sup>hi</sup> and CD16<sup>+</sup> monocyte subsets were identified by flow cytometry of PBMCs, as indicated in figure 4.5a, and absolute numbers of each subset calculated using full blood counts performed on whole blood on the same day as PBMC storage. Differences not significant for any comparison, Kruskal-Wallis test with Dunn's post-tests.



**Figure 4.5 CD1d expression on monocyte subsets.** (a) Example of gating from a patient with CLL, illustrating reduced CD1d expression on the CD16+ subset of monocytes, compared to CD14hi monocytes. Gates are indicated by gray lines. For CD1d expression histograms, the black line represents anti-CD1d PE, and solid gray represents isotype-matched control antibody. (b) comparison of CD1d expression on CD14hi and CD16+ monocyte subsets; \*\*\*  $p < 0.001$ , Wilcoxon matched pairs test. (c) Expression of CD1d on monocyte subsets was studied in controls (n = 12) and patients (n = 11) (bars represent medians, error bars represent range; n.s. = not significant, Mann Whitney test).



## 4.4 Summary

In this chapter, I have investigated the expression of CD1d on PBMC subsets in patients and controls.

CLL cells expressed CD1d at a similar level to that of normal B cells, with no evidence of a reduction in CD1d expression in those with more clinically advanced disease.

The highest levels of CD1d expression were found on mDCs. I found normal absolute numbers of circulating mDCs in untreated CLL patients, with a trend towards a reduction in those who had previously received fludarabine-based chemotherapy. The expression of CD1d on mDC was normal in patients, even among those with marked hypogammaglobulinaemia.

The numbers of the major monocyte subsets were similar in patients with CLL to healthy controls, although there was a non-significant trend towards higher numbers of 'inflammatory' CD16<sup>+</sup> monocytes in patients with CLL who had received a fludarabine-based chemotherapy. If confirmed with larger numbers, this would be in keeping with the finding of increased numbers of CD16<sup>+</sup> monocytes in patients with other malignancies, such as breast cancer and cholangiocarcinoma.<sup>453,454,457</sup>

Although overall monocyte CD1d expression levels were similar in patients with CLL compared to controls, the CD14<sup>hi</sup> and CD16<sup>+</sup> subsets of monocytes exhibited differential expression of CD1d. The CD14<sup>hi</sup> subset expressed CD1d at significantly a higher level than the minor population of CD16<sup>+</sup> monocytes in both healthy donors and patients.

Taken together, these results suggest that patients with fludarabine-treated CLL exhibit changes in myeloid APCs populations, with reduced mDC numbers and a trend towards higher CD16<sup>+</sup> monocyte numbers. However, the overall expression of CD1d on both mDCs and monocytes is largely intact in patients with both treated and untreated CLL. These results confirm that CLL cells themselves express CD1d, at a level similar to that of normal B cells.

Having established that phenotypically-normal iNKT cells can be detected in the peripheral blood of patients with CLL in chapter 3, and that CD1d expression on circulating APCs is intact in this chapter, I proceed to investigate the function of iNKT cells from patients with CLL in chapter 5.



## Chapter 5

# Function of iNKT cells from patients with CLL

### 5.1 Introduction

In chapters 3 and 4, I established that the iNKT cell and CD1d axis is essentially numerically and immunophenotypically intact in patients with untreated CLL, although absolute numbers of iNKT cells and mDCs were reduced in patients who had previously received a fludarabine-containing therapy. In this chapter, I aim to evaluate the function of iNKT cells from patients with CLL.

As discussed in section 1.3.3, properties of iNKT cells which might be exploited for use in immunotherapeutic strategies include the production of cytokines (resulting in transactivation of NK cells), the maturation of dendritic cells (resulting in enhanced T cell responses to peptide antigens), and the lysis of CD1d-expressing target cells.

Section 1.1.3.5 details the functional defects of T cells described in patients with CLL, which include increased IL-4 production,<sup>95</sup> defective immunological synapse formation,<sup>97</sup> and impaired *in vitro* expansion in response to mitogenic stimuli.<sup>94</sup>

Abnormalities of iNKT cell function have been described in patients with other malignancies. The production of IFN- $\gamma$  by iNKT cells is reduced in patients with advanced myeloma and prostate cancer.<sup>304,307</sup> In patients with advanced prostate cancer, *ex vivo* iNKT cell proliferation was impaired,<sup>304</sup> while in contrast, iNKT cell expansion from patients with myeloma was maintained.<sup>307</sup>

The function of iNKT cells from patients with CLL has not previously been described. In view of the wide-ranging defects of T cell function described in patients with CLL,<sup>334</sup> and the abnormal iNKT cell function observed in patients with other malignancies, I hypothesised that, despite possessing a normal immunophenotype, iNKT cells from patients with CLL would exhibit abnormalities in cytokine production, proliferation or cytotoxicity.

### 5.2 Aims

To assess function of iNKT cells from patients with CLL, I performed a series of experiments to assess iNKT cell cytokine production and *in vitro* proliferation in whole PBMCs cultures. To enable analysis of iNKT cell cytotoxicity, I generated and characterised the function of iNKT cell lines from patients and controls.

The specific aims of the experiments detailed in this chapter were:

- To evaluate cytokine production by iNKT cells from patients with CLL
- To determine the capacity of iNKT cells from patients with CLL to proliferate *in vitro*
- To generate and characterise iNKT cell lines from patients with CLL

### 5.3 Results

#### 5.3.1 Cytokine production by iNKT cells

Invariant natural killer T cells are potent producers of cytokines. Among others, iNKT cells are capable of producing both Th1-type cytokines (such as IFN- $\gamma$  and IL-2) and Th2-type cytokines (such as IL-4, IL-5, IL-10 and IL-13). Defects of iNKT cell cytokine production have been described in patients with advanced myeloma and prostate cancer.<sup>304,307</sup>

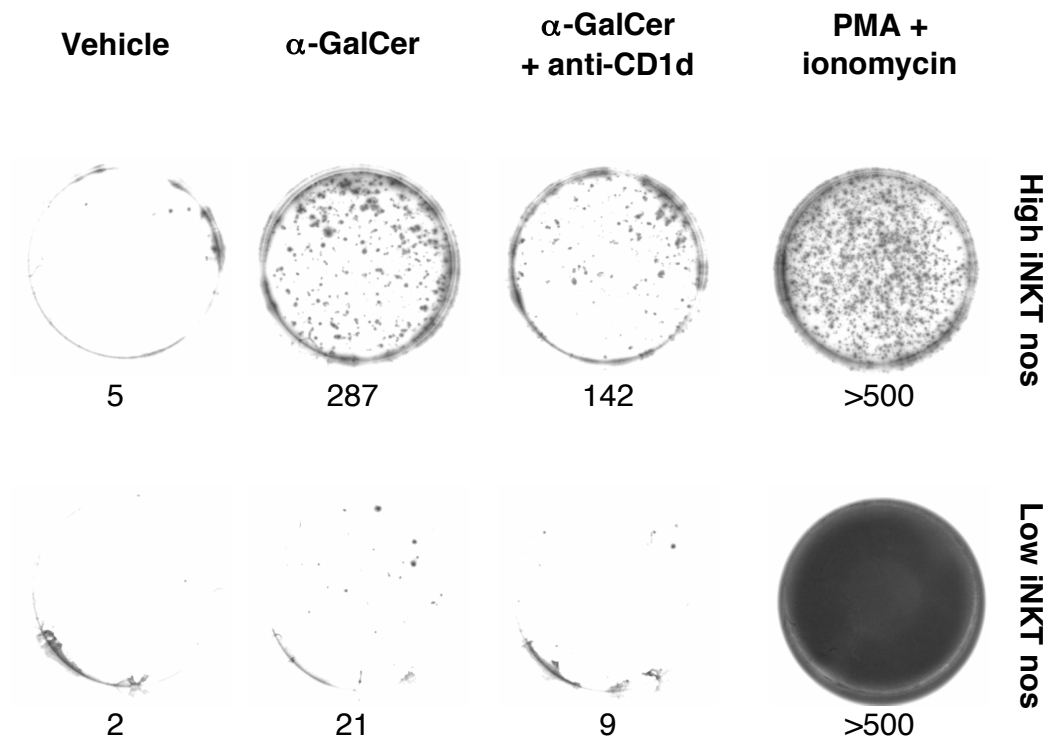
Assessment of cytokine production is challenging because iNKT cells constitute a very small proportion of circulating T cells in humans (typically 0.01% in patients with CLL, as determined in chapter 3). I therefore employed two sensitive techniques: an ELISpot assay, and intracellular cytokine staining (ICS).

Preliminary experiments demonstrated that the addition of  $\alpha$ -GalCer to PBMCs enabled specific detection of iNKT cells: the numbers of  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpots exceeded those produced in the presence of vehicle alone, and were inhibited by the addition of anti-CD1d antibody (figure 5.1).

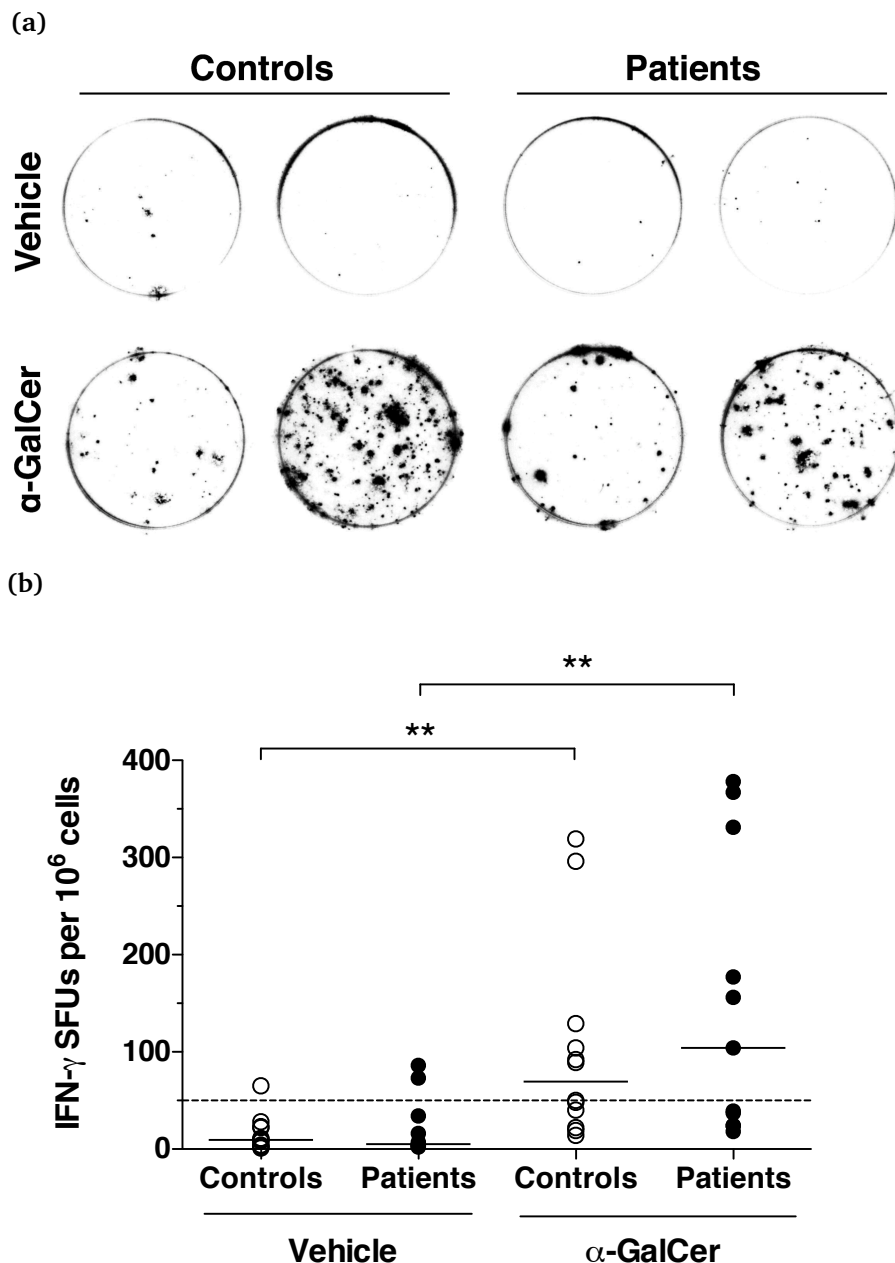
I performed IFN- $\gamma$  ELISpot assays on B-cell depleted PBMCs from patients with CLL and controls (figure 5.2). A significant increase in ELISpots over background was observed with the addition of  $\alpha$ -GalCer in both groups. In the presence of  $\alpha$ -GalCer, at least 50 IFN- $\gamma$  ELISpots were observed, and the number of IFN- $\gamma$  ELISpots was at least double that of control, in six out of twelve healthy donors and in six out of eleven patients. The difference between the number of  $\alpha$ -GalCer induced ELISpots observed in patient and healthy donor samples was not statistically significant.

Although this indicates that  $\alpha$ -GalCer-induced IFN- $\gamma$  production is intact in CLL, the ELISpot assay does not determine which cell type is releasing IFN- $\gamma$ . The cytokine could be produced by iNKT cells themselves, or by neighbouring cell types (such as NK or T cells). To directly assess cytokine production by iNKT cells, I used flow cytometry with ICS. This technique allows simultaneous evaluation of multiple surface and intracellular markers. The results of ICS of T cells have previously been reported in CLL: reported findings include enhanced T cell production of IFN- $\gamma$  and IL-2 in patients with early CLL,

**Figure 5.1  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpot assay.** PBMCs from individuals with high or low numbers of circulating iNKT cells were incubated with  $\alpha$ -GalCer,  $\alpha$ -GalCer with anti-CD1d antibody, vehicle (negative control) or PMA and ionomycin (positive control) at  $5 \times 10^5$  cells per well. The number of IFN- $\gamma$  ELISpots is given below each image.



**Figure 5.2 IFN- $\gamma$  ELISpot production by patient and control PBMCs in response to  $\alpha$ -GalCer.**  $1 \times 10^6$  B-cell depleted PBMCs were incubated for 24 hours in the presence of  $\alpha$ -GalCer or vehicle control. ELISpots were developed and enumerated by automated counter. (a) Representative  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpots from two controls and two patients with CLL. (b) ELISpots from patients with CLL (n = 11) and age-matched healthy controls (n = 11). Dashed line represents a threshold value of 50 ELISpots per well. \*\* p < 0.01, Wilcoxon matched-pairs test.





and in one study, a Th2 bias with increased IL-4 production in patients with progressive CLL.<sup>458-460</sup>

Expression of intracellular IFN- $\gamma$  and IL-4, the archetypal Th1 and Th2 cytokines respectively, was assessed in PMA and ionomycin-stimulated PBMCs from healthy donors and controls, gating separately on iNKT and T cell populations. In comparison to normal T cells, iNKT cells produced significantly more IFN- $\gamma$  (figure 5.3). Moreover, compared to 'conventional' T cells, a significantly higher proportion of iNKT cells were dual cytokine producers, with some individual iNKT cells producing both IFN- $\gamma$  and IL-4, as previously reported.<sup>461</sup> Separate analysis of CD4+ and CD4- iNKT cell subsets in healthy donors confirmed the previously reported finding that the CD4+ subset of iNKT cells produces more IL-4 than the CD4- subset (figure 5.4).<sup>218</sup>

Significantly higher T cell IFN- $\gamma$  production was observed in patients compared to controls, consistent with previous reports (figure 5.5a).<sup>458,460</sup> Although there was a trend towards higher IFN- $\gamma$  production from patient iNKT cells, this was not statistically significant, and iNKT cell IL-4 expression was the same in patient and control groups (figure 5.5b).

Taken together, the ELISpot and ICS assays performed indicate that circulating iNKT cells in patients with CLL have an intact potential to produce IFN- $\gamma$ , and do not exhibit a Th2 bias compared with iNKT cells from healthy controls.

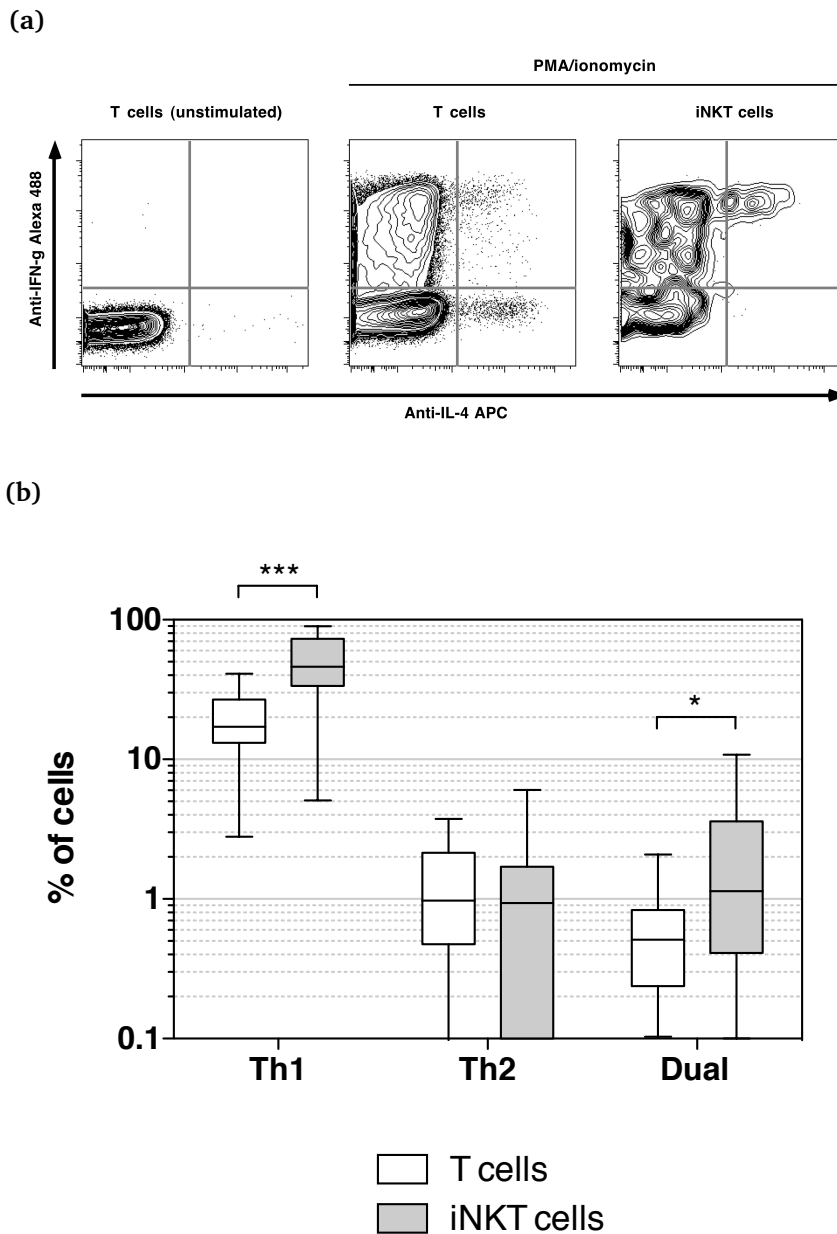
### 5.3.2 Granzyme B expression by iNKT cells

The serine protease granzyme B is expressed by cytotoxic CD8+ T cells and NK cells, and contributes to target cell lysis. Granzyme B appears to play a role in iNKT cell cytotoxicity: in one study,  $\alpha$ -GalCer-induced cell lysis of primary human myelomonocytic leukemia cells by iNKT cells was perforin- and granzyme B-dependent.<sup>229</sup>

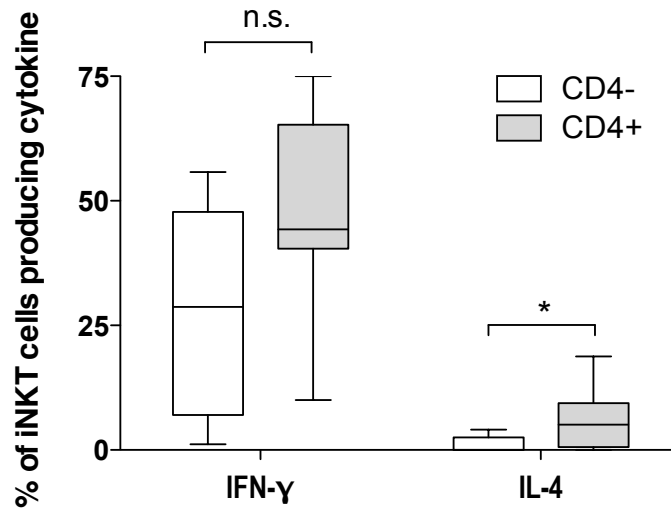
The expression of granzyme B is modulated by cytokines, particularly by IL-2 and its related cytokines IL-15 and IL-21, which enhance granzyme B expression.<sup>462</sup> CLL cells express the IL-2 receptor alpha (CD25) and can take up IL-2 directly, depriving T cells of this signal.<sup>106</sup> Moreover, supernatant from cultures of CLL cells actively inhibits T cell IL-2 production.<sup>91</sup> I therefore wished to determine granzyme B expression of iNKT cells from patients with CLL as a first step towards establishing their cytotoxic function.

I used intracellular staining to determine granzyme B expression levels on NK, T and iNKT cells in patients and controls. Granzyme B expression of iNKT cells was lower than on NK cells, and at a similar level to T cells overall (figure 5.6a and 5.6b). Comparing patients with controls, I found no major differences between granzyme B expression levels on NK, T or iNKT subsets (figure 5.6c). As with the ICS data above, iNKT cell granzyme B expression data should be interpreted with caution, as fewer iNKT cell events than NK or T cell events were available for analysis.

**Figure 5.3 Intracellular cytokine staining of iNKT cells.** Patient and control PBMCs were incubated with PMA and ionomycin for 6 hours then permeabilised and stained for intracellular IFN- $\gamma$  and IL-4. (a) Representative intracellular cytokine staining showing cytokine profiles of T cells and iNKT cells. (b) Comparison of cytokine profiles of T and iNKT cells (n = 22). ‘Th1’ refers to cells producing IFN- $\gamma$  alone; ‘Th2’ refers cells producing IL-4 alone; ‘Dual’ refers to cells expressing both IFN- $\gamma$  and IL-4 producing. Bars within boxes represent medians, boxes represent interquartile range, and whiskers represent range. \*\*\* p < 0.0001, \* p < 0.05, Wilcoxon signed rank test.



**Figure 5.4 Differential cytokine production by CD4<sup>+</sup> and CD4<sup>-</sup> subsets of iNKT cells.** ICS was performed on PBMCs from healthy donors (n = 6), gating on the CD4<sup>-</sup> and CD4<sup>+</sup> subpopulations of iNKT cells. \* p < 0.05, Mann Whitney test..



### 5.3.3 Proliferative capacity of iNKT cells

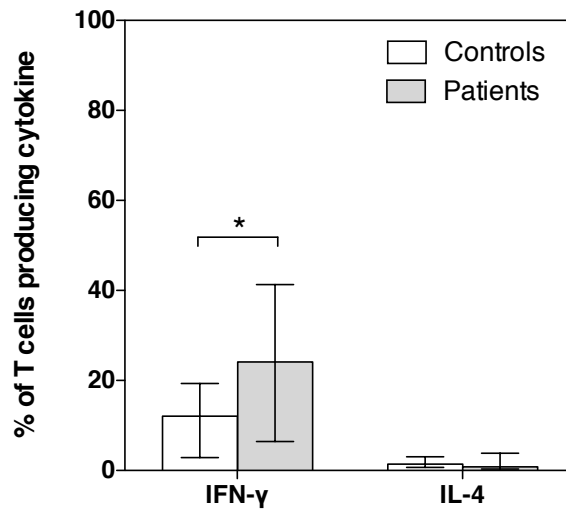
Next, I wished to establish whether iNKT cells from patients with CLL were able to proliferate when PBMCs were stimulated with  $\alpha$ -GalCer. I reasoned that proliferation of iNKT cells in response to  $\alpha$ -GalCer within whole PBMC cultures would be a useful marker of iNKT cell function, as it would require  $\alpha$ -GalCer presentation by patient APCs, recognition of the  $\alpha$ -GalCer/CD1d complex by patient iNKT cells, and the presence of functional iNKT cell signalling pathways downstream of the TCR.

Preliminary experiments using healthy donor samples indicated that proliferation of iNKT cells could be induced by culturing whole PBMCs with  $\alpha$ -GalCer in the presence of 100 U/mL IL-2 (figure 5.7a). An  $\alpha$ -GalCer concentration of 100 ng/mL was chosen for subsequent experiments. Although IL-2, IL-7 and IL-15 were able to support iNKT cell proliferation, IL-21 was not (figure 5.7b). Used at 100 U/mL, IL-2 appeared at least as effective as IL-7 or IL-15 in promoting iNKT cell proliferation in the presence of  $\alpha$ -GalCer (figure 5.7c). A further experiment indicated that delaying the addition of IL-2 until 24 hours after the start of the cultures significantly improved iNKT cell proliferation (figure 5.7d). On the basis of these data, I chose to study iNKT cell proliferation in the presence of 100 ng/mL  $\alpha$ -GalCer, with addition of 100 U/mL IL-2 24 hours after start of culture.

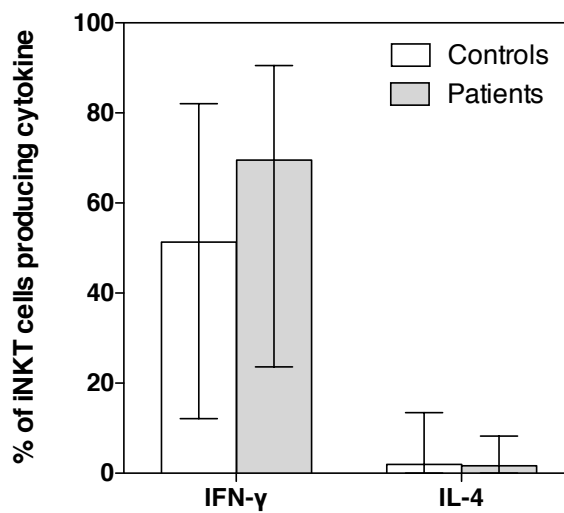
To allow comparison between patient and control samples, PBMCs were depleted of B-cells using anti-CD19 immunomagnetic beads before incubation with  $\alpha$ -GalCer and then IL-2. An  $\alpha$ -GalCer-dependent increase in iNKT cell numbers (figure 5.8a) was observed in all 20 controls and in 22 out of 25 patients studied (figure 5.8b). The median fold increase in iNKT cells over the seven-day culture period was 37.9 in controls, 26.5 in untreated patients, and 74.1 in fludarabine-treated patients (difference not significant;

**Figure 5.5 Cytokine production by T and iNKT cells in patients and controls.** Intracellular cytokine staining was performed on PBMCs from healthy donors (n = 11) and patients with CLL (n = 11). (a) Cytokine profile of T cells. (b) Cytokine profile of iNKT cells. \* p < 0.05, Mann Whitney test.

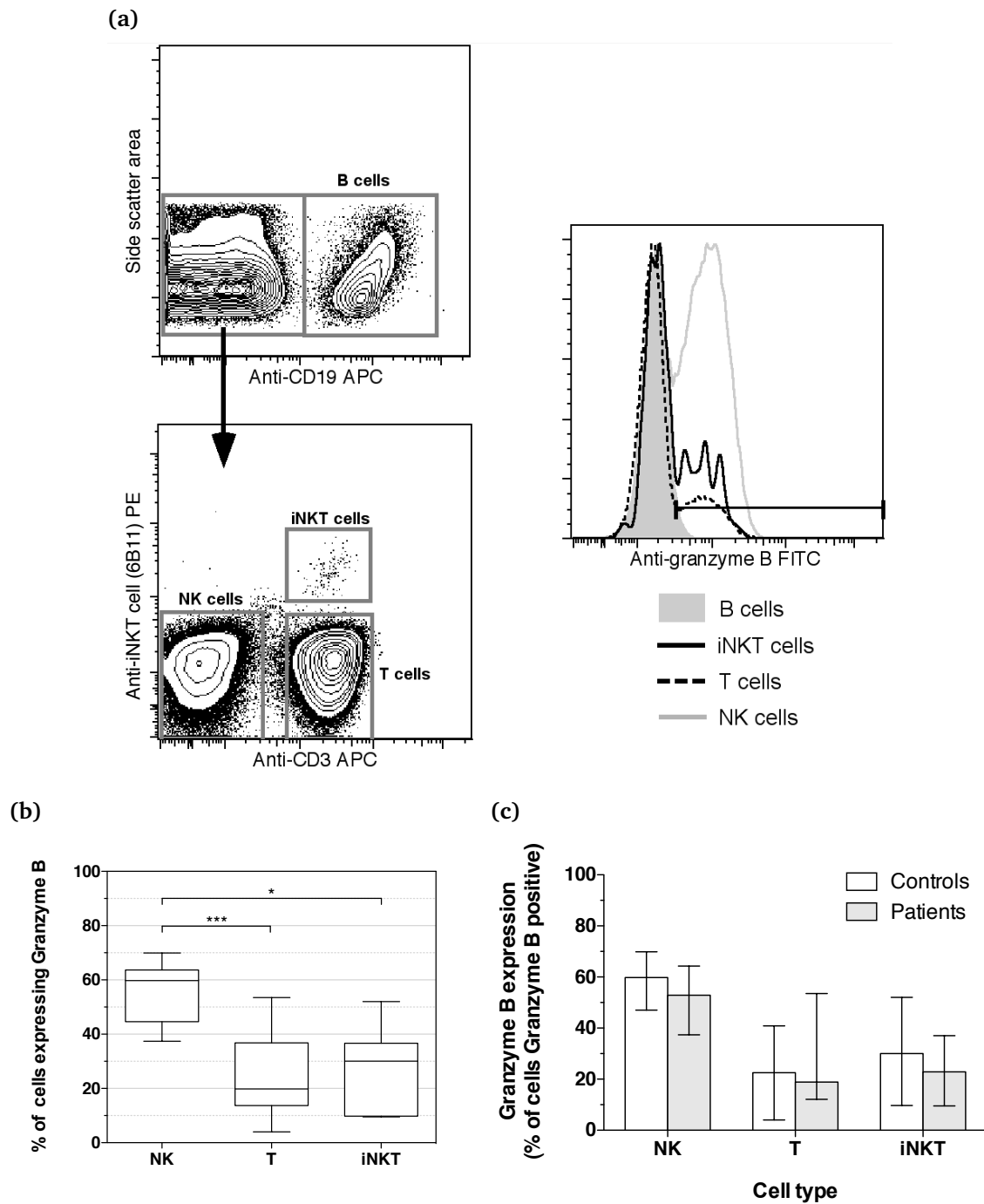
(a)



(b)



**Figure 5.6 Intracellular granzyme B expression of iNKT cells.** PBMCs from healthy donors ( $n = 4$ ) and patients with CLL ( $n = 4$ ) were stained for intracellular granzyme B alongside surface markers. (a) Illustrative flow cytometry plot showing granzyme B expression on B cells, T cells and iNKT cells. (b) Comparison of granzyme B expression levels in NK, T and iNKT cells. \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; Friedman test with Dunn's post-tests. (c) Graph summarising the proportions NK, T and iNKT cells expressing granzyme B in patients and controls.



**Figure 5.7 Optimising cytokine conditions for iNKT cell proliferation.** (a)  $1 \times 10^6$  healthy donor PBMCs were incubated with the indicated concentration of  $\alpha$ -GalCer and 100 U/mL IL-2. Cells were harvested after 7 days, and iNKT cells enumerated by flow cytometry. (b)  $1 \times 10^6$  PBMCs from two healthy donors were incubated with 100 ng/mL  $\alpha$ -GalCer and the indicated cytokines at 100 U/mL for IL-2 and 10 ng/mL for the other cytokines. Cells were harvested after 7 days of culture, and live iNKT cell numbers determined by flow cytometry. \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; two way ANOVA with Bonferroni post-tests. (c)  $2 \times 10^5$  PBMCs from a healthy donor were cultured in triplicate in the presence of  $\alpha$ -GalCer and the indicated concentration of cytokine for 7 days, then harvested, counted, and analysed by flow cytometry to determine the numbers of iNKT cells. Bars represent means, error bars represent standard error of the mean (SEM). \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ , one-way ANOVA with Tukey post-test. (d) Effect of delayed addition of IL-2 on iNKT cell expansion. PBMCs from three healthy donors were cultured with 100 ng/mL  $\alpha$ -GalCer in triplicate. 100 U/mL IL-2 was added immediately or 24 hours after start of culture. Cells were harvested after 7 days, and iNKT cells enumerated by flow cytometry.  $p < 0.01$  for effect of IL-2 timing, two-way ANOVA.

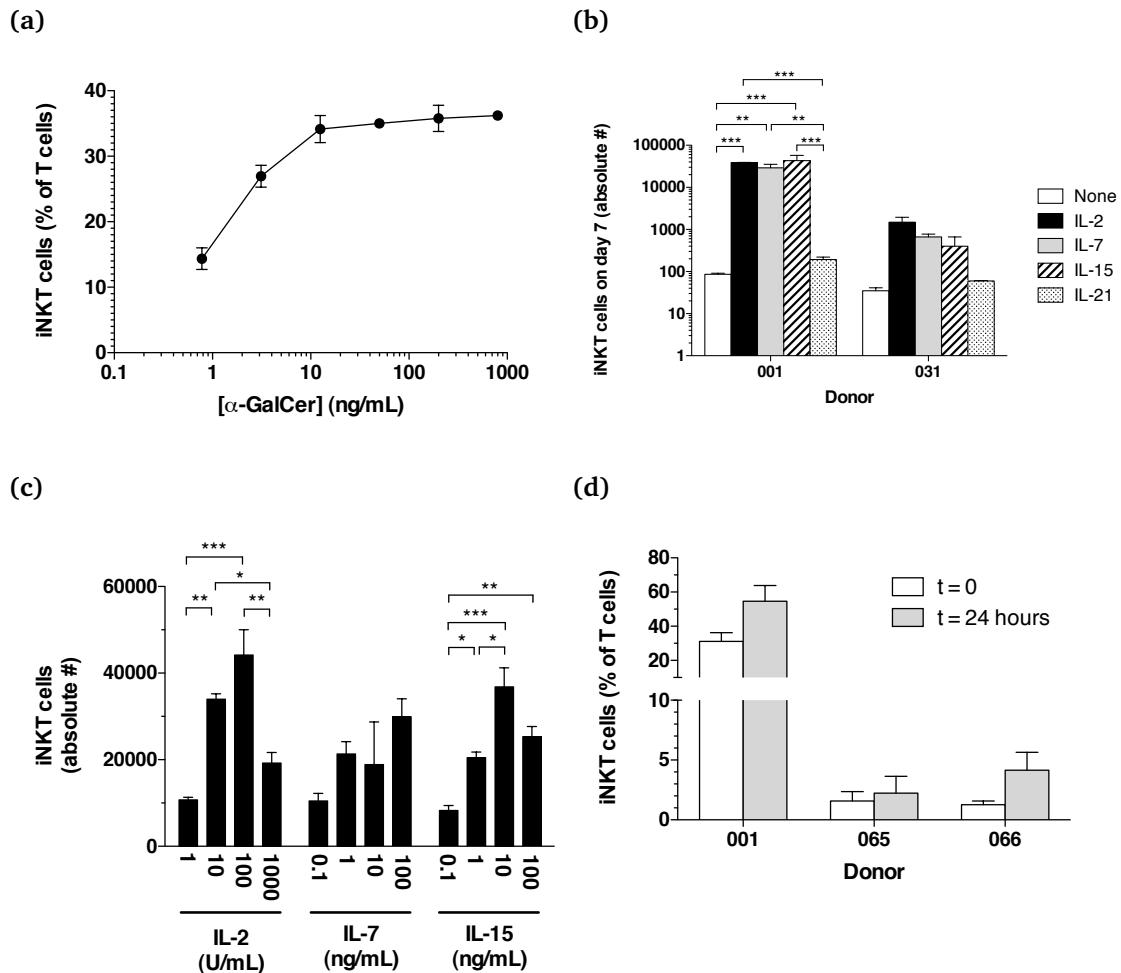
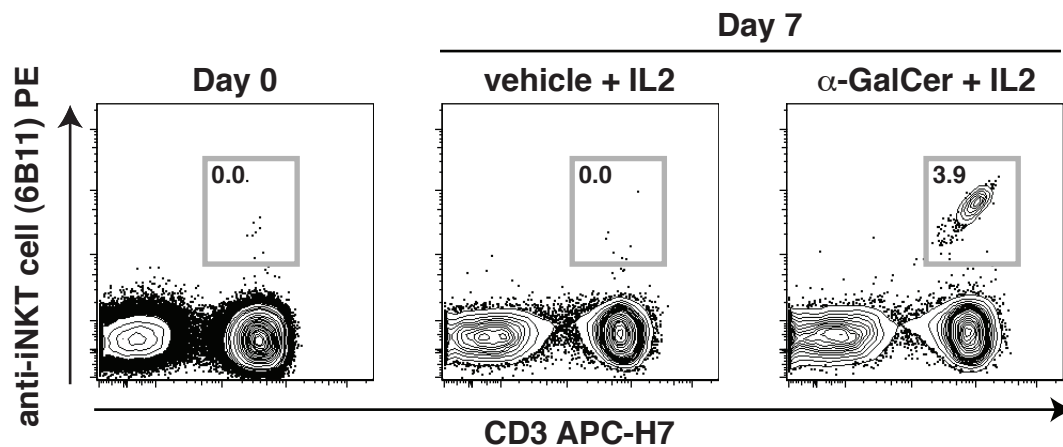


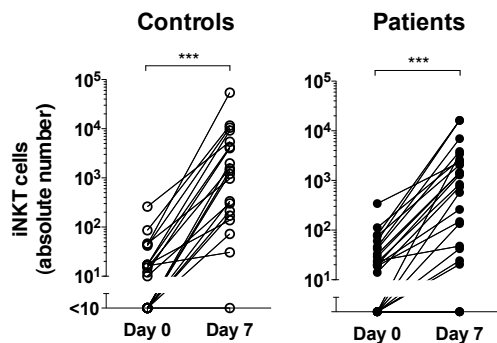
figure 5.8c).

**Figure 5.8 iNKT cell proliferation from patients and controls.** (a) Representative flow cytometry plots demonstrating expansion of iNKT cells after culture of  $5 \times 10^5$  patient B-cell depleted PBMCs with  $\alpha$ -GalCer and IL-2 for seven days. (b) Expansion of iNKT cells from patients ( $n = 25$ ) and healthy controls ( $n = 20$ ). \*\*\*  $p < 0.001$ , Wilcoxon matched-pairs. (c) The same data as in (b) presented as fold expansion of iNKT cells over seven days in untreated ( $n = 20$ ) and fludarabine-treated ( $n = 5$ ) patients compared to controls ( $n = 20$ ). Difference not significant for any comparison, Kruskal Wallis test with Dunn's post-tests.

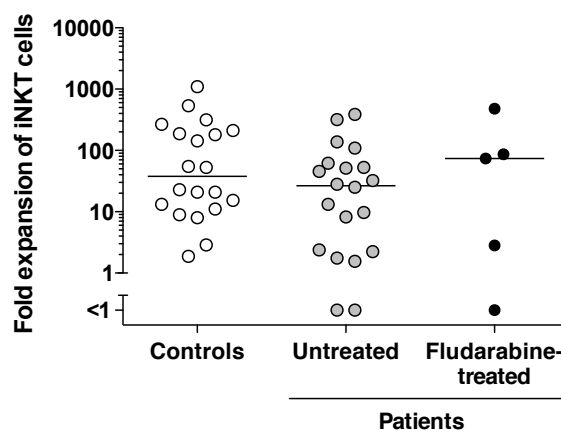
(a)



(b)



(c)



### 5.3.4 Immunophenotype of iNKT cells after expansion

The CD4<sup>-</sup> subpopulation of human iNKT cells is known to exhibit a Th1-type cytokine profile, while the CD4<sup>+</sup> subpopulation produces both IFN- $\gamma$  and IL-4 upon stimulation. <sup>218,219</sup>

In mice, it is the CD4<sup>-</sup> subset of iNKT cells which mediates anti-tumour immunity.<sup>222</sup> I assessed the CD4 status of iNKT cells following *in vitro* culture, to determine whether proliferating iNKT cells acquire a CD4<sup>+</sup> bias.

The proportion of iNKT cells that were CD4<sup>+</sup> increased following the culture of PBMCs with  $\alpha$ -GalCer and IL-2 (figure 5.9a and 5.9b). This was observed in both patient and control groups, was statistically significant, and was accompanied by a reciprocal decrease in the proportion of CD8<sup>+</sup> and double negative iNKT cells (figure 5.9c and 5.9d). The difference in the proportion of iNKT cells CD4<sup>+</sup> between patients and controls was not statistically significant (Mann Whitney test).

The culture of human iNKT cells in the presence of IL-7 or IL-15, rather than IL-2, has been reported to favour proliferation of the CD4<sup>-</sup> subset.<sup>463</sup> Therefore, I compared IL-2 with two other gamma chain cytokines, IL-7 and IL-15 again, assessing the proportion of iNKT cells that were CD4<sup>+</sup> following a seven day culture of healthy control PBMCs with  $\alpha$ -GalCer and each cytokine (figure 5.10). I found no clear evidence that IL-7 or IL-15 favoured the proliferation of CD4<sup>-</sup> iNKT cells in the four donors studied. Rather, the final proportion of CD4<sup>+</sup> iNKT cells appeared to be related to the starting proportion with the exception of one of the four donors, in whom the addition of low doses of IL-2 led to a decline in CD4<sup>+</sup> iNKT cells and an increase in the CD4-CD8<sup>-</sup> iNKT cell population.

### 5.3.5 Effect of leukaemic cells on iNKT cell proliferation

In the experiments above, I had depleted patient PBMCs of B cells before culture. In patients with CLL, a marked excess of leukaemic cells over T cells in the blood, bone marrow and lymph nodes is typical. Leukaemic CLL cells can impair T cell function *in vitro*,<sup>91</sup> and in a mouse model, the adoptive transfer of CD1d-expressing B cells impairs iNKT cell proliferation.<sup>246</sup> Therefore, I wished to determine whether the presence of CLL cells impaired human iNKT cell proliferation *in vitro*. To determine this, I used autologous CLL cells, as allogeneic CLL cells would be likely to provide NK and T cell signals in these mixed PBMC cultures, which might complicate interpretation of the data.

First, whole PBMCs from eight patients with CLL (constituting a median of 55.2% malignant B cells, range 20.6% – 87.7%) were cultured with  $\alpha$ -GalCer and IL-2 for 7 days (figure 5.11). An increase in iNKT cell numbers was seen in all cases, indicating that leukaemic cells do not completely prevent iNKT cell proliferation *in vitro*.

To quantify the effect of CLL cells on iNKT cell proliferation, I immunomagnetically depleted patient PBMCs of B cells before adding back positively-selected autologous CLL cells in defined numbers. The addition of CLL cells did not impair *in vitro* iNKT cell proliferation. In contrast, the addition of autologous CLL cells to B cell-depleted PBMCs at a ratio of 2:1 resulted in a significant increase in iNKT cell proliferation ( $p < 0.05$  compared to no CLL cells added, Wilcoxon matched pairs test; figure 5.12a). The addition of CLL cells did not affect the final proportion of iNKT cells which were CD4<sup>+</sup> (figure 5.12b).

These experiments indicate that iNKT cells from patients with CLL retain their ability to proliferate, and that under the *in vitro* conditions studied, leukaemic cells do not inhibit, and may promote, iNKT cell proliferation.



**Figure 5.9 Immunophenotype of iNKT cells after expansion.** Illustrative flow cytometry plots showing iNKT cell numbers and CD4 and CD8 status before (a) and after (b) 7 days of culture of healthy donor PBMCs with  $\alpha$ -GalCer and IL-2. Numbers within gates indicate percentages.

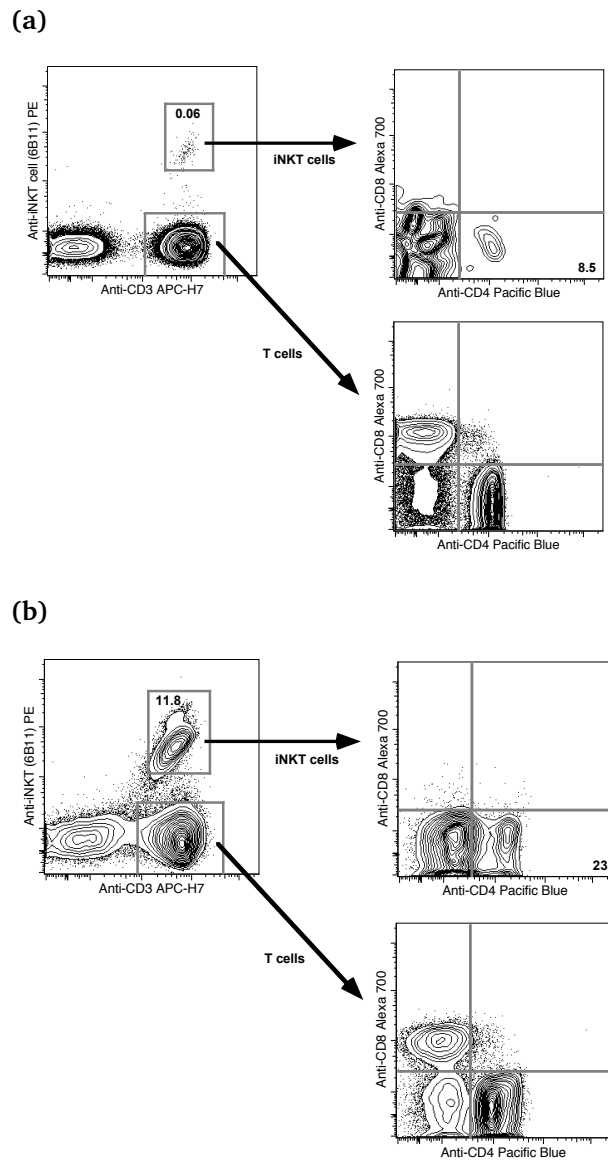
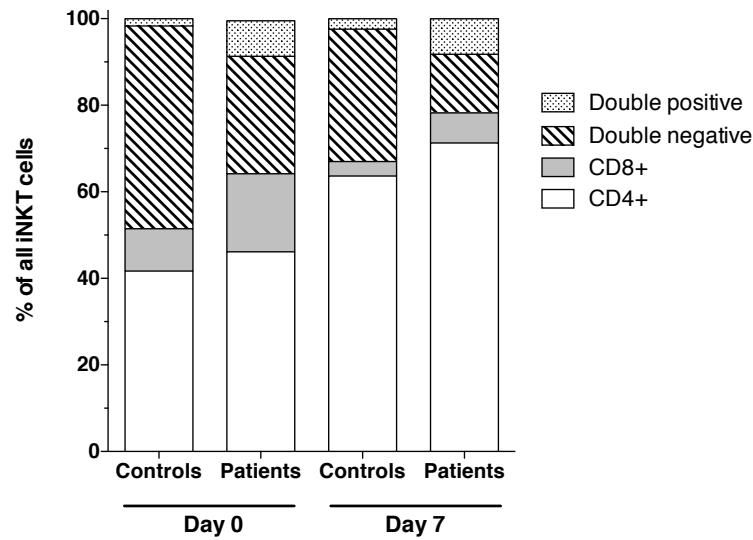
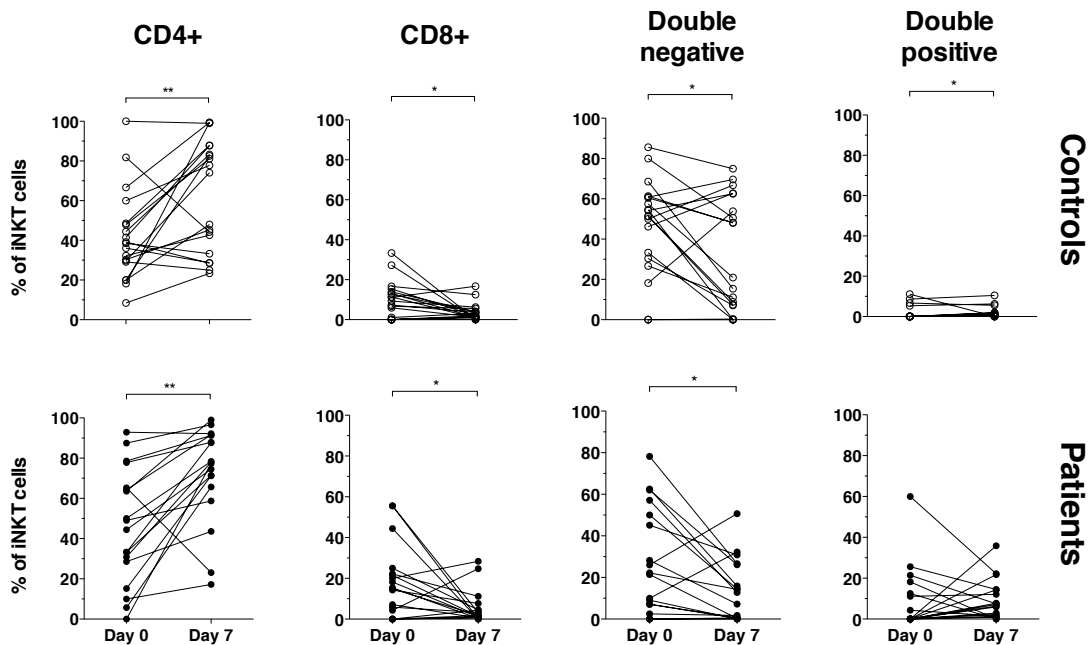


Figure 5.9 (continued) Immunophenotype of iNKT cells after expansion. (c) Summary graph showing changes in CD4 and CD8 status with iNKT cell expansion in patients (n = 21) and controls (n = 20) (bars represent medians). (d) Differences in iNKT cell phenotype before and after expansion in patients and controls. \*\* p < 0.01, \* p < 0.05, Wilcoxon signed rank test.

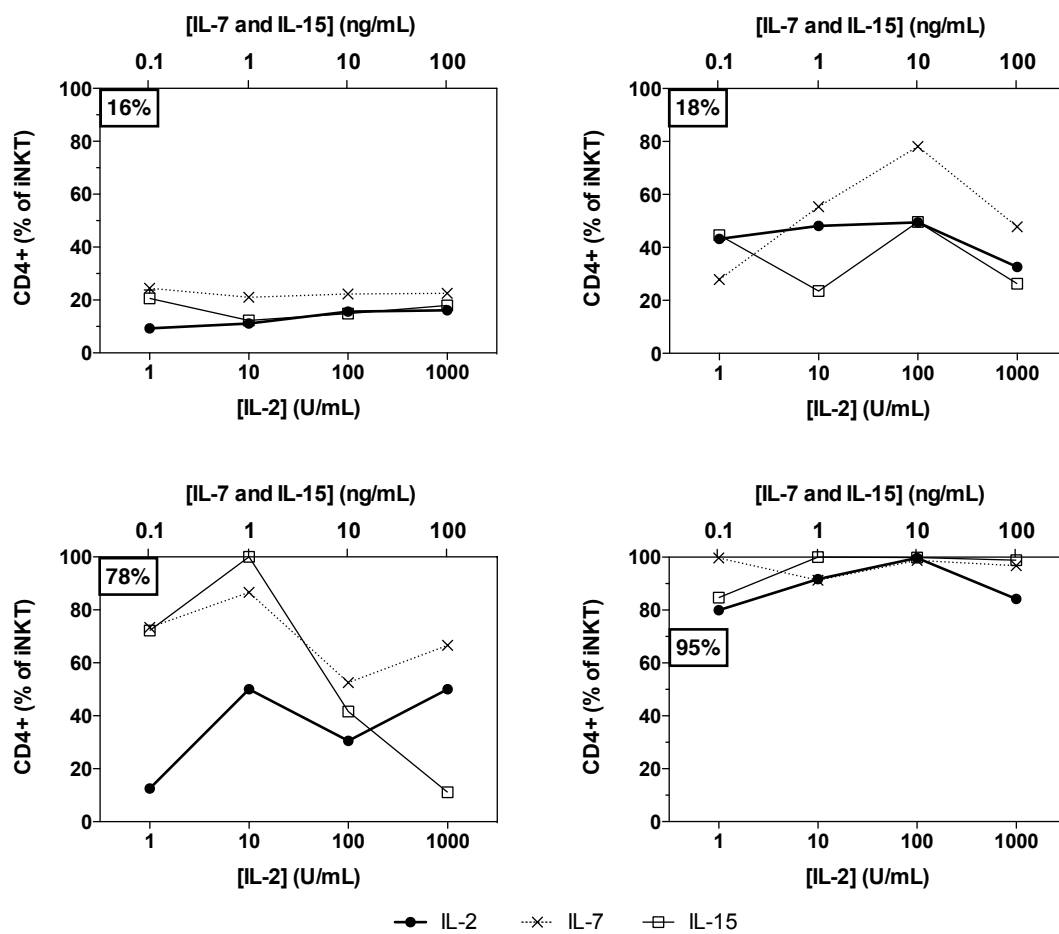
(c)



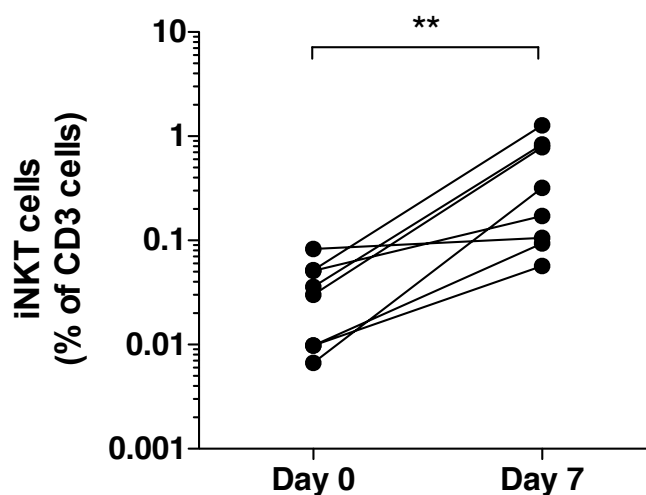
(d)



**Figure 5.10 Effect of IL2, IL7 and IL15 on iNKT subsets.** PBMCs from four healthy donors were cultured with 100 ng/mL  $\alpha$ -GalCer in the presence of the indicated concentration of each cytokine for seven days. CD4 status of the proliferating iNKT cells was assessed by flow cytometry. IL-2 concentration is indicated on lower x axis; IL-7 and IL-15 concentrations are indicated on the upper x axis. The starting proportion of iNKT cells which were CD4+ is given in the box in each chart. Points represent means of triplicate analyses.



**Figure 5.11 Proliferation of iNKT cells in the presence of leukaemic cells.** Whole PBMCs (without prior B cell depletion) from 8 patients with CLL were cultured at  $2 \times 10^6$ /well with  $\alpha$ -GalCer 100 ng/mL and 100 U/mL IL-2 added at 24 hours. After 7 days, wells were harvested, and iNKT cells enumerated. \*\*  $p < 0.01$ , Wilcoxon signed rank test.



### 5.3.6 Generation of iNKT cell lines

Having established that iNKT cells from patients with CLL retain the capacity to proliferate *in vitro*, I attempted to generate iNKT cell lines from patients. The purpose of this was twofold:

1. To provide a pure population of iNKT cells for further analyses, including for cytotoxicity assays, and
2. To investigate the feasibility of *ex vivo* iNKT cell expansion from patients with CLL, for potential therapeutic purposes

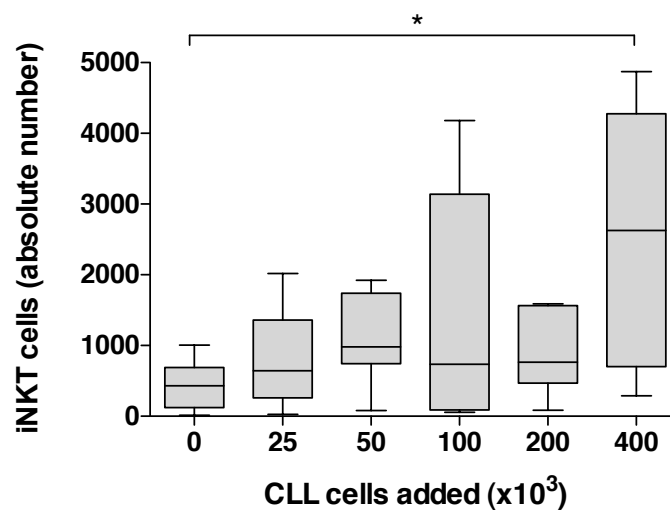
In order to generate iNKT cell lines, PBMCs were initially cultured in the presence of  $\alpha$ -GalCer and IL-2 as for the proliferation experiments above. FACS was used to select cells staining positively with both the anti-iNKT cell antibody (clone 6B11) and with anti-CD3.

In a preliminary experiment, I tested various irradiated cells as 'feeder cells' to support ongoing proliferation of sorted iNKT cells in the presence of IL-2 (figure 5.13). The HLA Class I-low human lymphoblastoid cell line, C1R, was at least as effective as autologous PBMCs in leading to iNKT cell expansion. Surprisingly, an untransfected C1R cell line was as effective as one transfected to express CD1d. Nonetheless, I used the CD1d-transfected C1R (C1R-CD1d) cell line for restimulation of iNKT cells.

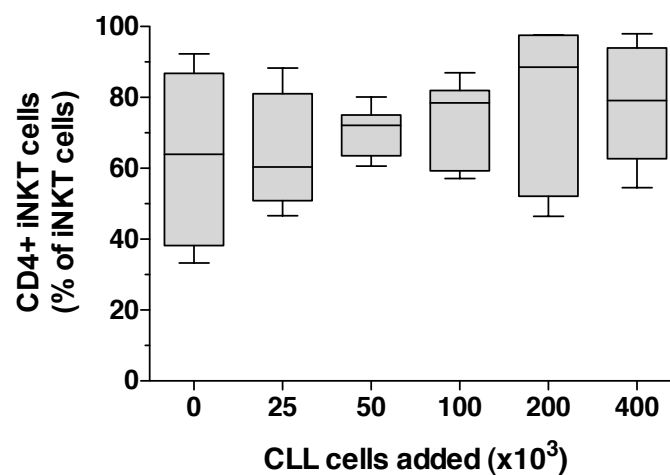
Four patient and four control samples were selected for generation of iNKT cell lines, based on the presence of sufficient iNKT cells after seven day culture to enable cell sorting (median 2.73% iNKT cells after culture; range 0.73% – 5.74%). Using flow cytometric cell sorting, iNKT cells were enriched to a median of 89.0% of live cells (figure 5.14). How-

**Figure 5.12 Effect of addition of autologous CLL cells on iNKT cell expansion.**  $2 \times 10^5$  B-cell depleted PBMCs from six patients with CLL were cultured in the presence of 100 ng/mL  $\alpha$ -GalCer and 100 U/mL IL-2, with the addition of purified B cells (> 95% with the CLL phenotype CD19+CD5+) at the indicated numbers. After 7 days, iNKT cell numbers were evaluated by flow cytometry. (a) Absolute numbers of iNKT cells at day seven, according to number of CLL cells added per well. Bars represent medians, boxes represent interquartile range, and whiskers represent range. \*  $p < 0.05$ , Wilcoxon matched pairs test. (b) Proportion of iNKT cells expressing CD4+, according to number of CLL cells added. Bars represent medians, boxes represent interquartile range, and whiskers represent range.

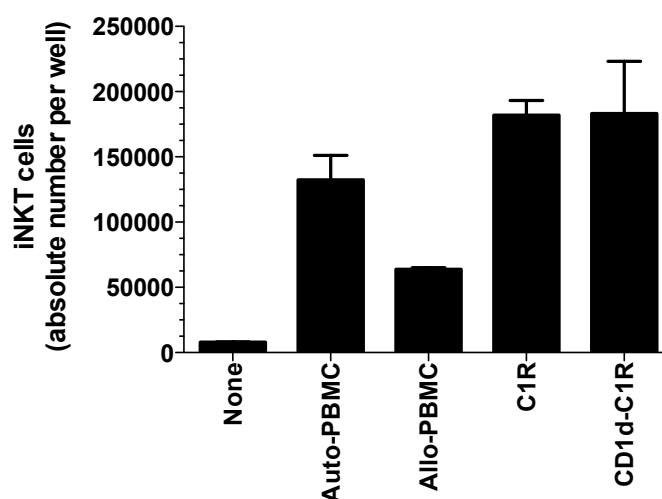
(a)



(b)



**Figure 5.13 Effect of feeder cells on iNKT cell expansion.** Feeder cells were irradiated to 50 Gy and washed, then added to  $4 \times 10^4$  FACS-sorted iNKT cells at a 5:1 feeder:iNKT cell ratio, in the presence of  $\alpha$ -GalCer, in duplicate. 100 U/mL IL-2 was added at 24 hours. After one week, wells were harvested, and iNKT cells enumerated by flow cytometry. None = no feeder cells added; auto-PBMC = whole autologous PBMCs; allo-PBMC = mixed PBMCs from three different healthy donors; C1R = untransfected C1R cell line; C1R-CD1d = CD1d-transfected C1R cell line.



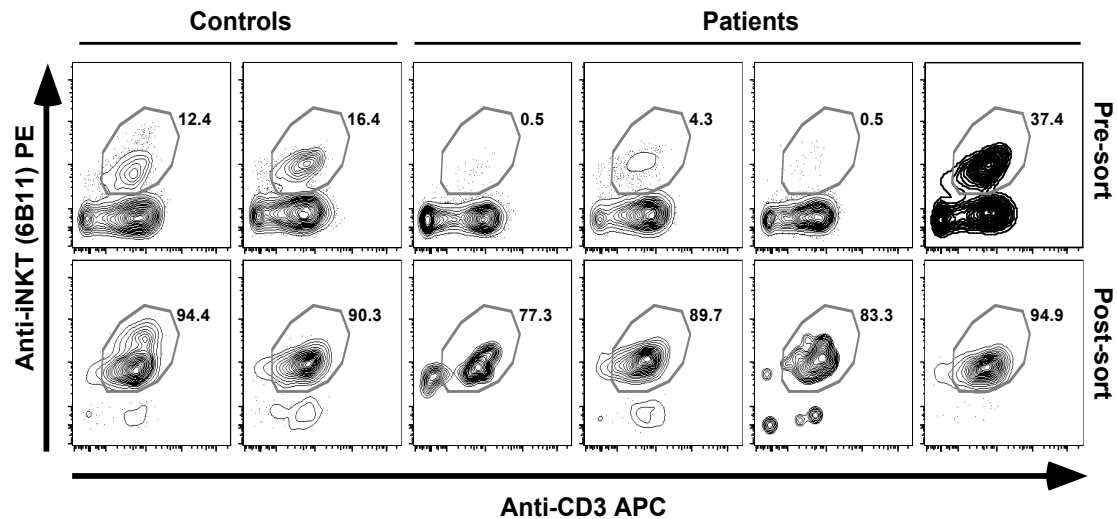
ever, iNKT cell purity declined after two restimulations with CD1d-C1R cells. A second sort was performed at this stage, to yield iNKT cell lines of median 99.3% purity (range 95.2% – 99.8%) (figure 5.15a). After two restimulations, the most frequent contaminating cell types were CD4+ ‘conventional’ T cells (median 7.2% of live cells), followed by CD8+ ‘conventional’ T cells (median 1.1%) and NK cells (median 0.5%).

The absolute numbers of iNKT cells rose rapidly, with a median 25.8-fold increase during the seven day culture of PBMCs with IL-2 and  $\alpha$ -GalCer and a further 44.1-fold increase over two further 2-weekly restimulations before approaching a plateau. Overall, there was a median 4186-fold increase in iNKT cell numbers by the end of the fourth restimulation, beyond which iNKT cell numbers fell, in some instances precipitously (figure 5.15b).

The immunophenotype of the iNKT cell lines generated was assessed by flow cytometry: the iNKT cell populations were overwhelmingly CD4 positive (median 99.8% of cells CD4+), and a minority expressed CD161 (figure 5.16). This contrasts with the phenotype of circulating iNKT cells, around half of which were CD4+, and the majority of which expressed CD161 (figure 3.11).

Having generated iNKT cell lines from patients with CLL and healthy controls, I proceeded to characterise their function.

**Figure 5.14 Flow cytometric cell sorting of iNKT cells.** Representative flow cytometry plots showing enrichment of iNKT cells using FACS, positively selecting for CD3 and 6B11 co-positive cells. Numbers represent percentage of events within iNKT cell gate (CD3+ 6B11+).



### 5.3.7 Cytokine profile of iNKT cell lines

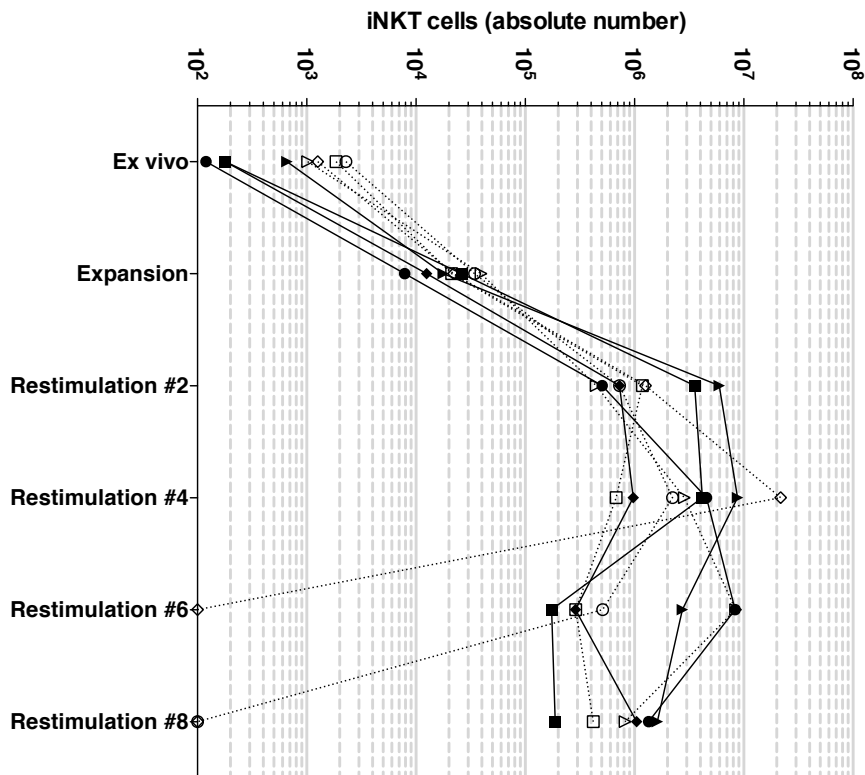
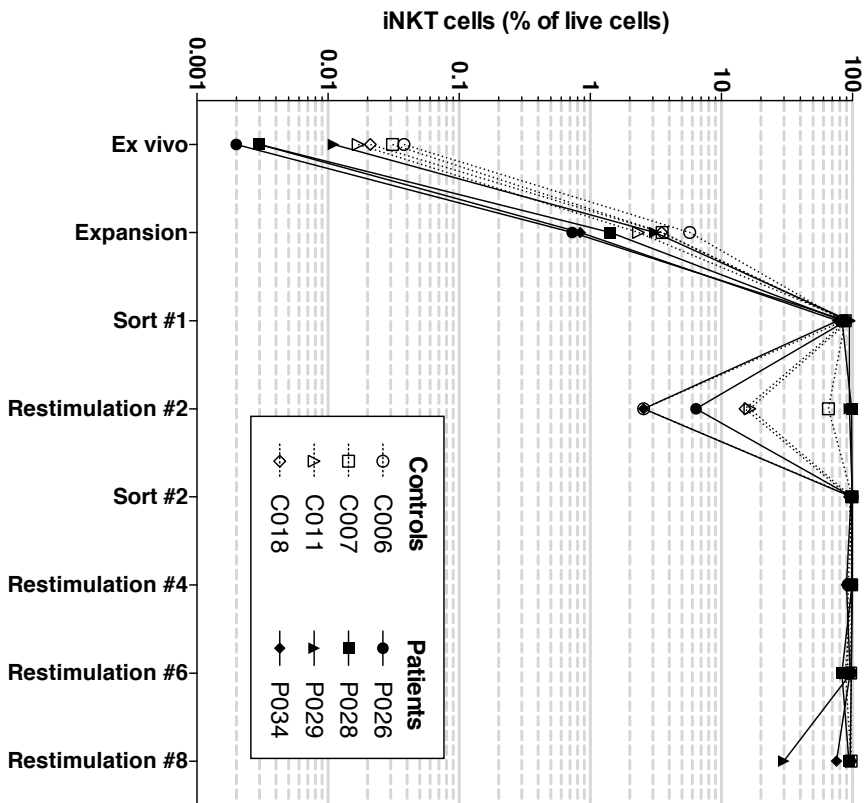
To determine the cytokine profile of the iNKT cell lines generated, I performed intracellular cytokine staining for IFN- $\gamma$  and IL-4 after stimulation with PMA and ionomycin (figure 5.17a). Patient and control iNKT cell lines were dual producers of both cytokines, consistent with their overwhelmingly CD4+ status (figure 5.17b).

To further assess the cytokine profile of the iNKT cell lines generated, I co-cultured iNKT cell lines with C1R-CD1d cells and either vehicle or  $\alpha$ -GalCer (figure 5.18). Background levels of cytokine production were observed in the presence of vehicle and CD1d-transfected C1R cells, but cytokine production was markedly enhanced in the presence of  $\alpha$ -GalCer, indicating at least a component of  $\alpha$ -GalCer specificity.

The cytokine profiles of control- and patient-derived iNKT cell lines were similar. Both produced both Th1-type cytokines such as IFN- $\gamma$  and TNF- $\alpha$  in combination with Th2-type cytokines such as IL-4 and IL-13. iNKT cell lines produced GM-CSF, a finding which has previously been reported.<sup>220</sup>  $\alpha$ -GalCer-induced IL-10 production was observed at a low level in a patient-derived cell line. The iNKT cell lines tested did not produce detectable quantities of IL-17.

### 5.3.8 Cytotoxicity of iNKT cell lines

Intracellular staining confirmed that iNKT cell lines expressed granzyme B (figure 5.19). An LDH release cytotoxicity assay confirmed that both a patient and a control iNKT cell line could lyse a C1R target cell, in a CD1d- and  $\alpha$ -GalCer dependent fashion (figure 5.20).



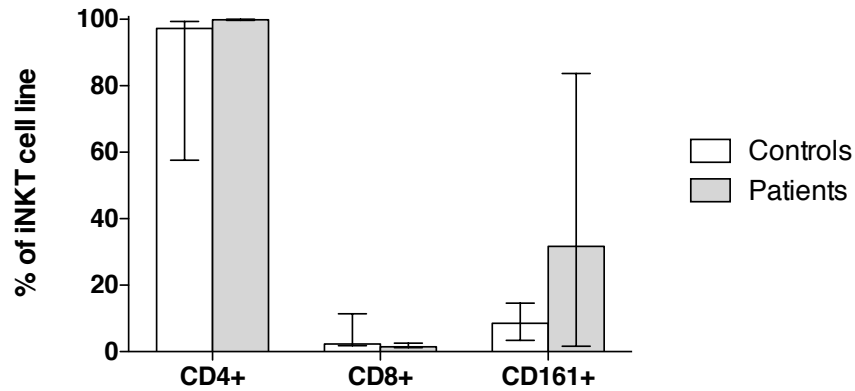
(a)

(b)

**Figure 5.15 Purity and numbers of iNKT cell lines.** (a) Purity of patient and control iNKT cell lines as assessed by flow cytometry (CD3 + 6B11+) before initial expansion, after expansion, before and after flow cytometric cell sorts, and after indicated numbers of restimulations. (b) Absolute numbers of iNKT cells.

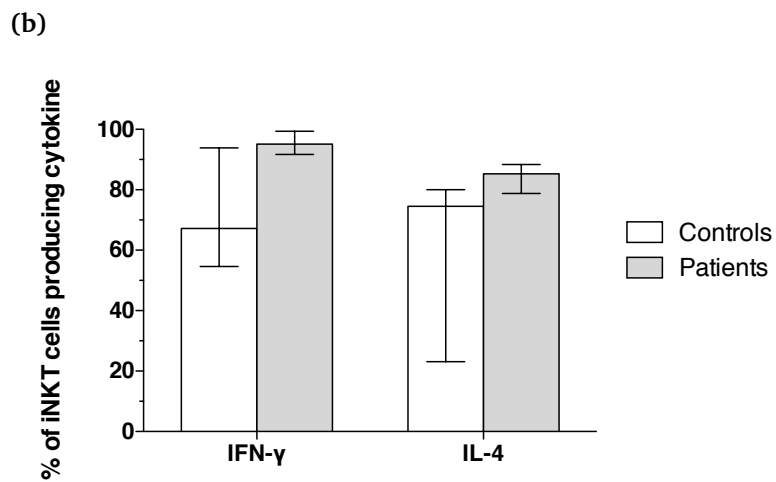
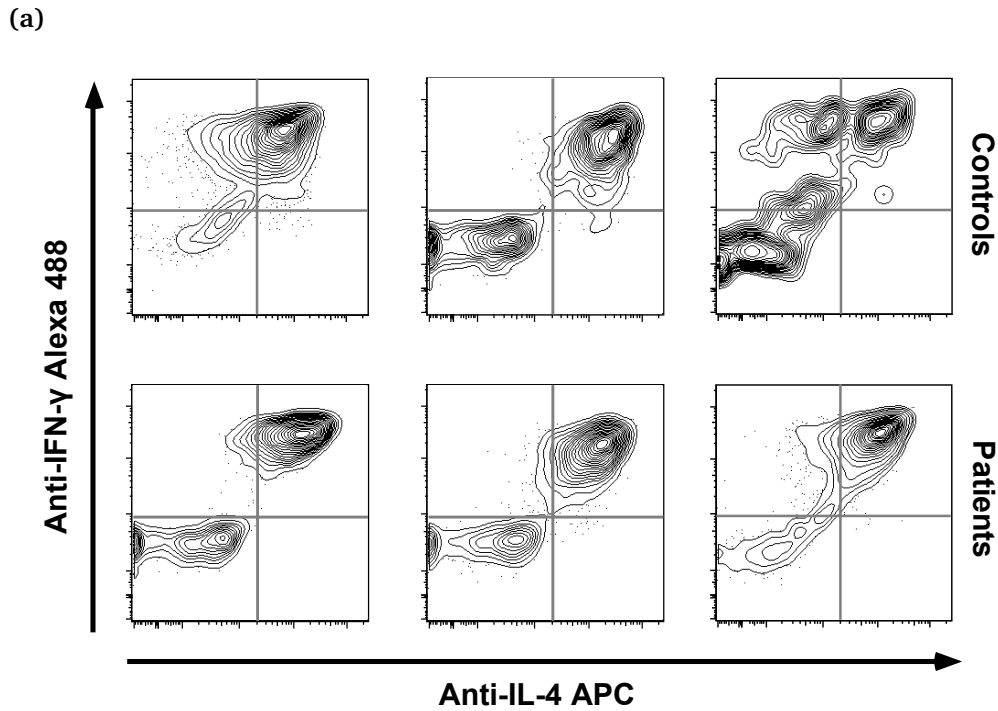


**Figure 5.16 Phenotype of iNKT cell lines.** Surface immunophenotype of patient (n = 4) and control (n = 4) iNKT cell lines. Bars represent medians, error bars represent range. Difference non significant for any comparison, Mann Whitney test.



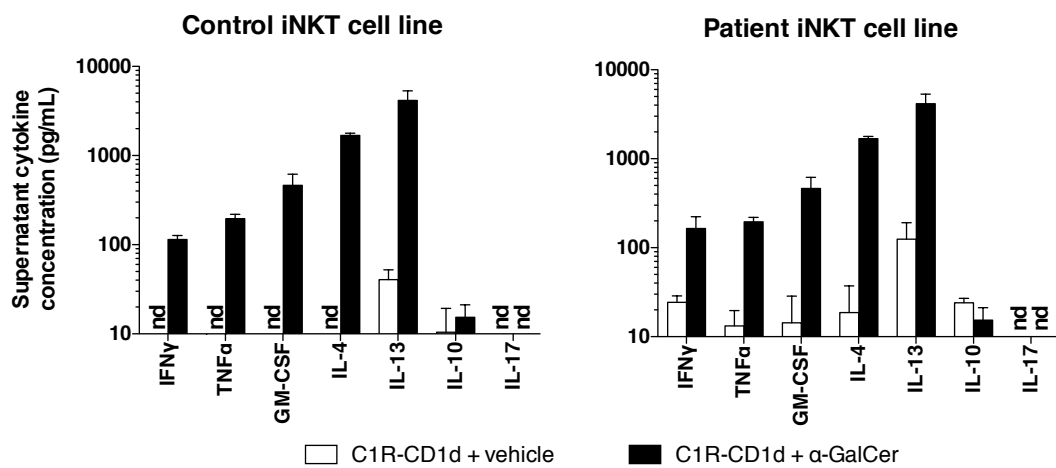
Together, these data indicate that iNKT lines derived from patients with CLL have similar functional properties to those from healthy donors: they produce both Th1 and Th2-type cytokines, exhibit intracellular granzyme B expression, and have the capacity to lyse a CD1d-expressing cell line in the presence of  $\alpha$ -GalCer.

**Figure 5.17 Intracellular staining of iNKT cell lines.** The iNKT cell lines generated from three patients and three controls were studied by intracellular cytokine staining. (a) Flow cytometry plots showing Intracellular cytokine staining. (b) Cytokine expression represented as a graph. Bars represent medians, error bars represent range.

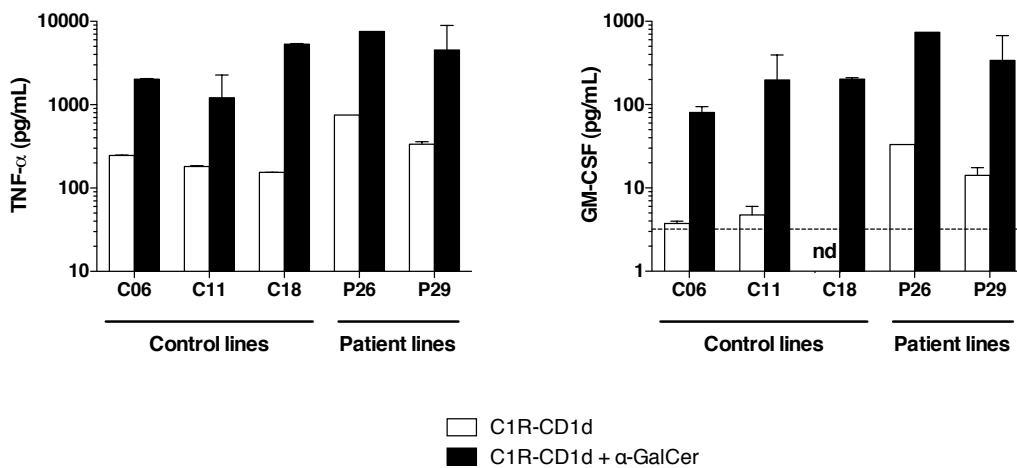


**Figure 5.18 Cytokine production by iNKT cell lines.** (a)  $\alpha$ -GalCer induced cytokine production by a representative control and patient iNKT cell line. iNKT cell lines were cultured with CD1d-expressing C1R cells and either  $\alpha$ -GalCer or vehicle. Supernatants were taken at 24 hours and cytokines assayed by bead array. nd = not detectable. (b) In a separate experiment, supernatant cytokine levels were measured after culturing three control and two patient iNKT cell lines with CD1d-expressing C1R cells in the presence or absence of  $\alpha$ -GalCer. Dashed line represents lower limit of detection for GM-CSF. nd = not detectable.

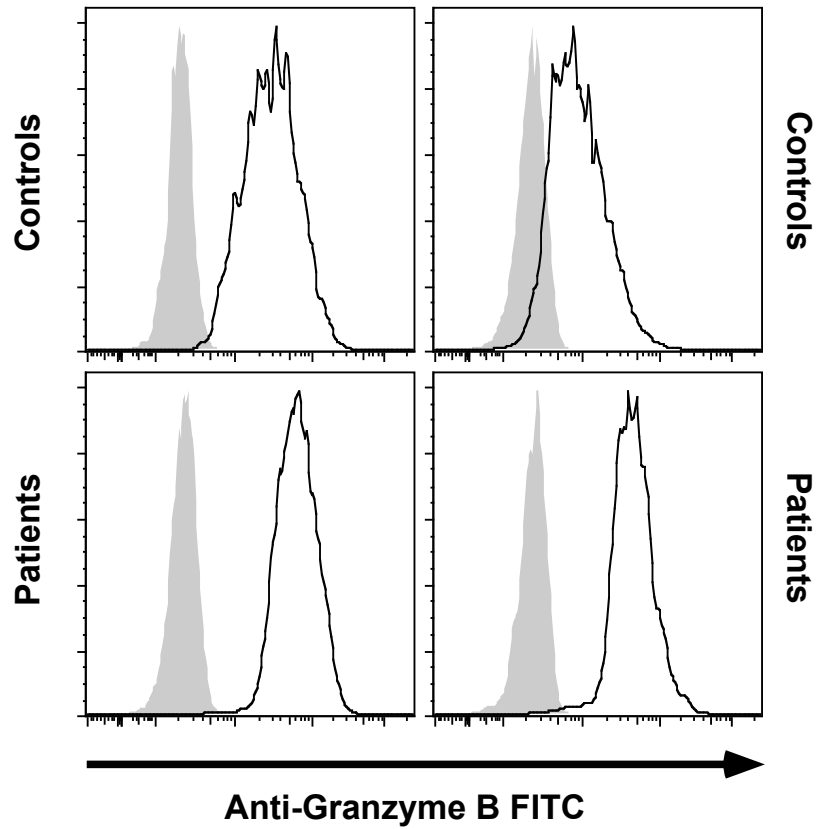
(a)



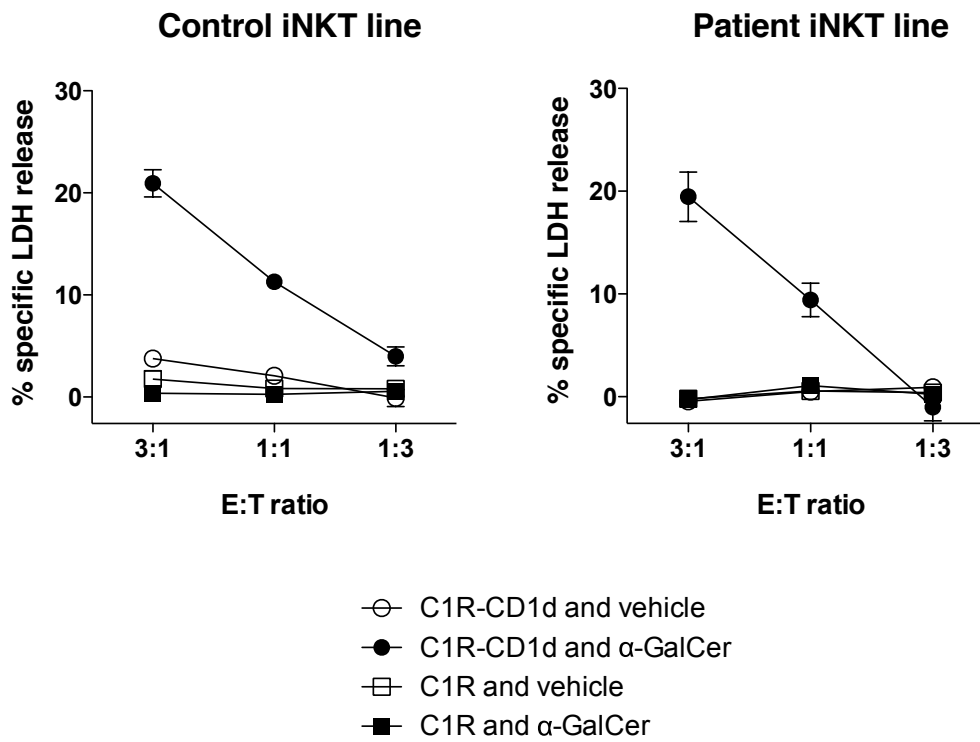
(b)



**Figure 5.19 Granzyme B expression of iNKT cell lines.** Intracellular granzyme B expression of iNKT cell lines from two patients and two controls, determined by flow cytometry. Black line represents anti-granzyme B FITC; shaded histogram represents isotype control.



**Figure 5.20 Cytotoxicity of iNKT cell lines.** Cytotoxicity of an iNKT cell line from a patient and control against untransfected and CD1d-transfected C1R cells, in the presence and absence of  $\alpha$ -GalCer, as assessed by LDH release assay.



### 5.4 Summary

In this chapter, I have assessed the function of iNKT cells from in patients with CLL.

Cytokine production by iNKT cells from patients with CLL is intact, as assessed by  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpots and by intracellular cytokine staining for IFN- $\gamma$  and IL-4. Both patient and control iNKT cells express granzyme B, at a level similar to that of T cells.

Proliferation of iNKT cells from mixed PBMC cultures was achieved using  $\alpha$ -GalCer and delayed addition of IL-2. The degree of iNKT cell expansion seen in PBMCs from patients was similar to that observed in healthy, age-matched controls. Moreover, the addition of autologous CLL cells to the cultures did not impair iNKT cell proliferation. The culture conditions employed appeared to favour expansion of the CD4<sup>+</sup> subset of iNKT cells in both patient and control groups.

I have demonstrated a method of generating iNKT cell lines from patients with CLL, and have determined the immunophenotype and cytokine profile of these lines. The iNKT cell lines produced were overwhelmingly CD4<sup>+</sup>, and consistent with this surface phenotype, expressed both IFN- $\gamma$  and IL-4 intracellularly. Cell lines from both patients and controls produced Th1 and Th2-type cytokines and GM-CSF upon exposure to  $\alpha$ -GalCer and a CD1d-expressing cell line. IL-17 was not produced.

Finally, I have shown that both patient- and control-derived iNKT cell lines express granzyme B, and have demonstrated the capacity of a CLL patient-derived iNKT cell line to lyse a target cell line in a CD1d- and  $\alpha$ -GalCer-dependent manner. The degree of cytotoxicity was modest in this experiment, a finding which will be discussed in section [7.2.4.5](#).

Together, these findings indicate that iNKT cell function is fundamentally intact in CLL. In chapter [6](#), I shall explore potential immunotherapeutic strategies using iNKT cells.

## Chapter 6

# Application of iNKT cells to CLL immunotherapy

### 6.1 Introduction

In chapters 3 to 5, I established that the iNKT cell and CD1d axis is essentially numerically, immunophenotypically and functionally intact in patients with CLL. In this chapter, I aim to explore potential immunotherapeutic strategies for CLL, which exploit the iNKT/CD1d axis.

There are three principal mechanisms by which iNKT cells might contribute to the immunological control of a malignancy: the transactivation of NK or other effector cells, direct cytotoxicity against tumour cells, or the provision of adjuvant activity to enhance T cell responses to peptide antigens.

**Transactivation of natural killer effector cells** The transactivation of NK cells by activated iNKT cells is a critical antitumour effector mechanism of  $\alpha$ -GalCer in some murine models of cancer.<sup>271,272</sup> Moreover, iNKT cell activation of NK cells has also been observed in human cell cultures *in vitro*.<sup>276</sup> In human clinical trials, the administration of intravenous  $\alpha$ -GalCer resulted in increases in circulating cytokines and NK cell activation in some individuals.<sup>303,404</sup>

**Direct cytotoxicity against CD1d-expressing target cells** As outlined in section 1.2.5, iNKT cells can exhibit direct cytotoxicity against CD1d-expressing target cells via a number of pathways including perforin/granzyme B and Fas/FasL. Human iNKT cells have been shown to lyse allogeneic CD1d-expressing haematopoietic malignancies *in vitro*, including chronic lymphocytic leukaemia, myeloid leukaemias and T cell acute lymphoblastic leukaemia.<sup>229,416,417</sup> In chapter 5 I demonstrated the feasibility of generation of iNKT cell lines from patients with CLL, raising the possibility that these might be used as effector cells. Although human iNKT cell lines can lyse allogeneic human CLL cell lines,<sup>417</sup> this has not been demonstrated in the autologous setting, where NK receptor mismatches are absent.

**Provision of adjuvant activity** The co-administration of  $\alpha$ -GalCer with a protein or peptide antigen results in enhanced ‘conventional’ CD4+ and CD8+ T cell responses to the protein,<sup>258–260</sup> as discussed in section 1.2.6. This interaction requires CD40/CD40L, and not IFN- $\gamma$ , and can result in protection against subsequent challenge with a tumour expressing the protein.<sup>258</sup> The adjuvant activity of  $\alpha$ -GalCer has also been exploited using  $\alpha$ -GalCer-pulsed whole tumour cells as a vaccine—this approach has proved effective against CD1d-expressing haematopoietic malignancies in preclinical studies<sup>422,424,425</sup>

In this chapter, I present a series of *in vitro* experiments using PBMCs from patients and healthy controls, aimed at exploring the potential applicability of each of these mechanisms to the immunotherapy of CLL.

## 6.2 Aims

The aims of the experiments presented in this chapter are to identify promising strategies for the incorporation of iNKT cells in the immunotherapy of CLL. Specifically, these experiments aim to:

- Assess the effect of  $\alpha$ -GalCer addition to whole PBMC cultures on supernatant cytokine levels and APC activation status
- Determine the capacity of patient-derived iNKT cell lines to lyse autologous CLL cells
- Investigate the effect of  $\alpha$ -GalCer on T cell responses to a co-administered protein antigen
- Explore the capacity of  $\alpha$ -GalCer-pulsed CLL cells to elicit expansion of tumour-reactive T cells

## 6.3 Results

### 6.3.1 $\alpha$ -GalCer-induced cytokine release in whole PBMC cultures

In mice, the systemic administration of  $\alpha$ -GalCer results in a rapid increase in circulating IFN- $\gamma$  levels, which is at least partly responsible for the transactivation of NK cells and for enhancement of NK cytotoxicity.<sup>273</sup> However, in clinical trials of intravenous  $\alpha$ -GalCer, systemic cytokine responses and NK cell activation were restricted to patients with the highest numbers of circulating iNKT cells.<sup>303,404</sup>

Having previously established that iNKT cell cytokine production is intact in patients with CLL using the highly-sensitive techniques of  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpot enumeration and intracellular cytokine staining (figures 5.3 and 5.5), I wished to determine whether the addition of  $\alpha$ -GalCer to patient PBMCs would induce appreciable levels of cytokines in PBMC culture supernatants. I reasoned that supernatant cytokine concentrations would



depend upon the degree of  $\alpha$ -GalCer-induced transactivation of other cell types (such as NK and T cells) as well as cytokines released by iNKT cells alone.

Cryopreserved supernatants of PBMC cultures used in the iNKT cell proliferation experiments outlined in section 5.3.3 were analysed for cytokines. In a preliminary experiment, PBMCs from three healthy donors aged 26 to 34 years old were cultured with  $\alpha$ -GalCer or vehicle. IL-2 100U/mL was added at 24 hours. Supernatants were analysed at various timepoints (figure 6.1). In the two donors with higher numbers of iNKT cells, the addition of  $\alpha$ -GalCer resulted in an increase in supernatant concentrations of IFN- $\gamma$  and IL-13 beyond 24 hours.

The effect of  $\alpha$ -GalCer addition on the cytokine levels of supernatants from cultured patient and control PBMCs was examined (figure 6.2). The addition of  $\alpha$ -GalCer led to a significant increase in supernatant IFN- $\gamma$  in controls only: an  $\alpha$ -GalCer-dependent induction of IFN- $\gamma$  was not observed in patient samples. The addition of  $\alpha$ -GalCer did not lead to significant induction of GM-CSF, TNF- $\alpha$ , IL-4, IL-10 or IL-13 in either patient or control samples.

Several explanations for this lack of a cytokine response in patients could be proposed, including the lower relative numbers of iNKT cells (median 0.01% of T cells) seen in patients than in age-matched healthy controls (median 0.02% of T cells), the possibility of inhibitory effects of residual CLL cells in the B-cell depleted patient samples, or defects in other cell types, such as NK cells, within the CLL patient PBMCs. Nonetheless, in this *in vitro* setting, the undirected administration of  $\alpha$ -GalCer did not elicit a major cytokine response in mixed patient PBMCs.

### 6.3.2 Effect of $\alpha$ -GalCer on APC co-stimulatory markers

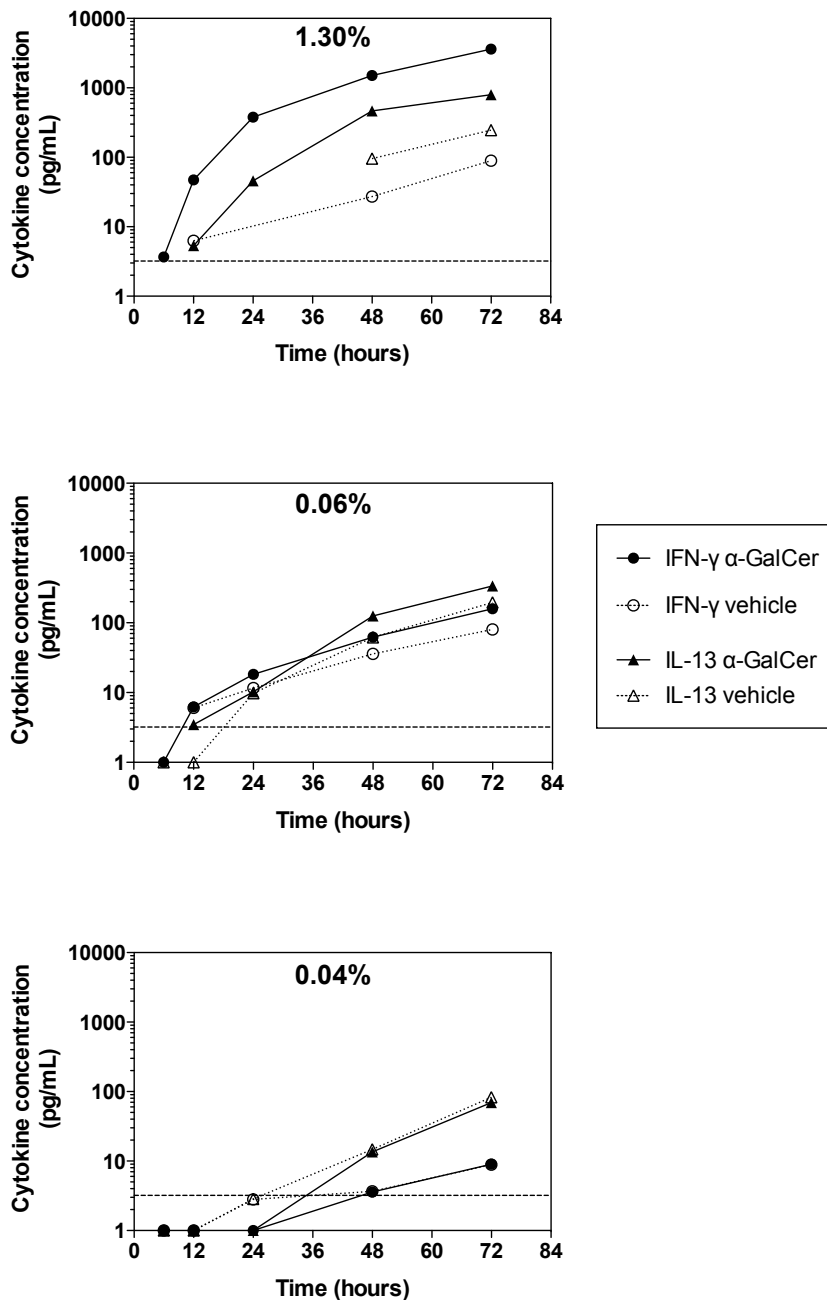
In mice, the systemic administration of  $\alpha$ -GalCer results in upregulation of co-stimulatory markers on splenic DC.<sup>258</sup> I wished to determine whether the addition of  $\alpha$ -GalCer to whole human PBMC cultures would result in a similar effect. To do so, I examined the expression of activation markers on monocytes and B cells, which are more frequent and therefore more readily analysed than DCs.

The incubation of human PBMCs with  $\alpha$ -GalCer led to significantly increased upregulation of monocyte MHC Class II only in a donor with a high percentage of iNKT cells (figure 6.3a). In donors with smaller proportions of iNKT cells, upregulation of the activation markers CD80, CD86 and class II was inconsistent (figure 6.3b).

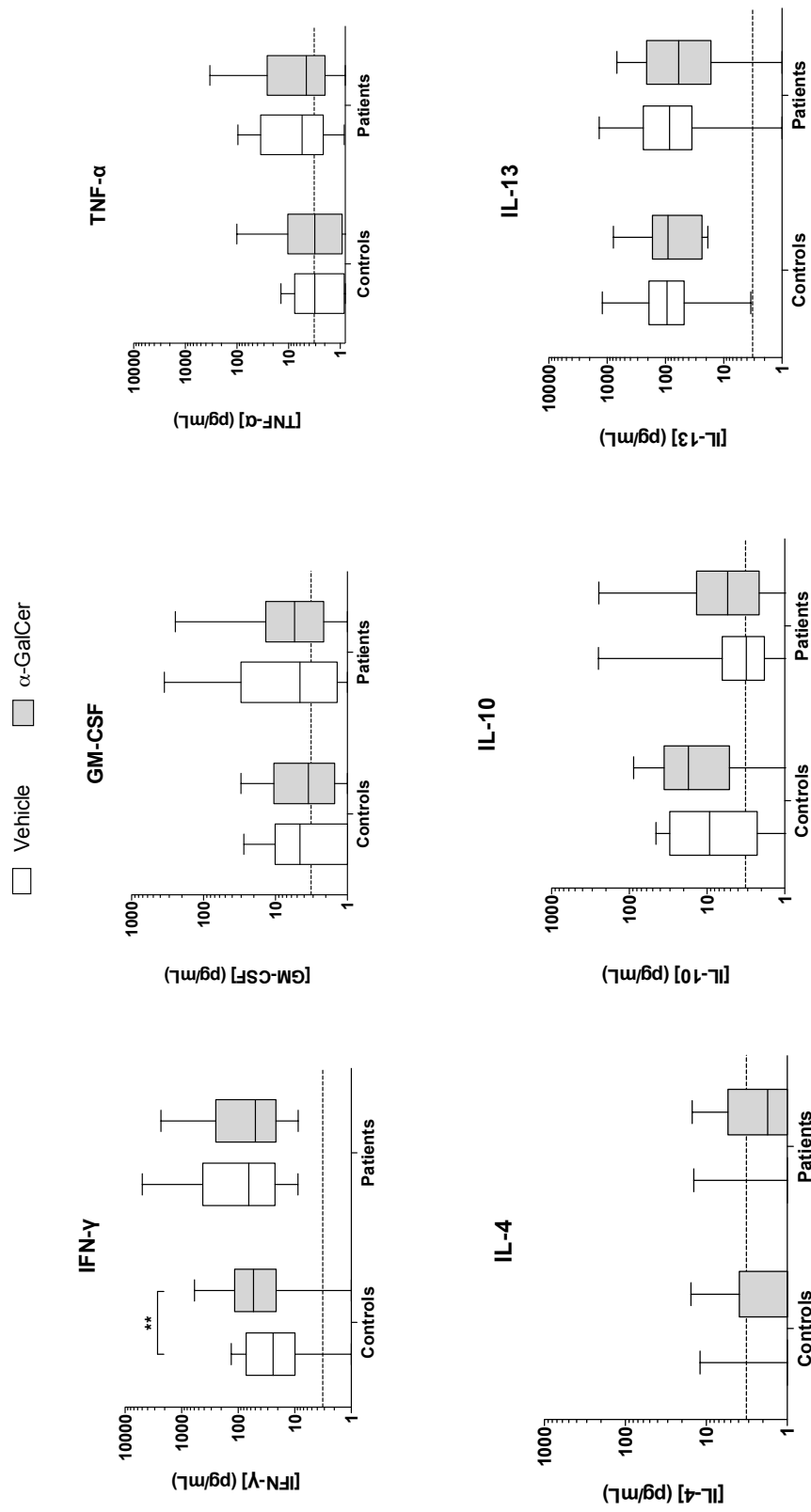
In mice, the systemic administration of  $\alpha$ -GalCer leads to upregulation of CD86 on circulating B cells. I investigated this using human PBMCs. Examining PBMCs from a healthy donor with a high number of iNKT cells, the culture of PBMCs with  $\alpha$ -GalCer led to slight upregulation of CD86 and CD54 on B cells at 60 hours. CD80, CD25, CD40 and HLA-DR did not appear to be upregulated under these conditions (figure 6.4a to 6.4f).

These data suggest that, although  $\alpha$ -GalCer might be capable of inducing human monocyte and B cell activation when iNKT cells are present in sufficiently high proportions, this phenomenon is unlikely to be observed in mixed PBMC cultures from most humans in whom the frequency of iNKT cells is low. Given the lack of effect of  $\alpha$ -GalCer on mono-

**Figure 6.1 Detection of cytokines in supernatants of cultured PBMCs.**  $2 \times 10^6$  PBMCs from each of three healthy donors, were cultured with  $\alpha$ -GalCer or vehicle and 100 U/mL IL-2. Supernatants were taken at the indicated times, and cytokine concentrations evaluated by cytokine bead array. Percentages in the charts indicate the number of iNKT cells (as a percentage of all T cells) before culture. Dashed lines represent lower limit of detection of assay.

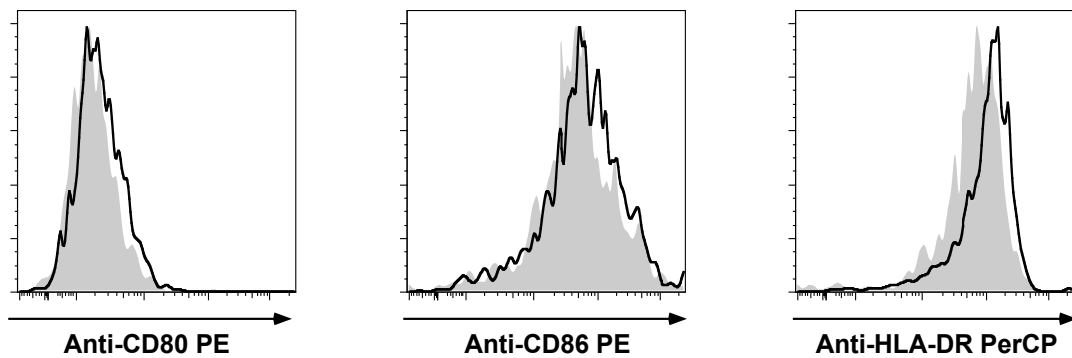


**Figure 6.2  $\alpha$ -GalCer induced cytokine production in patient and control PBMCs.**  $2 \times 10^6$  B-cell depleted control (n = 16) and patient (n = 14) PBMCs were cultured with vehicle or  $\alpha$ -GalCer. IL-2 100 U/mL was added at 24h. Supernatants were analysed at 68 hours by cytokine bead array. Dashed line represents lower limit of detection. Lines represent medians, bars represent quartiles, whiskers represent range. \*\*\* p < 0.01, Wilcoxon matched pairs signed rank test; all other comparisons not significant.

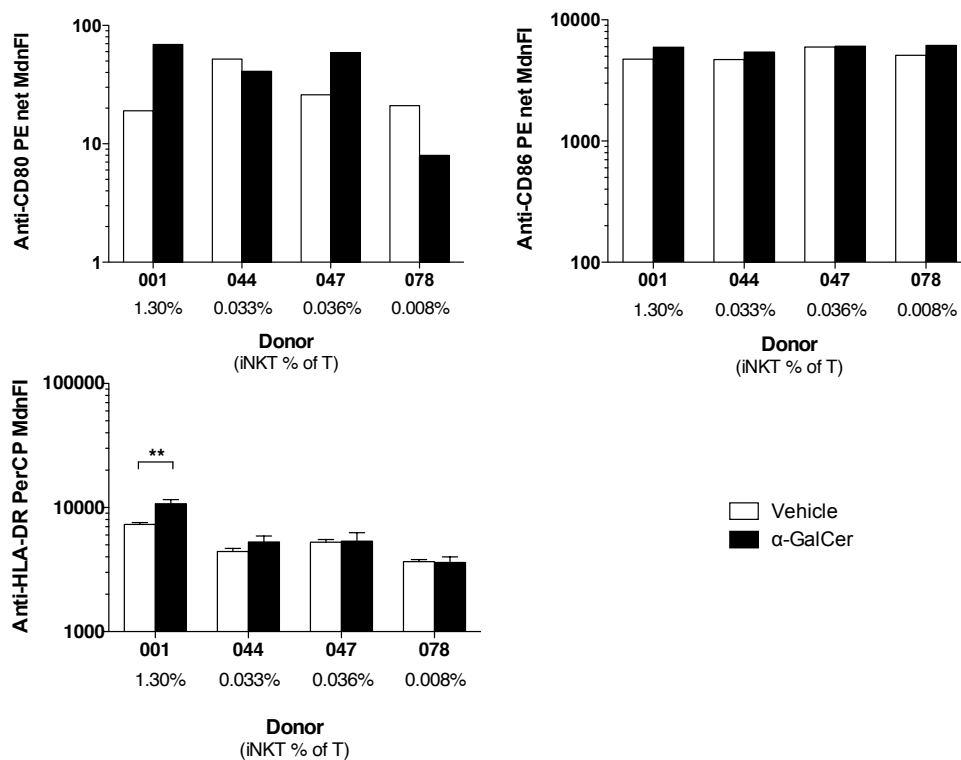


**Figure 6.3 Effect of  $\alpha$ -GalCer on monocyte activation in PBMC cultures.**  $2 \times 10^6$  PBMCs from each of four healthy donors were cultured with 100 ng/mL  $\alpha$ -GalCer or vehicle. After 18 hours, cells were harvested and APC maturation assessed by flow cytometry for monocyte CD80, CD86 and HLA-DR (MHC Class II) expression. (a) Histograms showing monocyte expression levels after culture with vehicle (shaded area) or  $\alpha$ -GalCer (black line) from a healthy donor with a high number of iNKT cells. (b) Graphs showing monocyte expression levels from four different healthy donors. Percentages beneath graph represent iNKT cell numbers as a % of all iNKT cells.

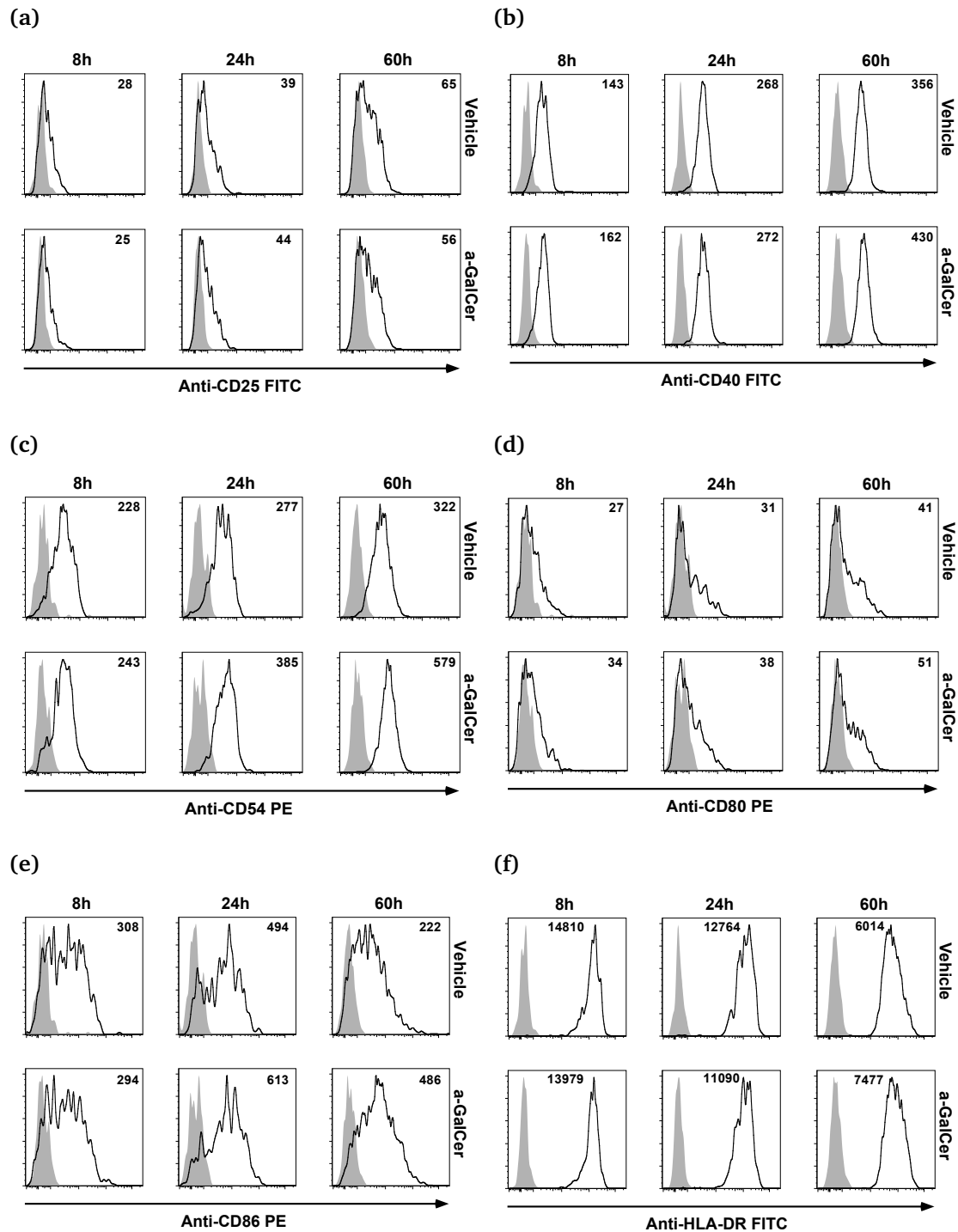
(a)



(b)



**Figure 6.4 Effect of addition of  $\alpha$ -GalCer on B cells.** PBMCs from a healthy donor with a high number of iNKT cells (1.3% of T cells) were cultured in the presence of vehicle or  $\alpha$ -GalCer, and expression of (a) CD25, (b) CD40, (c) CD54, (d) CD80, (e) CD86, and (f) HLA-DR on B cells assessed by flow cytometry at the indicated time points. Numbers in histograms indicate net median fluorescence intensity of the indicated marker on B cells.



cyte HLA-DR expression in healthy donors with an iNKT cell frequency of 0.03% of T cells, I considered it unlikely that I would be able to demonstrate this effect in cultures of PBMCs from CLL patients, where iNKT cells constitute a median of just 0.01% of T cells (figure 3.7).

### 6.3.3 Activation of CLL cells by patient-derived iNKT cell lines is not $\alpha$ -GalCer-dependent

Having shown that  $\alpha$ -GalCer-induced activation of B cells was weak in mixed PBMC cultures, even in a donor with a relatively high proportion of iNKT cells (figure 6.4), I wished to establish whether purified iNKT cells could interact directly with CLL cells to result in CLL cell activation.

Patient CLL cells were co-cultured with autologous patient-derived iNKT cell lines at various ratios in the presence or absence of  $\alpha$ -GalCer. Activation status of the CLL cells was determined by flow cytometry. The results indicate that co-culture of CLL cells with autologous iNKT cell lines results in the upregulation of CD86 and CD80 on the leukaemic cells, but that this effect was not dependent upon the addition of  $\alpha$ -GalCer (figure 6.5). Contamination with the IL-2 used during iNKT cell line generation is unlikely to explain this, as the iNKT cells had been washed four times during the iNKT cell cryopreservation, thawing and viable cell counting steps.

One explanation for the  $\alpha$ -GalCer-independent activation of CLL cells by autologous iNKT cell lines may be via a CD40/CD40L interaction. B cells express CD40, while CD40L (CD154) is upregulated by T cells upon activation and exposure to IL-2.<sup>464</sup> Flow cytometric analysis confirmed that leukaemic CLL cells expressed CD40 (figure 6.6a), and that a patient-derived iNKT cell line expressed surface CD40L (CD154) (figure 6.6b).

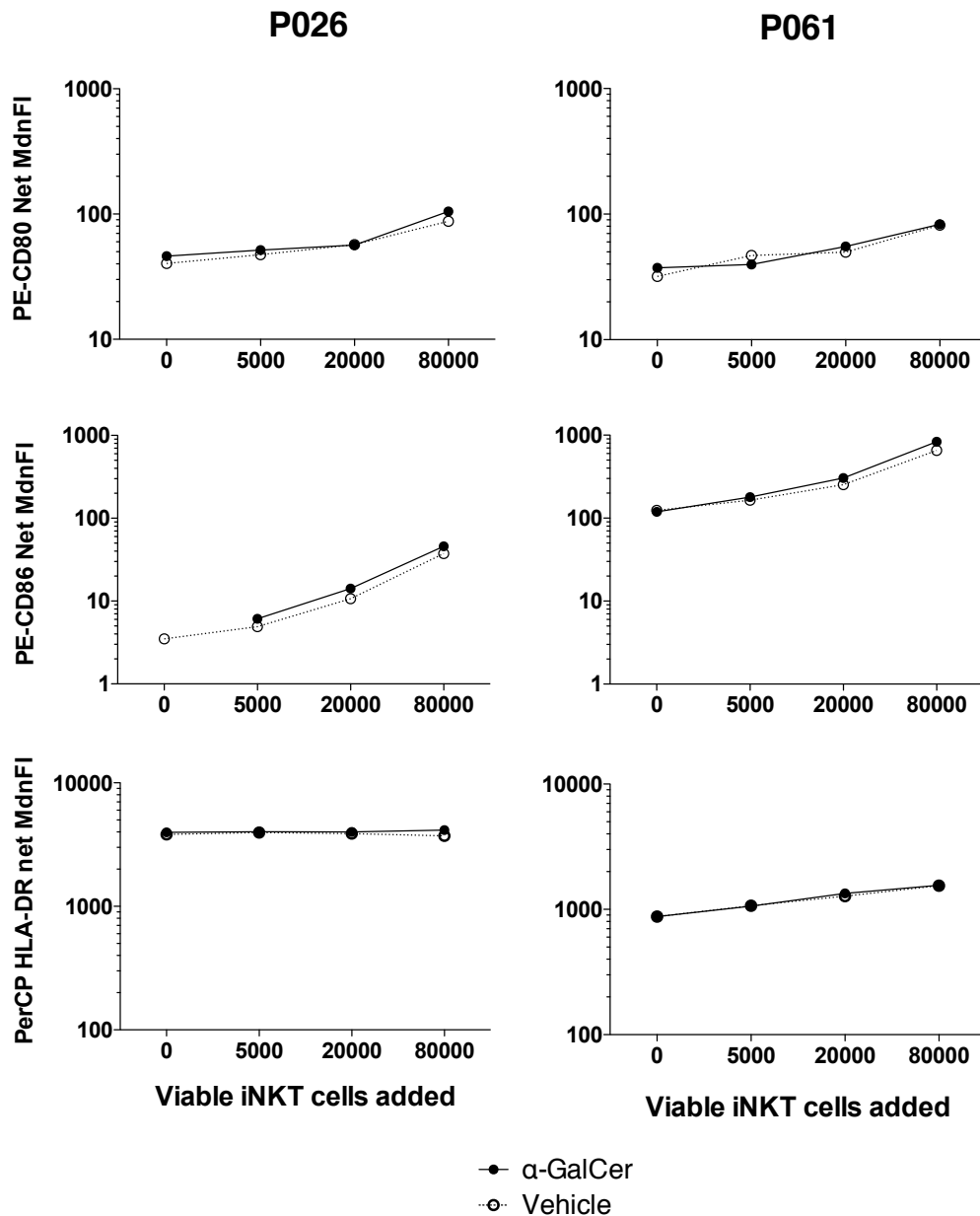
### 6.3.4 Lysis of autologous CLL cells by patient-derived iNKT cell lines

Healthy donor iNKT cell lines can lyse allogeneic CLL cell lines.<sup>417</sup> Having demonstrated that iNKT cell lines can be derived from patients with CLL (figure 5.15), and that a patient-derived iNKT cell line can lyse a CD1d-positive target cell (figure 5.20), I wished to determine whether patient-derived iNKT cells can lyse autologous CLL cells. Unlike allogeneic CLL cells or the MHC Class I-low C1R cell line, autologous leukaemic cells would not provide activating NK signals, and may provide inhibitory NK signals, to iNKT cells.

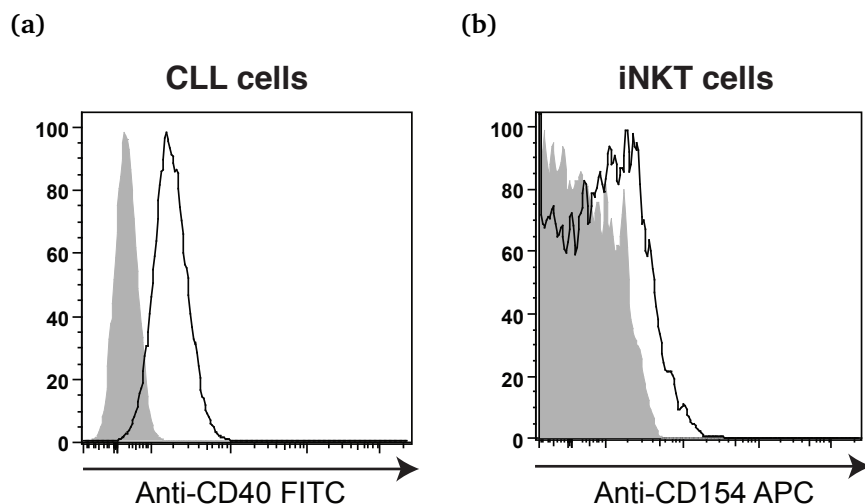
First, I examined killing of  $\alpha$ -GalCer pulsed target cells in whole PBMC cultures. I used the VITAL assay, which employs differential fluorescent labelling to determine the relative proportions of  $\alpha$ -GalCer and vehicle-pulsed CD1d-expressing target cells remaining following co-culture with whole PBMCs as effector cells.<sup>439</sup>

The VITAL assay enabled discrimination between  $\alpha$ -GalCer-pulsed and unpulsed target cells (figure 6.7a). When assessed using two healthy donors, up to 16% specific lysis of  $\alpha$ -GalCer-pulsed C1R cells was observed at an effector:target ratio of 20:1 (figure 6.7b).

**Figure 6.5 Activation of CLL cells by iNKT cell lines is  $\alpha$ -GalCer-independent.**  $2 \times 10^4$  immunomagnetically-selected patient CLL cells ( $> 95\%$  CD5+ CD19+) were cultured alone, or in the presence of the indicated numbers of cryopreserved, washed, viable autologous iNKT cells ( $> 98\%$  CD3+ 6B11+), in the presence of  $\alpha$ -GalCer or vehicle. After 20 hours, cells were harvested, and CLL cell expression of CD80, CD86 and MHC Class II determined by flow cytometry, gating on CD5+ CD19+ cells.



**Figure 6.6 Expression of CD40 on CLL cells and CD40L on iNKT cells.** (a) Immunomagnetically-selected patient CLL cells were stained with anti-CD40 antibody or isotype control, gated on CD19+ CD5+ cells. (b) A patient-derived iNKT cell line was stained with anti-CD154 (anti-CD40L) antibody or isotype control, gated on CD3+ 6B11+ cells. Solid black lines represent anti-CD40 or anti-CD154 antibody stains; filled gray histograms represent matched isotype control stains.



The low maximal specific lysis percentages observed may be because iNKT cells account for a small proportion of nucleated cells within whole PBMCs. Thus the ‘effective’ effector:target ratios were much lower than the ‘nominal’ effector:target ratios of up to 20:1.

I therefore focussed on using iNKT cell lines rather than whole PBMCs as effector cells, having already demonstrated that iNKT cell lines could lyse a CD1d-transfected target cell at effector:target ratios of 3:1 *in vitro* (figure 5.20).

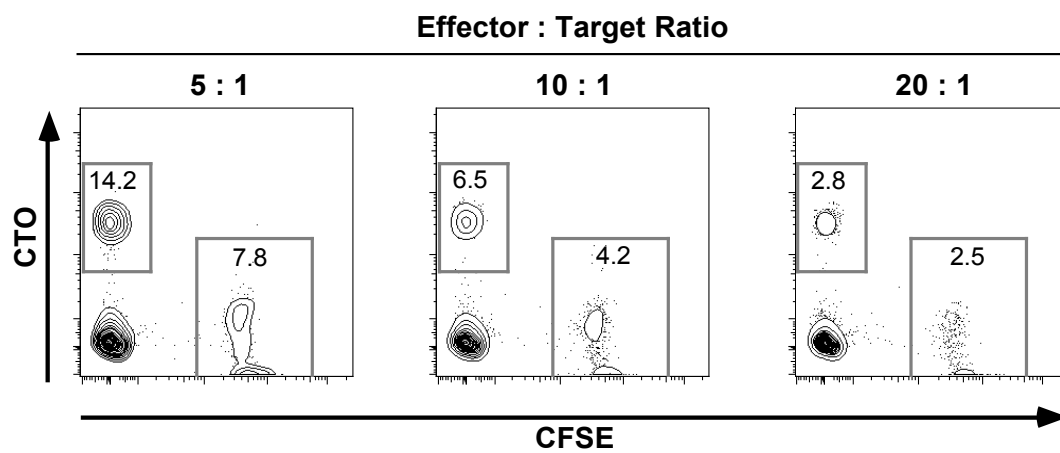
A patient-derived iNKT cell line was incubated with autologous  $\alpha$ -GalCer-pulsed or unpulsed CLL cells at various effector:target ratios, and target cell lysis determined by LDH release. This indicated that a patient-derived iNKT cell line was capable of lysis of autologous CLL cells in an  $\alpha$ -GalCer-dependent manner (figure 6.8a). A further experiment showed inconsistent results, with  $\alpha$ -GalCer-dependent lysis of autologous and allogeneic CLL cells by patient-derived iNKT cells in three out of six target and effector cell pairs (figure 6.8b).

The degree of specific lysis was low, and varied between target and effector cell pairs (figure 6.8b), lysis of autologous CLL cells being demonstrated with one donor (P026) but not another (P060). This may be due to differences in susceptibility of the three patients’ CLL cells to lysis, differences in function of the two patient-derived iNKT cell lines, and to differences in NK receptor mismatches in each effector:target pair. Availability of the patient-derived iNKT cell lines limited my ability to explore these factors further: compared with the lymphoblastoid C1R cell line, CLL cells exhibited a lower maximal LDH release. This necessitated relatively large numbers of CLL cell target cells, and therefore of iNKT cell effector cells, to obtain detectable LDH release signals.

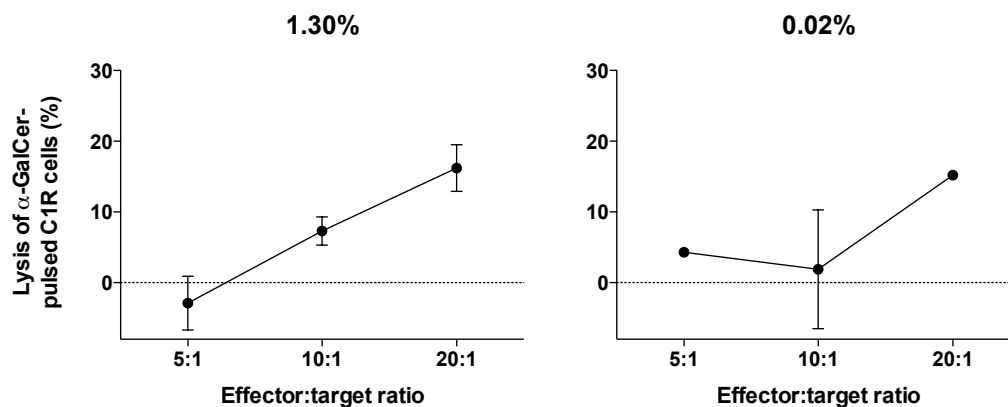


**Figure 6.7 VITAL assay to assess iNKT cell target killing in whole PBMCs.** CD1d-transfected C1R target cells were pulsed with  $\alpha$ -GalCer and labelled with CTO dye, or were unpulsed and labelled with CFSE.  $5 \times 10^3$  of each of the  $\alpha$ -GalCer-pulsed and unpulsed target cells were added to whole PBMCs from two healthy donors at the indicated effector:target cell ratios, in duplicate. After a six hour incubation, the ratio of  $\alpha$ -GalCer-pulsed to vehicle-pulsed target cells was determined by flow cytometry, and was used to determine the percentage specific lysis of  $\alpha$ -GalCer-pulsed targets. (a) Example of flow cytometry plots, demonstrating changing ratios of  $\alpha$ -GalCer pulsed and vehicle-pulsed target cells. (b) Specific lysis of  $\alpha$ -GalCer pulsed target cells by PBMCs from two healthy donors. The percentage above each graph represents the frequency of iNKT cells (as a proportion of T cells) in that donor's PBMCs

(a)

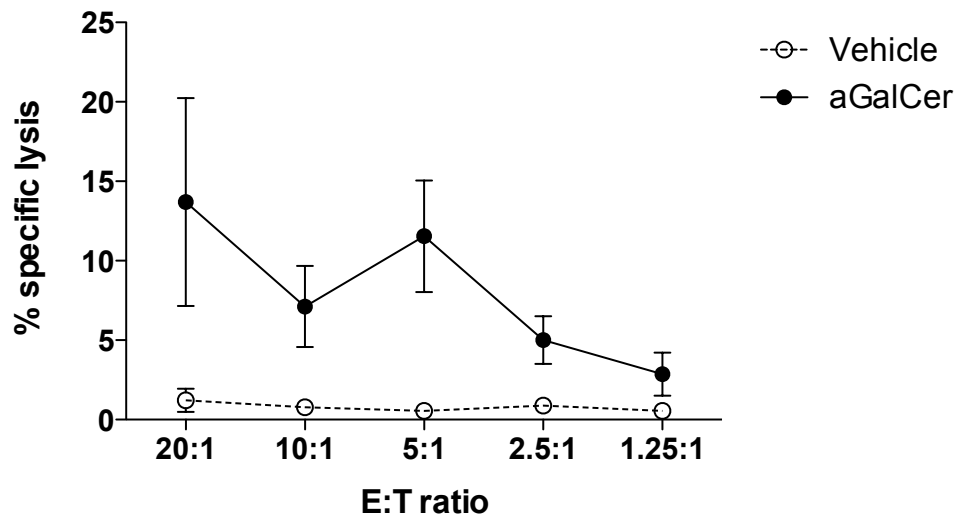


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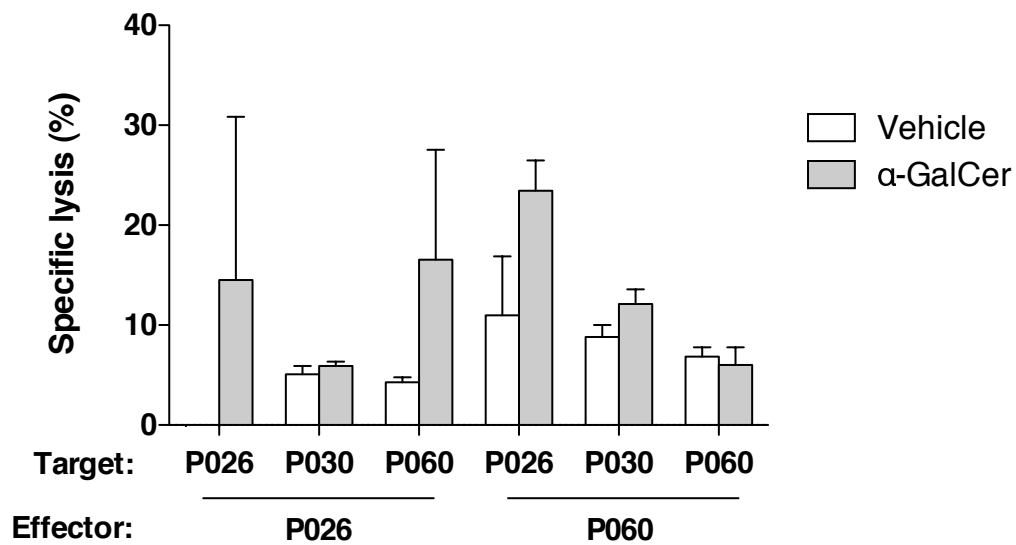


**Figure 6.8 Lysis of CLL cells by patient-derived iNKT cell lines.** Patient-derived iNKT cell lines were incubated for six hours at 37 °C with  $3 \times 10^4$  vehicle- or  $\alpha$ -GalCer-pulsed CLL cells at the indicated effector:target ratios. Lysis was assessed by LDH release assay. (a) Lysis of autologous CLL cells by a patient-derived iNKT cell line. Error bars represent standard error of means of triplicate repeats. (b) Separate experiment showing lysis of autologous and allogeneic CLL cells by two patient-derived iNKT cell lines, all at a 5:1 effector:target ratio. Bars and error bars represent means and standard error of means of triplicate repeats.  $p < 0.05$  for  $\alpha$ -GalCer treatment effect, 2-way ANOVA.

(a)



(b)



These data suggest that while patient-derived iNKT cell lines may be able to lyse CLL cells in the presence of  $\alpha$ -GalCer *in vitro*, the degree of lysis is variable, and is not efficient, requiring high effector:target cell ratios. Moreover, an earlier experiment had indicated that following a twenty-hour co-culture of CLL cells with iNKT cells at effector:target ratios of up to 4:1, viable target CLL cells remained readily detectable by flow cytometry (figure 6.5).

### 6.3.5 Expression of MHC Class I, but not CD1d, is upregulated by CLL cell exposure to IFN- $\gamma$

Killing of CD1d-expressing C1R cells by an iNKT cell line was detectable at a lower effector:target ratios than killing of CLL cells (figure 5.20, figure 6.8a). Expression levels of CD1d are higher on the CD1d-transfected C1R cell line than on CLL cells (figure 6.9a). As the capacity of IFN- $\gamma$  to induce upregulation of MHC Class I has long been recognised,<sup>465</sup> I hypothesised that expression of the MHC Class I-like molecule CD1d would also be increased upon culture of CLL cells in the presence of IFN- $\gamma$ .

To test this hypothesis, CLL cells were cultured in the presence of IFN- $\gamma$  or vehicle for 48 hours. Significant upregulation of MHC Class I was observed in the presence of IFN- $\gamma$  (figure 6.9b). In contrast, CLL cells did not upregulate CD1d upon exposure to IFN- $\gamma$  (figure 6.9c).

### 6.3.6 Effect of $\alpha$ -GalCer on vaccine recall responses

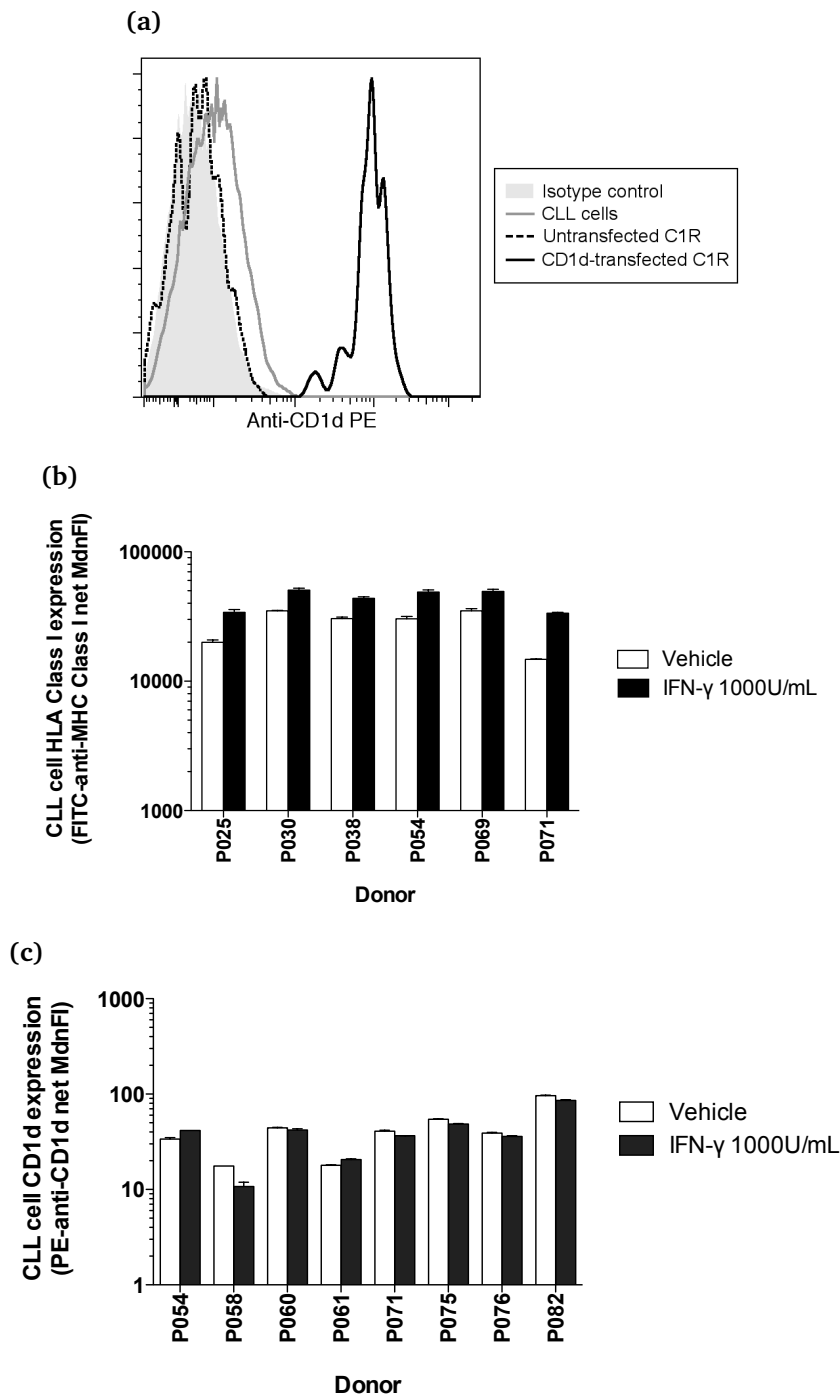
In mice,  $\alpha$ -GalCer can act as a powerful adjuvant to protein vaccination.<sup>258</sup> I wished to explore similar potential adjuvant effects in human PBMC samples. To do this, I examined the effect of  $\alpha$ -GalCer on vaccine recall responses using influenza and tetanus/diphtheria toxoid vaccines, to which many healthy donors have previously been exposed. Proliferation of PBMCs, determined by <sup>3</sup>H thymidine incorporation, was used to assess the vaccine recall response.

The addition of  $\alpha$ -GalCer significantly enhanced PBMC proliferation in response to influenza vaccine in an influenza-vaccinated donor with a high number of iNKT cells, but not in an influenza-vaccinated donor with a lower proportion of iNKT cells (figure 6.10a). Similar findings were observed when the tetanus/diphtheria toxoid vaccine recall response was assessed (figure 6.10b).

This <sup>3</sup>H thymidine incorporation assay has two important limitations. First, it does not indicate which cells are proliferating:  $\alpha$ -GalCer could be acting as an adjuvant to enhance proliferation of conventional CD4+ and CD8+ T cells in response to the vaccine, or the vaccine could act as an adjuvant to enhance proliferation of iNKT cells in response to  $\alpha$ -GalCer. Second, the assessment of proliferation by <sup>3</sup>H thymidine incorporation is dependent upon the timing of thymidine addition: if thymidine is added before or after the peak of the proliferative response, the response may be underestimated or missed.

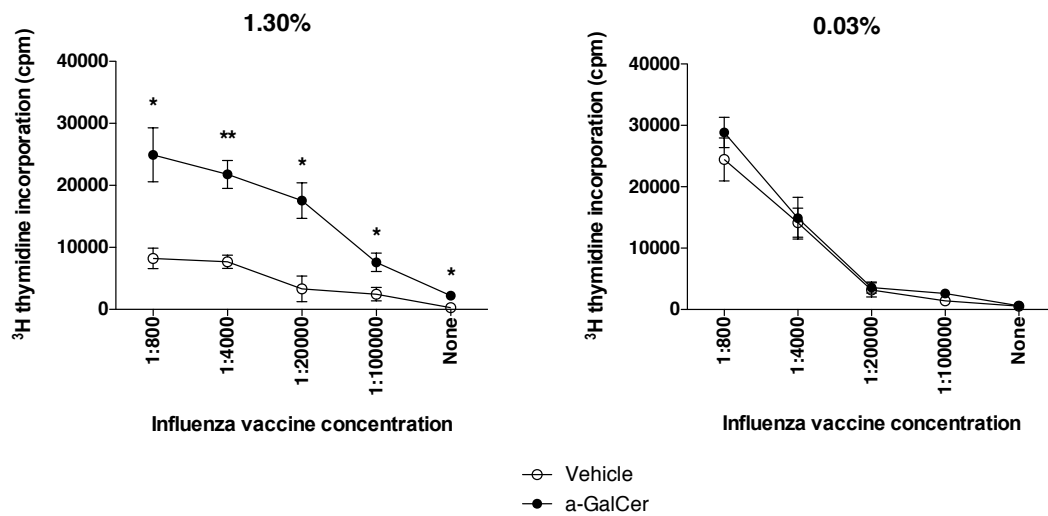
To overcome both of these limitations, a flow cytometric assay incorporating CFSE dilution was used to measure proliferation. This allows immunophenotypic identification

**Figure 6.9 Effect of IFN- $\gamma$  on CLL MHC Class I and CD1d expression.** (a) CD1d expression levels on CLL cells and CD1d-transfected and on untransfected C1R cell lines. (b), (c) Immunomagnetically purified CLL cells ( $\geq 95\%$  CD19+) were cultured for 48 hours with 1000 U/mL IFN- $\gamma$  or vehicle control. Cells were harvested, and expression of MHC Class I and CD1d was determined by flow cytometry.  $p < 0.0001$  for IFN- $\gamma$  effect on CLL cell MHC Class I expression (two-way ANOVA); difference not significant for IFN- $\gamma$  effect on CLL cell CD1d expression.

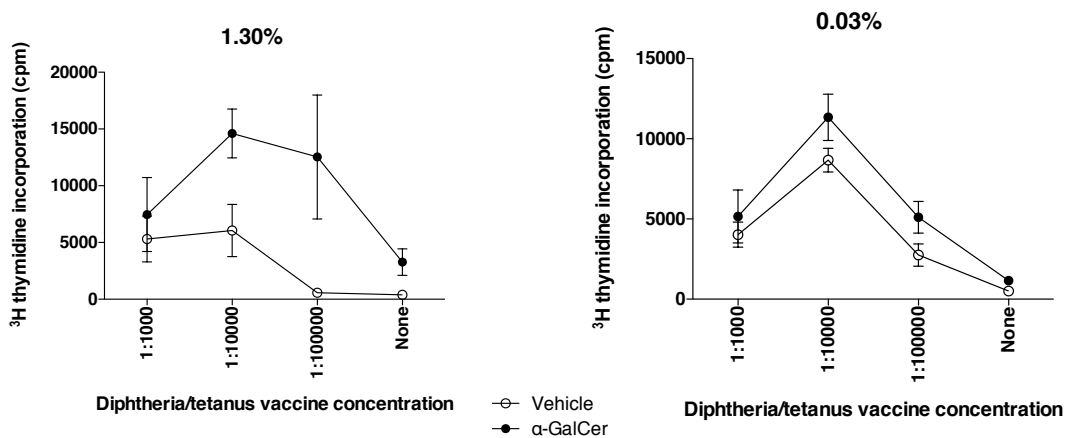


**Figure 6.10 Effect of  $\alpha$ -GalCer on vaccine recall responses.** The PBMCs of vaccinated healthy donors were cultured for five days with human influenza vaccine (a) or diphtheria and tetanus toxoid vaccine (b) at the indicated concentrations, in the presence of  $\alpha$ -GalCer or vehicle.  $^3\text{H}$  thymidine was added for the final 12 hours of culture, and proliferation was assessed by  $^3\text{H}$  thymidine incorporation. Numbers above each graph represent donor iNKT cell numbers (as a percentage of all T cells). \*  $p < 0.05$ , \*\*  $p < 0.01$ , unpaired T test.

(a)



(b)



of the divided cells. Moreover, once a cell has divided, the CFSE content of its progeny remains low even if the cells are no longer actively proliferating at the time of analysis.

This assay indicated that the addition of  $\alpha$ -GalCer resulted in an increase in the proliferation of both 'conventional' T cells and iNKT cells (figure 6.11a). The experiment was repeated using an  $\alpha$ -GalCer-loaded CD1d tetramer instead of the 6B11 antibody to identify iNKT cells, to exclude the possibility that non-invariant but CD1d-reactive iNKT cells were responsible for the apparent increase in divided 'conventional' T cells in the presence of  $\alpha$ -GalCer. Application of the  $\alpha$ -GalCer-loaded CD1d tetramer confirmed the enhancement of 'conventional' T cell proliferation in the presence of  $\alpha$ -GalCer (figure 6.11b).

Interestingly, these experiments indicated that the presence of the influenza vaccine enhanced the proliferation of iNKT cells at least as much as the presence of  $\alpha$ -GalCer enhanced the proliferation of 'conventional' T cells, suggesting that co-administration of peptide and glycolipid antigens may result in a reciprocal adjuvant effect.

Having demonstrated the adjuvant effect of  $\alpha$ -GalCer on an influenza vaccine recall response in a healthy donor with a high proportion of iNKT cells, I assessed this in healthy donors and patients with lower proportions of iNKT cells. Using a titrated concentration of the influenza vaccine, I assessed the effect of  $\alpha$ -GalCer addition on recall responses in vaccinated healthy controls and patients with CLL (figure 6.12). This did not yield consistent results, with a statistically significant increase in the recall response after addition of  $\alpha$ -GalCer in one of six controls, and a decrease in one of four patients.

These data suggest that although  $\alpha$ -GalCer can act as an adjuvant to support vaccine recall responses when iNKT cells are present at a high frequency, this effect is not observed in whole PBMCs from many human donors. The very low frequency of iNKT cells typically present in mixed PBMC cultures may account for this finding.

### 6.3.7 Pulsing CLL cells with $\alpha$ -GalCer induces allogeneic iNKT cell expansion

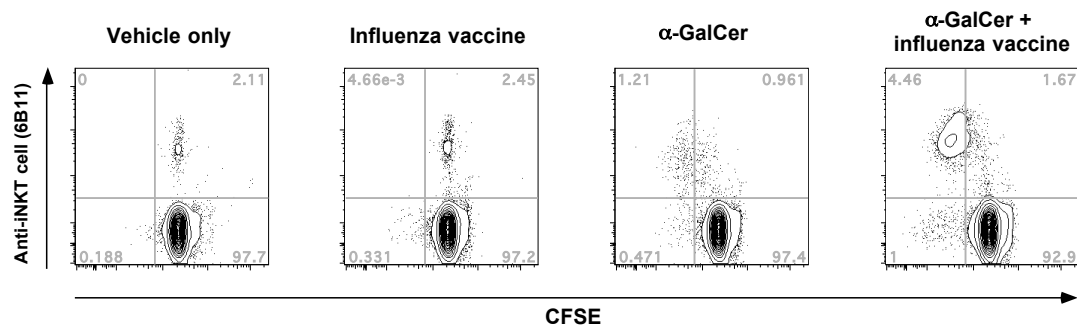
Given the low proportion of iNKT cells in patient PBMCs, a strategy to both expand iNKT cells and to deliver tumour antigen would appear attractive. Since CLL cells are readily obtained and express CD1d, I explored the possibility of loading CLL cells with  $\alpha$ -GalCer directly, utilising the tumour cells themselves to expand iNKT cell numbers and deliver tumour antigen.

CLL cell lines can present  $\alpha$ -GalCer to allogeneic iNKT cell lines,<sup>417</sup> but the capacity of CLL cells to induce iNKT cell proliferation in mixed PBMC cultures, where iNKT cells are present at very low frequencies, has not been established.

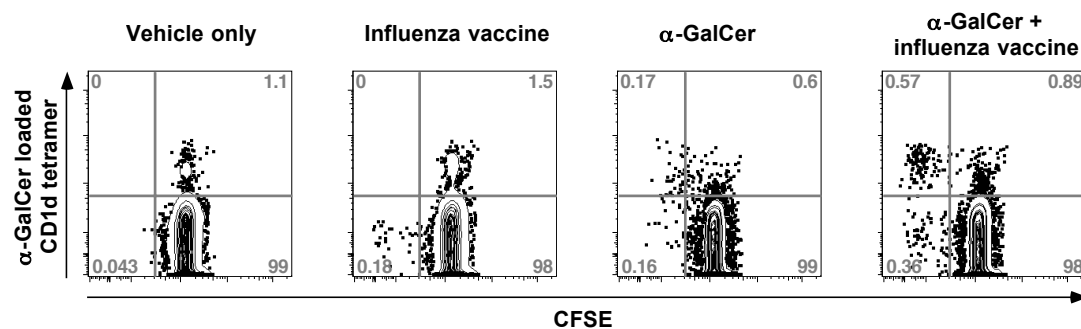
Immunomagnetically purified CLL cells were pulsed with  $\alpha$ -GalCer or vehicle, washed, then added to healthy donor PBMCs. Expansion of iNKT cells was observed in eight of nine responder/stimulator pairs tested (figure 6.13a and 6.13b), indicating that CLL cells are able to retain  $\alpha$ -GalCer and to elicit proliferation of allogeneic iNKT cells within mixed PBMC cultures.

**Figure 6.11 Immunophenotype of divided cells after  $\alpha$ -GalCer-enhanced vaccine recall response.** PBMCs from an influenza-vaccinated donor with a high percentage of iNKT cells (1.3% of T cells) were pulsed with CFSE then cultured for five days in the presence of influenza vaccine,  $\alpha$ -GalCer, both, or vehicle alone. After harvest, surface stains were applied, and flow cytometry performed, gating on live CD3+ lymphocyte events. CFSE low, 6B11+ or tetramer+ cells represent divided iNKT cells. CFSE low, 6B11- or tetramer- cells represent divided 'conventional' T cells. (a) iNKT cells identified using anti-iNKT cell antibody (clone 6B11). (b) Separate experiment, identifying iNKT cells using  $\alpha$ -GalCer-loaded CD1d tetramer. Numbers on flow cytometry plots indicate percentage of events within each quadrant.

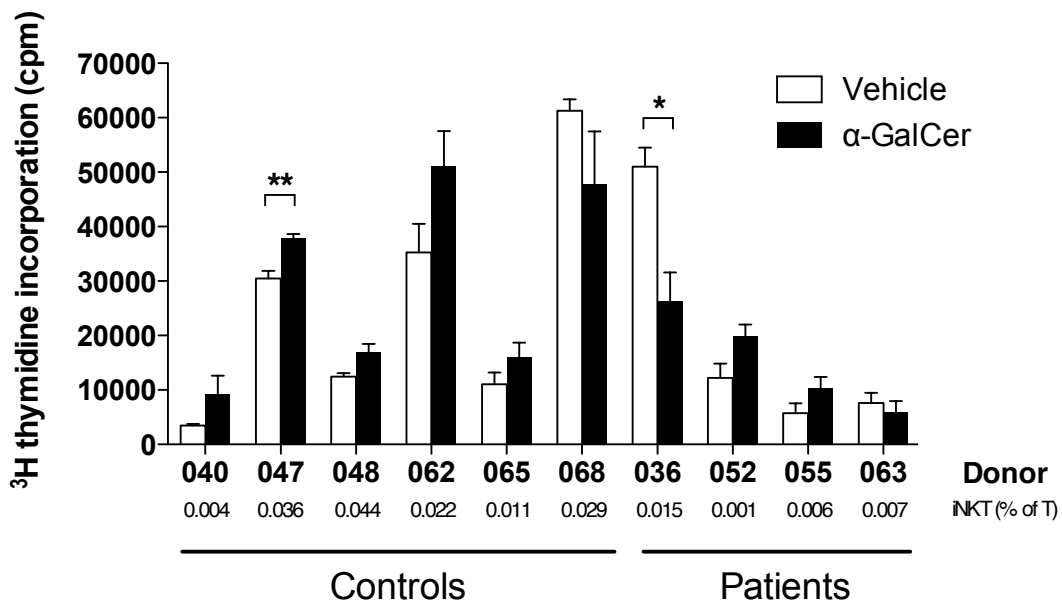
(a)



(b)

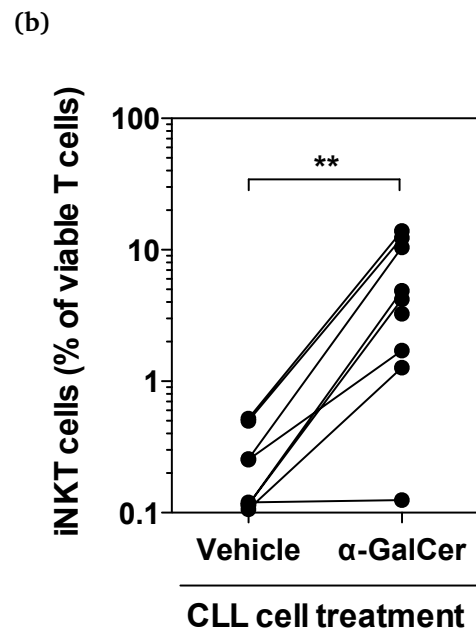
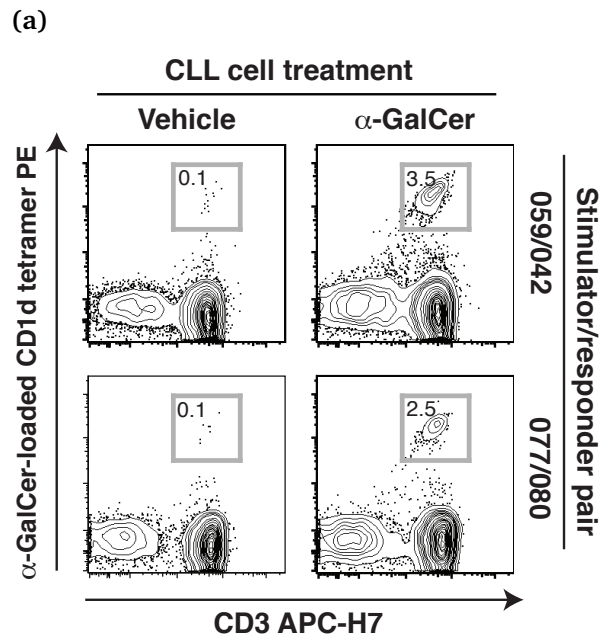


**Figure 6.12 Influenza vaccine recall responses with and without  $\alpha$ -GalCer.**  $2 \times 10^5$  B-cell depleted PBMCs from vaccinated patients and controls were cultured for five days in the presence of 2009 influenza vaccine (1:1000 dilution) with 100 ng/mL  $\alpha$ -GalCer or vehicle. \*\*  $p < 0.01$ , \*  $p < 0.05$ , two-tailed T test of triplicate repeats.





**Figure 6.13 Proliferation of allogeneic iNKT cells in response to  $\alpha$ -GalCer pulsed CLL cells.** Immunomagnetically-selected B cells from patients with CLL ( $\geq 95\%$  CD5+CD19+) were pulsed overnight with vehicle or  $\alpha$ -GalCer, then washed four times before addition at  $4 \times 10^4$ /well to  $2 \times 10^5$  allogeneic healthy donor PBMCs. After five days, iNKT cell proportions were assessed by flow cytometry. (a) Representative flow cytometry plots demonstrating increased iNKT cell numbers after addition of  $\alpha$ -GalCer-pulsed CLL cells. (b) Before/after graph showing results for nine patient and healthy donor pairs. \*\*  $p < 0.01$ , Wilcoxon matched pairs test.



### 6.3.8 Autologous $\alpha$ -GalCer-pulsed CLL cells induce proliferation of iNKT cells

Functional NK receptors are expressed by iNKT cells, and NK receptor mismatches can lead to greater iNKT cell proliferation in the allogeneic compared to the autologous setting.<sup>228</sup> Therefore, I wished to establish whether  $\alpha$ -GalCer-pulsed CLL cells can support the proliferation of *autologous* iNKT cells proliferation, without the potential effect of NK receptor mismatches.

CLL cells were pulsed with  $\alpha$ -GalCer or vehicle and used to stimulate autologous B-cell depleted PBMCs once weekly, in the presence of 1 ng/mL IL-7. After three restimulations, iNKT cells were enumerated. Pulsing CLL cells with  $\alpha$ -GalCer resulted in iNKT expansion in six out of eight patients tested (figure 6.14a and 6.14b). This indicates that  $\alpha$ -GalCer-pulsed CLL cells can support iNKT cell proliferation in the autologous, as well as in the allogeneic, setting.

### 6.3.9 $\alpha$ -GalCer treatment of CLL cells leads to increased proliferation of allogeneic PBMCs

Unmodified CLL cells are poor stimulators in autologous and allogeneic mixed lymphocyte reactions<sup>466,467</sup> As concomitant activation of iNKT cells can enhance the human *in vitro* alloresponse,<sup>468</sup> I explored whether  $\alpha$ -GalCer treatment could enhance the *in vitro* proliferation of PBMCs induced by allogeneic CLL cells.

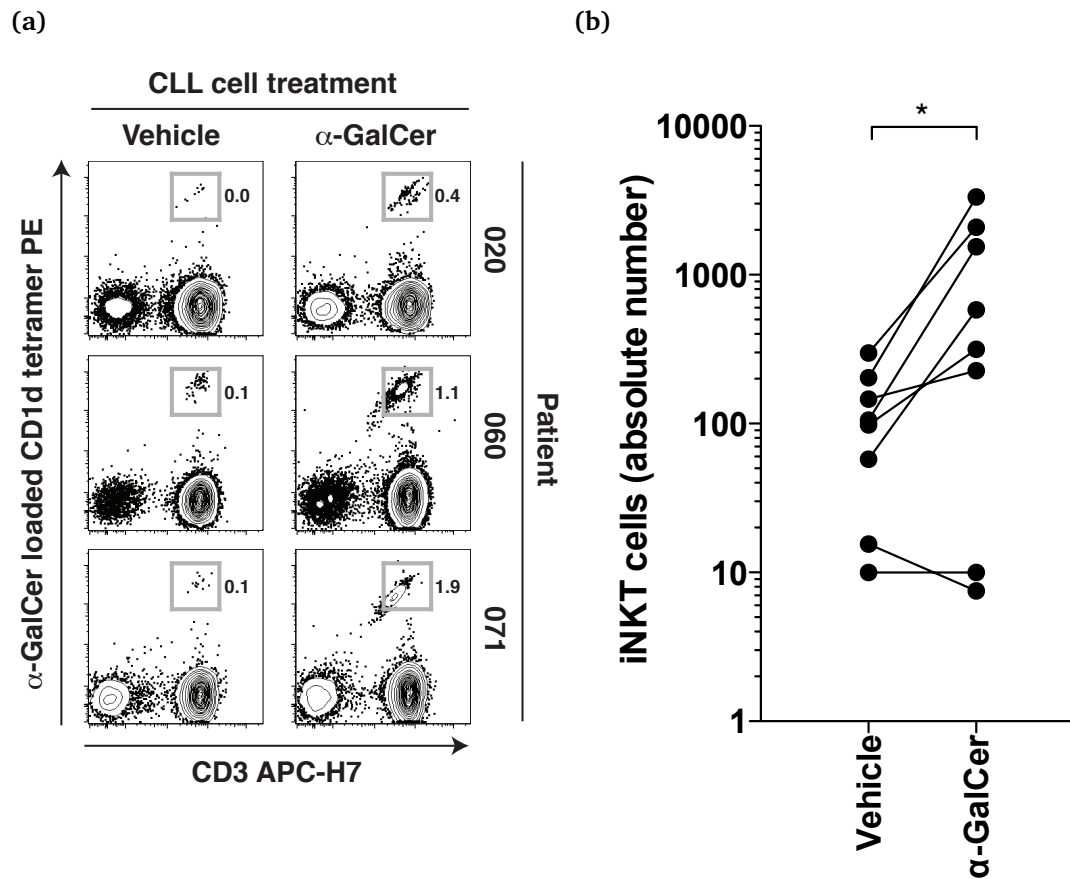
CLL cells were purified by FACS to high purity, to reduce the possibility that residual monocytes or myeloid DCs may be mediating the  $\alpha$ -GalCer effect. After overnight treatment with vehicle or  $\alpha$ -GalCer, CLL cells were irradiated and added to healthy donor PBMCs, and the proliferative response was measured by thymidine incorporation at day five. In five of six stimulator/responder pairs tested,  $\alpha$ -GalCer treatment of leukemic cells resulted in significantly increased proliferation of responding PBMCs (figure 6.15;  $p < 0.001$  for  $\alpha$ -GalCer treated versus untreated comparison, two-way ANOVA).

### 6.3.10 Effect of $\alpha$ -GalCer treatment is additional to that of TLR 7/8 ligation

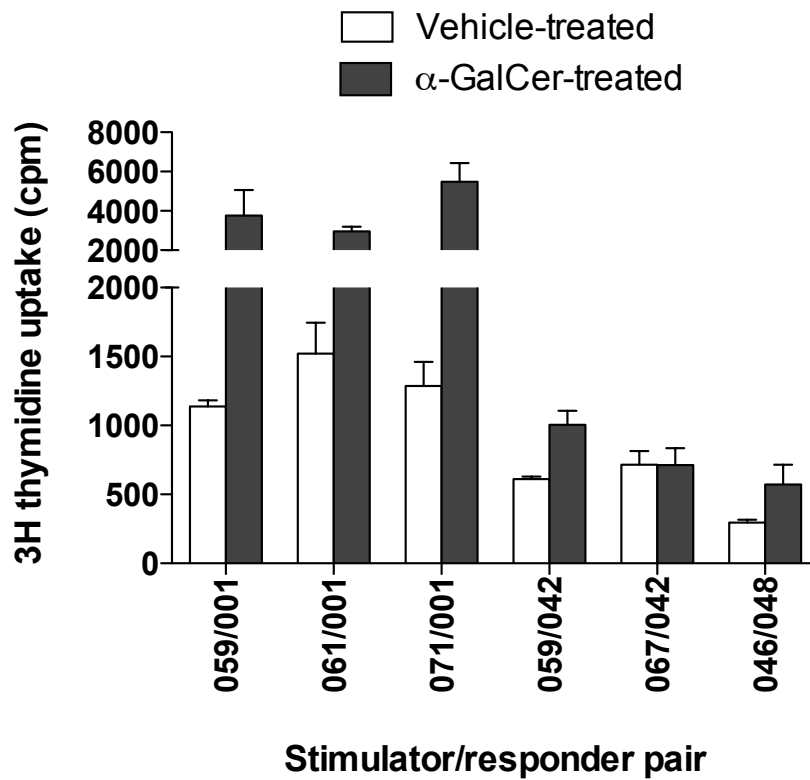
TLR agonists can co-operate with  $\alpha$ -GalCer to further enhance antigen-specific T cell responses *in vivo*.<sup>311</sup> CLL cells express TLR 7,<sup>469</sup> and a TLR 7 agonist leads to co-stimulatory marker up-regulation on leukemic cells and enhancement of their allostimulatory capacity.<sup>470</sup> I confirmed that treatment of CLL cells with the TLR 7/8 agonist resiquimod leads to upregulation of CD86 (figure 6.16a and 6.16b). Furthermore, culture of CLL cells with resiquimod, but not with  $\alpha$ -GalCer, resulted in upregulation of MHC class I and II on leukemic cells (figure 6.17a and 6.17b).

The proliferative responses of healthy donor PBMCs against CLL cells treated with vehicle,  $\alpha$ -GalCer, resiquimod or both compounds were assessed. Results from two responders with a high frequency of iNKT cells indicated that the  $\alpha$ -GalCer-induced increase in proliferation was additional to that of resiquimod (figure 6.18a). In contrast, results from

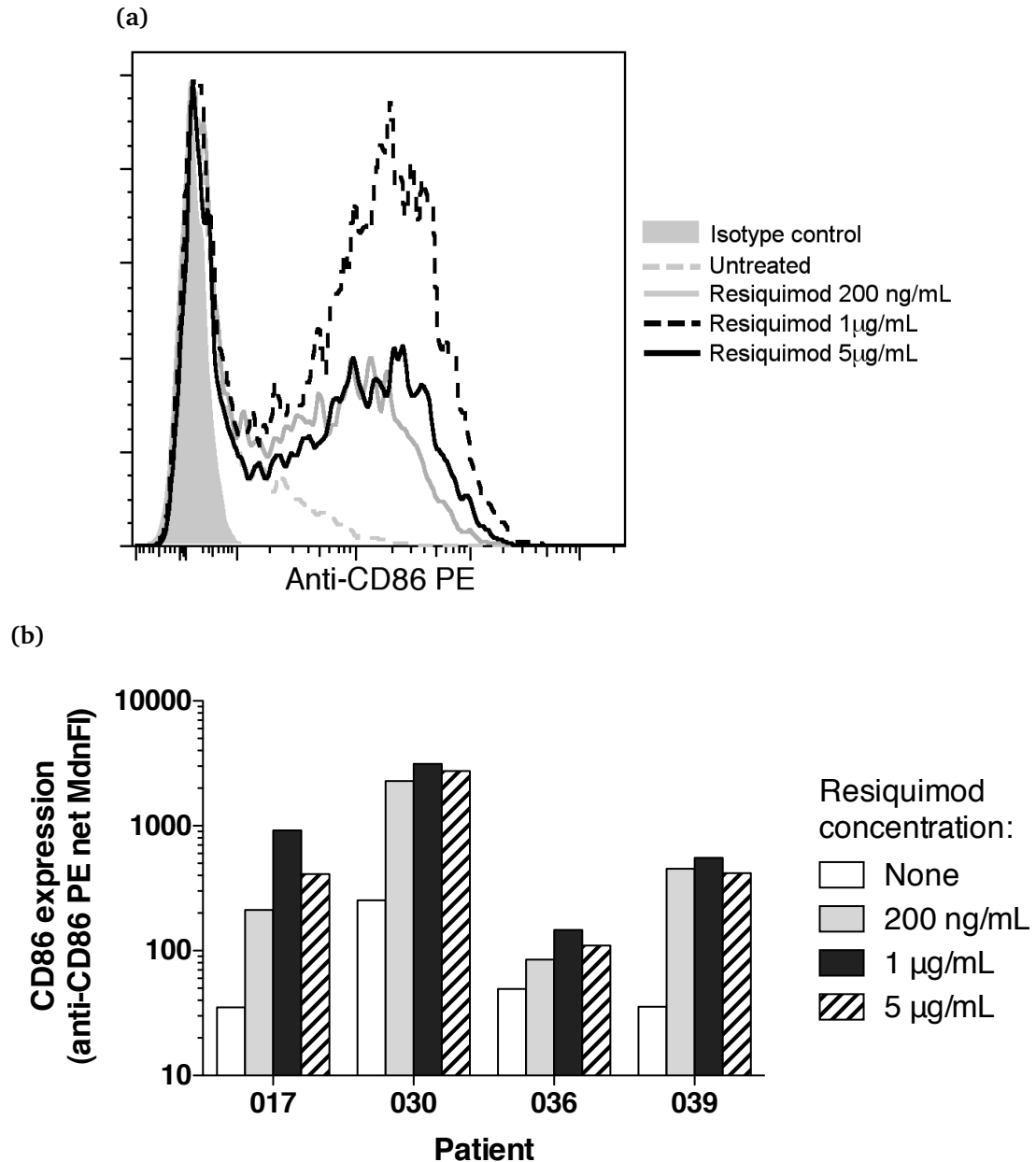
**Figure 6.14  $\alpha$ -GalCer-pulsed CLL cells support proliferation of autologous iNKT cells.** Immunomagnetically selected B cells from patients with CLL ( $\geq 95\%$  CD5+CD19+) were pulsed overnight with  $\alpha$ -GalCer or vehicle, irradiated, washed four times, then cryopreserved. Aliquots of  $1 \times 10^5$  to  $1 \times 10^6$  CLL cells were thawed, washed, and added back to  $1 \times 10^6$  autologous B cell-depleted PBMCs ( $< 5\%$  residual CD19+ cells), with the addition of 1 ng/mL IL-7. After two further weekly stimulations, flow cytometry was performed to assess iNKT cell numbers. (a) representative flow cytometry plots from three patients, demonstrating proliferation of iNKT cells in response to autologous  $\alpha$ -GalCer-pulsed CLL cells. (b) iNKT cell numbers following stimulation with autologous vehicle- or  $\alpha$ -GalCer-pulsed CLL cells in eight patients. \*  $p < 0.05$ , Wilcoxon matched pairs test.



**Figure 6.15  $\alpha$ -GalCer treatment of CLL cells leads to increased proliferation of allogeneic PBMCs.** FACS-purified CLL cells (> 98% CD19+CD5+) were pulsed overnight with  $\alpha$ -GalCer or vehicle, irradiated to 50 Gy, washed four times, then added to healthy donor PBMCs for five days.  $^3\text{H}$  thymidine was added during the final 12 hours of culture and  $^3\text{H}$  thymidine incorporation determined by scintillation counter.  $p < 0.001$  for  $\alpha$ -GalCer treatment effect, 2-way ANOVA.

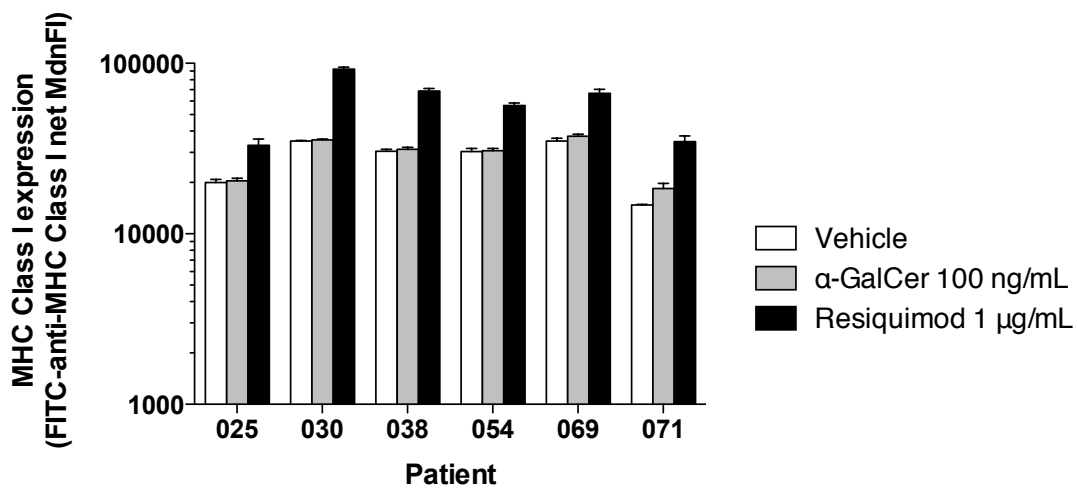


**Figure 6.16 Upregulation of CD86 on CLL cells by resiquimod.** Immunomagnetically selected CLL cells ( $\geq 95\%$  CD19+CD5+) were cultured for 24 hours in the presence of the indicated concentration of resiquimod before flow cytometric analysis of CD86 expression. (a) Representative flow cytometry histogram showing CD86 expression on CLL cells from a single patient. (b) CD86 expression on CLL cells from four patients.

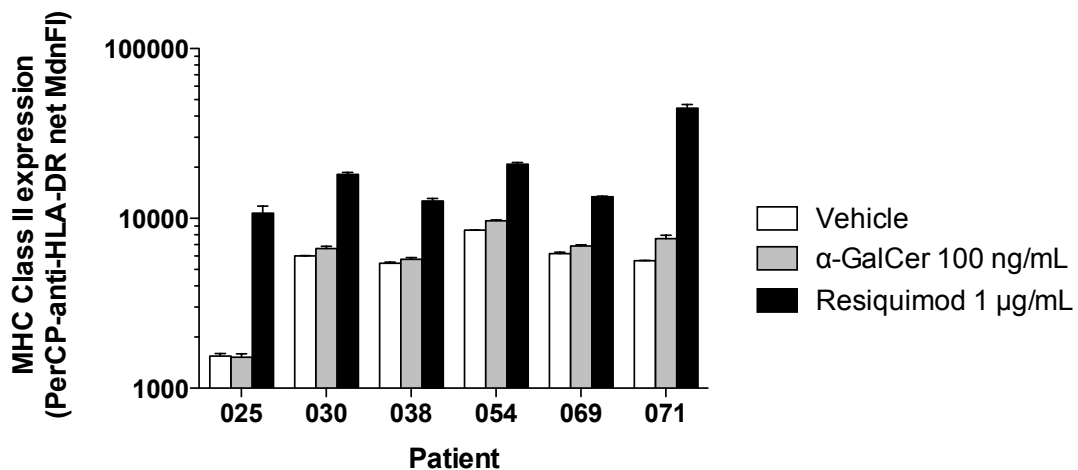


**Figure 6.17 Resiquimod, but not  $\alpha$ -GalCer, leads to upregulation of MHC Class I and II on CLL cells.** Immunomagnetically-selected B cells from six patients with CLL ( $\geq 95\%$  CD5+CD19+) were cultured for 48 hours with 100 ng/mL  $\alpha$ -GalCer, 1  $\mu$ g/mL resiquimod or vehicle in duplicate, before assessment of MHC Class I and Class II expression by flow cytometry. (a) MHC Class I expression.  $p < 0.001$  for vehicle versus resiquimod and for  $\alpha$ -GalCer versus resiquimod comparisons, not significant for vehicle versus  $\alpha$ -GalCer comparison, two-way ANOVA with Bonferroni post-tests. (b) MHC Class II expression.  $p < 0.001$  for vehicle versus resiquimod and for  $\alpha$ -GalCer versus resiquimod comparisons, not significant for vehicle versus  $\alpha$ -GalCer comparison, two-way ANOVA with Bonferroni post-tests.

(a)



(b)



two responders with low iNKT cell frequencies showed no effect of  $\alpha$ -GalCer treatment of CLL cells.

Together, these results suggest that  $\alpha$ -GalCer enhancement of allogeneic PBMC proliferation in response to CLL cells is independent of co-stimulatory marker upregulation, and requires sufficient iNKT cells. This is consistent with a published finding that iNKT cells can augment the human *in vitro* alloresponse.<sup>468</sup>

### 6.3.11 Increased allogeneic PBMC proliferation in response to $\alpha$ -GalCer-pulsed CLL cells is primarily due to iNKT cell proliferation

Given the observation that in vaccine recall responses,  $\alpha$ -GalCer and a protein antigen could co-operate to enhance the proliferation of both iNKT and 'conventional' T cells (figure 6.11), I aimed to separately assess the proliferation of 'conventional' T and iNKT cells in response to resiquimod and  $\alpha$ -GalCer-treated CLL cells.

CLL cells from three patients were treated with  $\alpha$ -GalCer, resiquimod, both or vehicle, were washed, then added to CFSE-labelled healthy donor PBMCs as responder cells. The proliferation of T cell subsets was assessed by CFSE dilution after five days. This experiment indicated that, as previously demonstrated (figure 6.13), the addition of  $\alpha$ -GalCer resulted in enhanced proliferation of iNKT cells. The addition of resiquimod led to enhanced proliferation of 'conventional' CD4+ and CD8+ T cells. However,  $\alpha$ -GalCer treatment of CLL cells did not consistently result in enhanced proliferation of 'conventional' T cells in this short term culture (figure 6.19).

### 6.3.12 Autologous $\alpha$ -GalCer-pulsed CLL cells induce proliferation of conventional T cells

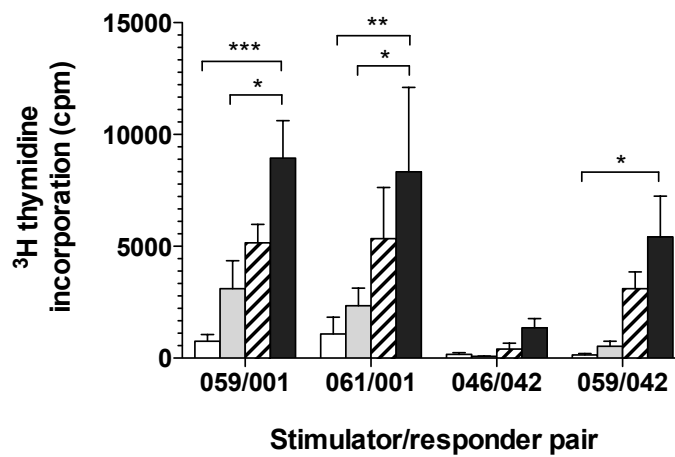
Leukaemic cells treated with  $\alpha$ -GalCer can induce proliferation of autologous iNKT cells (figure 6.14). To determine whether proliferation of autologous 'conventional' T cells was also enhanced by  $\alpha$ -GalCer treatment of leukaemic cells, vehicle- or  $\alpha$ -GalCer-treated irradiated CLL cells were added back to B cell-depleted, CFSE-labelled PBMCs and cultured for 5 days in the absence of exogenous cytokines.

As expected, divided iNKT cells (CD3+, CD1d tetramer+, CFSE low) were detected only where CLL cells had been treated with  $\alpha$ -GalCer (figure 6.20a). However, as well as inducing iNKT cell proliferation,  $\alpha$ -GalCer-treatment of CLL cells resulted in a modestly increased proportion of divided conventional T cells (CD3+, CD1d tetramer negative, CFSE low) in seven out of ten patients tested ( $p < 0.05$ ; figure 6.20b). These divided non-CD1d restricted T cells comprised both CD4+ and CD8+ cells (constituting a median of 43% and 24% of divided T cells in the  $\alpha$ -GalCer-treated samples, respectively).

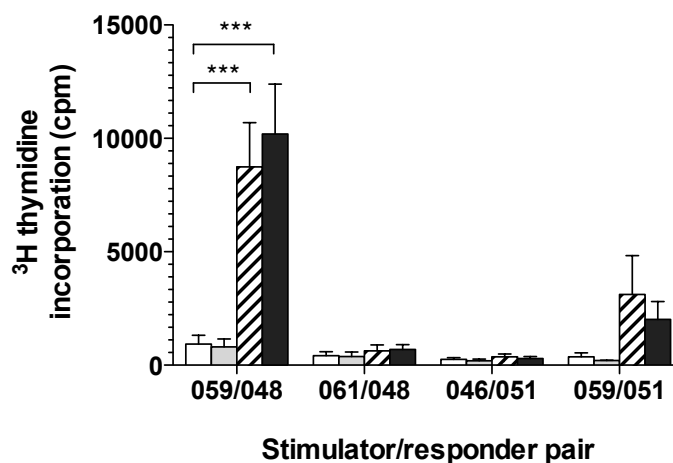
These data confirm that  $\alpha$ -GalCer-treated CLL cells induce proliferation of autologous iNKT cells. Moreover, they suggest that  $\alpha$ -GalCer treatment of autologous leukaemic cells leads to enhanced proliferation of 'conventional' T cells.

**Figure 6.18 Effect of  $\alpha$ -GalCer on allogeneic PBMC proliferation in response to CLL cells is additional to that of resiquimod.** FACS-purified CLL cells (> 98% CD19+CD5+) were pulsed overnight with  $\alpha$ -GalCer, resiquimod, both or vehicle,  $\gamma$ -irradiated to 50Gy, washed four times, then added at  $4 \times 10^4$ /well to  $2 \times 10^6$ /well healthy donor PBMCs as responders for five days, in triplicate.  $^3\text{H}$  thymidine was added during the final 12 hours of culture and  $^3\text{H}$  thymidine incorporation determined by scintillation counter. (a) Results from two responders with a high frequency of iNKT cells (median 0.69% of T cells). (b) Results from two responders with a low frequency of iNKT cells (median 0.03% of T cells). Solid white bars represent untreated CLL cells, solid gray bars represent  $\alpha$ -GalCer-treated CLL cells; hatched bars represent resiquimod-treated CLL cells; solid black bars represent CLL cells treated with both  $\alpha$ -GalCer and resiquimod. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ , two-way ANOVA.

(a)

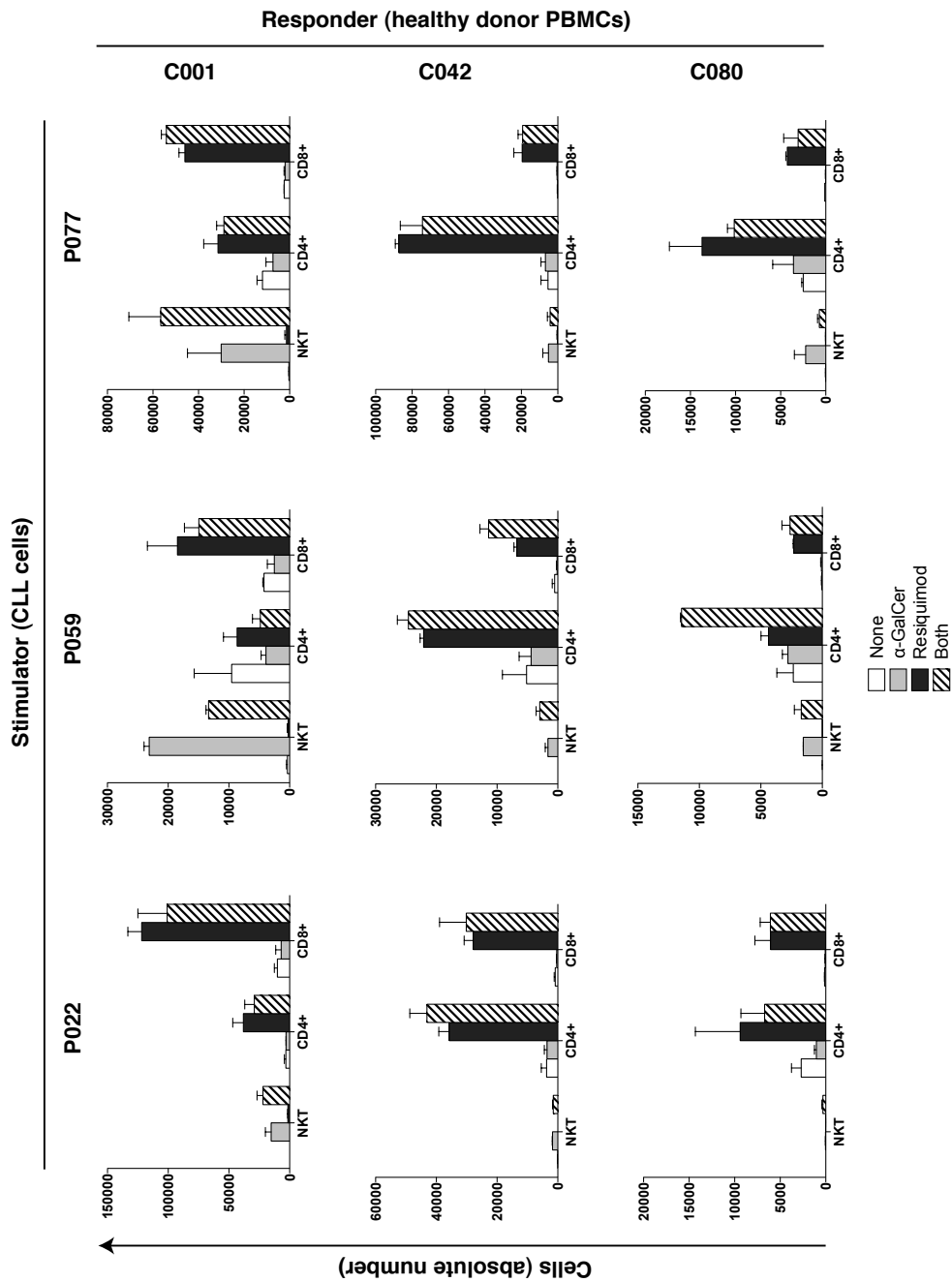


(b)

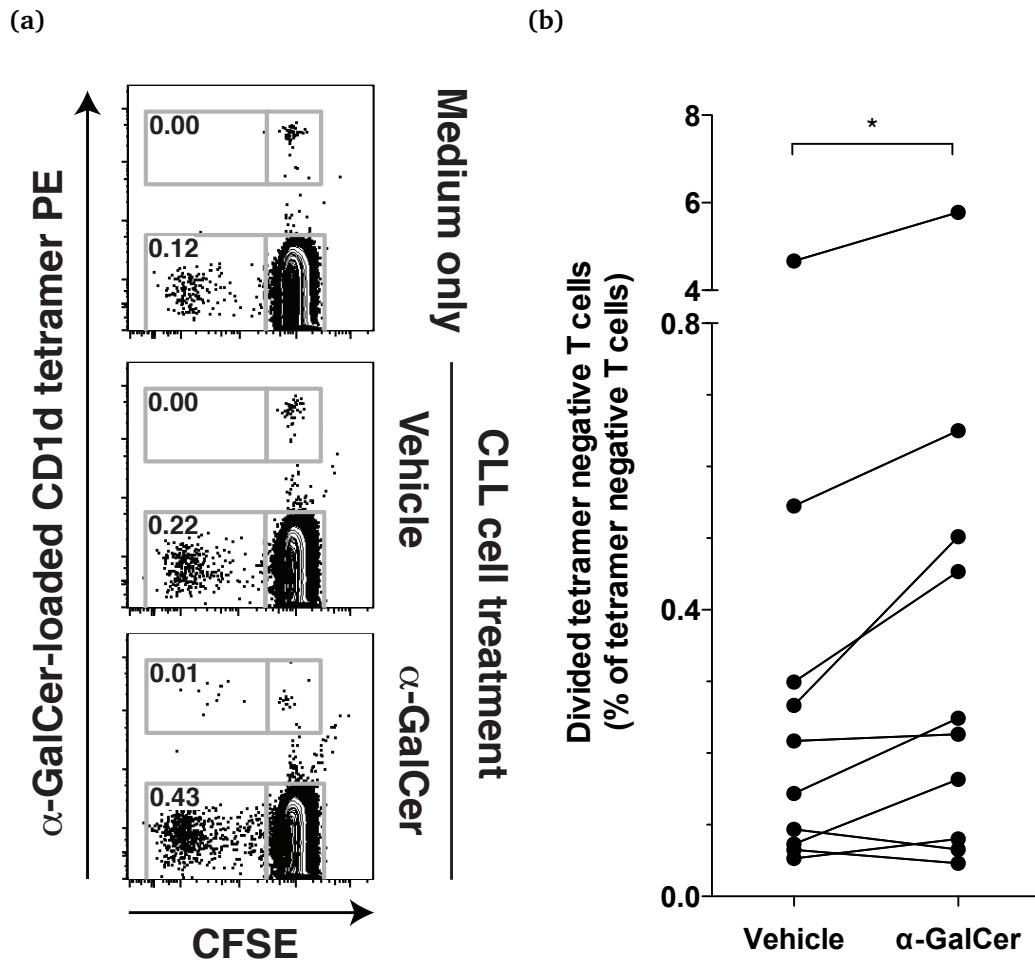




**Figure 6.19 Increased allogeneic PBMC proliferation in response to  $\alpha$ -GalCer treatment of CLL cells is due to iNKT cell, not 'conventional' T cell, proliferation.** Immunomagnetically selected CLL cells ( $\geq 95\%$  CD19+CD5+) were pulsed overnight with  $\alpha$ -GalCer, resiquimod, both or vehicle, irradiated to 50 Gy, washed four times, then added at  $4 \times 10^5$ /well as stimulators to  $2 \times 10^5$ /well CFSE-labelled healthy donor PBMCs as responders for five days, in duplicate. Flow cytometry was used to determine the numbers of divided iNKT and 'conventional' CD4+ and CD8+ T cells for each stimulator/responder pair.



**Figure 6.20  $\alpha$ -GalCer-pulsed CLL cells may support proliferation of ‘conventional’ T cells.** Immunomagnetically selected B cells from patients with CLL ( $\geq 95\%$  CD5+CD19+) were pulsed overnight with  $\alpha$ -GalCer or vehicle, irradiated, washed four times, and added at  $1 \times 10^6$ /well to  $5 \times 10^5$  CFSE-pulsed autologous B cell-depleted PBMCs ( $< 5\%$  residual CD19+ cells). After five days, flow cytometry was performed to identify divided (CFSE-low) iNKT (CD19- CD3+ CD1d tetramer+) and ‘conventional’ T cells (CD19- CD3+ CD1d tetramer-). (a) representative flow cytometry plots, demonstrating enhanced proliferation of non-CD1d restricted cells in response to autologous  $\alpha$ -GalCer-pulsed CLL cells. (b) numbers of divided ‘conventional’ T cells following stimulation with autologous vehicle- or  $\alpha$ -GalCer-pulsed CLL cells in ten patients. \*  $p < 0.05$ , Wilcoxon matched pairs test.



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## 6.4 Summary

In this chapter, I have explored the potential utility of the iNKT cell/CD1d axis in the immunotherapy of CLL using a series of *in vitro* assays. I have investigated several potential methods of exploiting this axis:

**Administration of  $\alpha$ -GalCer to induce a cytokine response** In contrast with findings using a healthy donor with a high frequency of iNKT cells, I was unable to demonstrate significant  $\alpha$ -GalCer-induced cytokine production within the supernatants of PBMCs from patients with CLL. This may be because due to low frequency of iNKT cells in most patients. In a study using PBMCs from healthy human donors, the addition of  $\alpha$ -GalCer did not result in marked IFN- $\gamma$  production or NK cell activation unless previously-cultured activated iNKT cells were added to the cultures to a level of 0.1% of all cells.<sup>402</sup>

**Administration of  $\alpha$ -GalCer to induce APC maturation** While the administration of  $\alpha$ -GalCer led to upregulation of B cell and APC co-stimulatory molecules in a donor with a high frequency of iNKT cells, this effect was not consistently observed in donors with frequencies of iNKT cells closer to those of patients with CLL *in vitro*.

**Direct cytotoxicity against CLL cells by iNKT cells** Killing of allogeneic CLL cells by iNKT cell lines has previously been demonstrated.<sup>417</sup> I endeavoured to demonstrate this in the autologous setting, where potential activating NK signals are not present. While I was able to demonstrate lysis of autologous CLL cells by a patient-derived iNKT cell line, these experiments were limited by the number of effector iNKT cells available — large numbers of target cells were needed to detect lysis in the assay used, and high effector:target ratios were required. The lysis of CLL cells by iNKT cell lines appeared less efficient than lysis of the CD1d-transfected C1R cell line, which expresses CD1d at a much higher level. I therefore assessed the effects of IFN- $\gamma$  on CLL cell CD1d expression. I found that, although IFN- $\gamma$  resulted in increased CLL cell MHC class I expression, it did not result in upregulation of CD1d.

**$\alpha$ -GalCer as an adjuvant to protein vaccines** To investigate the potential role of  $\alpha$ -GalCer as an adjuvant to a protein vaccine, I assessed the impact of  $\alpha$ -GalCer on vaccine recall responses. These experiments demonstrated that  $\alpha$ -GalCer can enhance the vaccine recall response within whole PBMCs when iNKT cells are present at a high frequency, leading to enhanced proliferation of non-CD1d restricted T cells as well as of iNKT cells.

The demonstration of an adjuvant effect using human PBMCs is important, as the efficacy of  $\alpha$ -GalCer-treated tumour cells or  $\alpha$ -GalCer-treated tumour-loaded DCs as an immunotherapy relies upon the enhancement of peptide-specific CD4+ and CD8+ T cell responses via DC activation (see section 1.3.3.3).<sup>258,419,424,425</sup>

The restriction of the adjuvant effect to a donor with a high iNKT cell percentage is not surprising, given the low frequency of iNKT cells in the majority of individuals, and the limited cell numbers in each *in vitro* culture well. Nonetheless, it is possible that the low

frequency of iNKT cells in the majority of healthy donors, and particularly in older adults with CLL, may limit any adjuvant effect in clinical practice.

**Effect of  $\alpha$ -GalCer pulsed CLL cells in allogeneic setting** As CLL cells themselves express CD1d, pulsing CLL cells with  $\alpha$ -GalCer offers a simple method for simultaneously delivering tumour antigen and the glycolipid adjuvant. I demonstrated that washed, irradiated,  $\alpha$ -GalCer-pulsed CLL cells support the expansion of iNKT cells in both allogeneic and autologous healthy donor PBMCs.

**Combination of  $\alpha$ -GalCer with resiquimod** The TLR 7/8 ligand resiquimod results in enhanced CD86 and MHC class I and II expression on CLL cells. CLL cells treated with both  $\alpha$ -GalCer and resiquimod, elicited an enhanced alloresponse compared to the cells treated with each alone. The effect of  $\alpha$ -GalCer treatment of CLL cells was restricted to responders with a higher frequency of iNKT cells. When the proliferation of iNKT and T cells was examined by CFSE dilution, the effects of  $\alpha$ -GalCer (enhancing iNKT cell proliferation) and resiquimod (enhancing proliferation of non CD1d-restricted CD4+ and CD8+ T cells) appeared to be independent, without evidence of synergy.

Again, whether or not the restriction of the  $\alpha$ -GalCer treatment effect to donors with a high iNKT cell frequency *in vitro* would limit the applicability of iNKT cell-directed immunotherapies in clinical practice is not clear.

**Effect of  $\alpha$ -GalCer pulsed CLL cells in autologous setting** Using CFSE dilution, I was able to confirm that  $\alpha$ -GalCer-pulsed CLL cells induced expansion of autologous iNKT cells within PBMC cultures. Moreover, compared to vehicle-treated CLL cells,  $\alpha$ -GalCer-treated CLL cells appeared to elicit enhanced proliferation of 'conventional' non CD1d-restricted T cells, although effect was small.

Together, these findings offer some insight into the uses and limitations of the iNKT cell and CD1d axis in the immunotherapy of CLL. Many of the effects of *in vitro* iNKT cell stimulation were limited by the low frequency of iNKT cells present in most healthy donor and patient PBMC samples. However, when iNKT cells were present at sufficiently high frequencies,  $\alpha$ -GalCer could induce cytokine release, induce upregulation of co-stimulatory markers on APCs, and augment T cell proliferative responses to a protein vaccine. Importantly,  $\alpha$ -GalCer-treated CLL cells were able to induce proliferation of *autologous* iNKT cells, and  $\alpha$ -GalCer treatment led enhanced proliferation of 'conventional' CD4+ and CD8+ T cells.

The findings presented in this chapter, their relation to the published literature, and their implications for the exploitation of iNKT cells in the immunotherapy of CLL, will be discussed in chapter 7.

# Chapter 7

## Discussion

### 7.1 Introduction

In the course of this experimental programme, I have described the iNKT cell and CD1d axis in patients with CLL in numeric, immunophenotypic and functional terms, and have performed a series of *in vitro* experiments aimed at identifying ways to exploit this axis for the immunotherapy of CLL.

In this chapter, I shall summarise these results and discuss their significance and limitations, with reference to the published literature. I shall then place the findings in the broader context of immunotherapy for cancer, outlining the ideal characteristics of, and factors associated with, a successful immunotherapy. The potential benefits and drawbacks of exploiting the iNKT cell/CD1d axis for the cellular immunotherapy of cancer will be discussed. Finally, I shall outline a potential immunotherapeutic strategy for the treatment of CLL that makes use of the iNKT cell and CD1d axis.

### 7.2 Summary and discussion of results

#### 7.2.1 Numbers and immunophenotype of iNKT cells

In section 3.3.4, I enumerated, and determined the immunophenotype of, iNKT cells in patients with CLL and in healthy age-matched controls. Multiparameter flow cytometry was chosen for this purpose, as it allows the simultaneous analysis of multiple surface and intracellular markers on a 'per cell' basis. However, the very small number of iNKT cells present in most donors (a median of 0.01% of T cells in patients) posed technical challenges to their flow cytometric identification. Optimising detection of these rare events required a number of measures, including the immunomagnetic depletion of malignant B cells before analysis, the inclusion of channels to exclude non-viable and leukaemic B cells, the use of a bright fluorophore and a highly specific antibody for iNKT cell identification, the application of a stringent gating strategy during data analysis which includes exclusion of doublet events, and the acquisition of a large number of T cell events (median of  $> 10^5$  events per donor).

Together, application of these measures enabled a lower limit of detection of iNKT cells of approximately 1 in  $10^5$  T cells, which approaches the lower limit of detection for flow cytometric events.<sup>471</sup>

The specificity of staining for iNKT cells using the 6B11 antibody clone was confirmed by application of an isotype control antibody (figure 3.6a), concordance with results using an  $\alpha$ -GalCer-loaded CD1d tetramer (figure 3.6c), and by the finding that 6B11-positive iNKT cell lines exhibited  $\alpha$ -GalCer dependent cytokine release (figure 5.18) and cytotoxicity (figure 5.20).

### 7.2.1.1 Numbers of iNKT cells in healthy controls

The frequency of iNKT cells observed in healthy controls is towards the lower end of the ranges published in the literature (see table 7.1). This may be due to the older age of controls in this study compared to the majority of published studies.

An age-related decline in iNKT cell numbers is well-described,<sup>210,223</sup> and contrasts with age-related increases in NK cell numbers, and with stable overall T cell numbers with aging.<sup>210</sup> The iNKT cell frequency reported here is similar to that observed in a study of healthy donors with a similar median age.<sup>308</sup>

The effect of gender on iNKT cell frequencies is not well established. In one study of 90 healthy donors, iNKT cell frequency was not significantly different between males and females,<sup>218</sup> while in another, iNKT cell frequency was lower in males.<sup>210</sup> The data presented in this thesis show no significant effect of gender on iNKT cell number or frequency among healthy controls (section 3.3.4). A study examining *in vitro* iNKT cell proliferation found no effect of gender.<sup>472</sup>

### 7.2.1.2 Numbers of iNKT cells in patients with CLL

**Relative iNKT cell numbers** The frequency of iNKT cells I observed in patients with CLL are presented alongside other reports of iNKT cells in patients with cancer in table 7.2. The two studies including larger numbers of patients with cancer reported similar iNKT cell frequencies to those reported here in patients with CLL.<sup>303,308</sup> In contrast, studies examining smaller numbers of patients found higher iNKT cell frequencies.<sup>418,475,476</sup> Possible reasons for this discrepancy include differing disease biology, differences in donor age distribution, methodological factors, or sampling error in the latter studies due to small patient numbers.

Considering the two larger series, compared to healthy controls, median iNKT cell numbers were  $\sim$  2-fold reduced in patients with head and neck squamous cell carcinoma,<sup>308</sup> and median iNKT cell frequency was  $\sim$  3-fold reduced in patients with a range of advanced cancers.<sup>303</sup> The 2-fold reduction in iNKT cell frequency that I observed in this study of patients with CLL is of similar magnitude.

I sought, but did not find, any trend towards lower iNKT cell frequencies in patients with more clinically advanced disease (figure 3.8), although given the relatively small numbers

Table 7.1 Circulating iNKT cell numbers reported in healthy donors

Donor characteristics	n	iNKT cell identification	Median iNKT cell numbers		Reference
			% of T cells	Absolute number per mL blood	
<b>Median age 64y</b>	<b>31</b>	<b>CD3+6B11+</b>	<b>0.022</b>	<b>195</b>	<b>This study</b>
Age range 21 – 52y	37	V $\alpha$ 24+V $\beta$ 11+	—	1013	303
Median age 54y	31	CD3+V $\alpha$ 24+V $\beta$ 11+	0.053	—	473
Mean age 44y	49	CD3+V $\alpha$ 24+V $\beta$ 11+	—	857	474
Median age 65y	33	CD3+V $\alpha$ 24+V $\beta$ 11+	0.021	373	308
Median age 29y	50	CD3+6B11+	—	2910	285
Mean age 30y	49	CD3+V $\alpha$ 24+V $\beta$ 11+	0.044 <sup>*</sup>	—	223
Mean age 78y	73	CD3+V $\alpha$ 24+V $\beta$ 11+	0.004 <sup>*</sup>	—	223
Age range 15 – 52y	90	CD3+6B11+	0.17 <sup>*,†</sup>	—	218
Ages not stated	20	CD3+Tetramer+ <sup>‡</sup>	0.034	—	220
Mean age 24y	31	CD3+Tetramer+ <sup>‡</sup>	0.086 <sup>§</sup>	—	286

<sup>\*</sup> iNKT cells expressed as a percentage of lymphocytes, not as a percentage of T cells

<sup>†</sup> Number of iNKT cells expressed as mean, not median

<sup>‡</sup> 'Tetramer' refers to  $\alpha$ -GalCer-loaded CD1d tetramer

<sup>§</sup> iNKT cells expressed as a percentage of total blood mononuclear cells, not as a percentage of T cells

of patients with advanced CLL in this study, a true difference could have been missed (a type II statistical error).

The preclinical and clinical data suggesting a role for iNKT cells in cancer immunosurveillance have already been discussed in section 1.2.7.3. However, whether the reductions in circulating iNKT cell frequencies observed in some patients with cancer is a cause or effect of the malignancy, or simply an epiphenomenon, remains unclear. Although this study adds to the body of data, it does not help to answer this question. Since lower iNKT cell numbers predict poor prognosis in patients with head and neck squamous cell carcinoma,<sup>308</sup> a longitudinal study examining the impact of iNKT cell numbers on CLL prognosis may be of interest. Given the indolent nature of CLL, long follow-up would be required, so such a study may be best performed using previously cryopreserved PB-MCs.

**Absolute iNKT cell numbers** In contrast to the reduction in the relative frequency of iNKT cells, I observed a trend towards increased *absolute* numbers of iNKT cells in patients with untreated CLL. This has to be interpreted in the context of the long-recognised phenomenon of increased T cell numbers in patients with untreated CLL.<sup>83,84</sup> The data presented in figure 3.10 confirm the presence of a statistically significant increase in the major T cell subsets in patients with untreated CLL in this study. The pathologic significance of this increase in T cell numbers has been discussed in section 1.1.3.5.

In this study, absolute numbers of PBMC subsets were calculated from absolute lymphocyte counts determined on a commercial blood count analyser, before PBMC separation. As this involves several steps, this could lead to a wider coefficient of variation for the determination of absolute cell counts, and could introduce sources of error, compared to the direct analysis of blood counts. Absolute counts could also be determined in a single step by flow cytometric analysis of whole blood spiked with a known number of 'counting beads', polystyrene beads with distinct forward and side scatter properties on flow cytometric analysis. This type of analysis was not performed in the current study, and cannot be applied retrospectively.

The discordance between reduced relative iNKT cell numbers but normal or increased absolute numbers suggests that iNKT cells do not fully participate in this T cell expansion. Whether this is because iNKT cells are resistant to the CLL cell / T cell interactions which support increased numbers of 'conventional' T cells, or because iNKT cell frequencies are lower as part of the aetiology or pathophysiology of CLL is not clear.

**Effect of fludarabine chemotherapy** Fludarabine-based chemotherapies are known to result in a prolonged depletion of all T cell subsets, particularly of CD4+ T cells, with full T cell reconstitution remaining incomplete at three years after treatment. In contrast, NK cell numbers are relatively preserved.<sup>175</sup> As the first candidates for any investigational therapy of CLL are likely to be patients who have relapsed following, or who are refractory to, fludarabine-based treatments, I recruited a cohort of patients who had received fludarabine treatment, and evaluated iNKT cell numbers in this group.

Among those who had received fludarabine treatment, the numbers of all T cell subsets were reduced compared to untreated patients (figure 3.10). This included an absolute



Table 7.2 Circulating iNKT cell numbers reported in patients with cancer

Donor characteristics	n	iNKT cell identification	Median iNKT cell numbers			Reference
			% of T cells	Absolute number per mL blood		
<b>Chronic lymphocytic leukaemia (median age 63y)</b>	<b>40</b>	<b>CD3+6B11+</b>	<b>0.010</b>	<b>244</b>		<b>This study</b>
Advanced cancer (median age 54y)	24	V $\alpha$ 24+V $\beta$ 11+	—	333		303
Head and neck squamous cell carcinoma (mean age 65y)	47	CD3+V $\alpha$ 24+V $\beta$ 11+	0.009	103		308
Advanced non small cell lung cancer (median age 66y)	6	CD3+V $\alpha$ 24+V $\beta$ 11+	0.03	—		418
Myeloma (ages not given)	7	CD3+V $\alpha$ 24+V $\beta$ 11+	0.01 *	—		477
Renal cell carcinoma (ages not given)	11	CD3+V $\alpha$ 24+V $\beta$ 11+	0.05	—		475
Glioma (median age 40y)	9	CD3+V $\alpha$ 24+V $\beta$ 11+	0.07 *,†	—		476

\* Number of iNKT cells expressed as a mean, not median

† iNKT cells expressed as a percentage of lymphocytes, not as a percentage of T cells

(but not relative, see figure 3.8) reduction in the numbers of iNKT cells. This suggests that the magnitude of iNKT cell depletion after fludarabine is similar to that of depletion of other T cell subsets.

This was a cross-sectional study, so confounding factors such as differing disease or immunological characteristics among patients who required treatment could be responsible for the reduction in absolute iNKT cell numbers observed. As discussed in section 7.2.1.1, a gender difference in iNKT cell numbers has been reported,<sup>210</sup> although another study did not confirm this,<sup>218</sup> and I did not find significant differences in iNKT cell number or frequency between healthy male and female donors in my own data. Nonetheless, as there was an excess of males among fludarabine-treated patients, I cannot exclude the possibility that gender contributed to the lower iNKT cell numbers observed in this group.

Fully defining the effect of fludarabine-based chemotherapy on the iNKT cell population would be more reliably achieved in a longitudinal study, examining iNKT cell numbers before and after fludarabine treatment in the same individuals.

### 7.2.1.3 Immunophenotype of iNKT cells

**Healthy donor iNKT cell immunophenotype** I examined the CD4, CD8, CD161 and CD25 expression of circulating iNKT cells in patients and controls (figure 3.11). The results in this study are compared with those reported elsewhere in table 7.3.

The proportions of CD4+ and CD161+ iNKT cells I observed in healthy donors were similar to those previously published. Expression levels of CD25 on T cells lie in a continuum rather than forming discrete positive and negative populations, so are sensitive to the positioning of gates during analysis. The CD25 expression levels I recorded are not therefore readily comparable with those of the one other study that reported them. However, I did observe a consistently lower proportion of CD8+ iNKT cells than that reported in other studies.

Of note, the *level* of CD8 expression on CD8+ iNKT cells was lower than that of ‘conventional’ CD8+ T cells in this study (figure 3.11a). This was a consistent finding across all experiments, and was not due to inconsistent staining, because the difference between the two cell subsets was observed within the same sample. The CD8 expressed by iNKT cells is reported to be in the form of the CD8 $\alpha\alpha$  homodimer, rather than the functional CD8 $\alpha\beta$  heterodimer more commonly expressed by ‘conventional’ T cells.<sup>220</sup> The antibody clone I employed (RPA-T8) is said to recognise CD8 $\alpha$ , so should have detected the CD8 $\alpha\alpha$  homodimer on iNKT cells. Nonetheless, antibody clones could differ in their relative detection of the homodimeric and heterodimeric forms of CD8. Thus, possible reasons for the lower iNKT cell CD8 levels I observed include a genuine reduction in surface expression, or an effect of the selected antibody clone. This could be explored further using alternative anti-CD8 antibody clones. However, since the CD8 $\alpha\alpha$  homodimer is not believed to be functional,<sup>478</sup> and because, unlike CD4 expression, no functional significance of CD8 expression on iNKT cells has been reported, I did not pursue this observation further.

**Patient iNKT cell immunophenotype** Comparing patients with controls, I did not find statistically significant differences in iNKT cell immunophenotype. However, a non-significant trend towards a higher proportion of CD4+ iNKT cells in patients was observed. This may relate to the lower relative frequency of iNKT cells in patients, as I confirmed that there was a negative correlation between the proportion of iNKT cells which were CD4+ and overall iNKT cell frequency (figure 3.12), as previously reported.<sup>218</sup>

I found no significant difference in the CD4 status of iNKT cells between patients who had and who had not received fludarabine chemotherapy (figure 3.11c). This could imply that fludarabine does not disproportionately deplete CD4+ iNKT cells, but as this is a cross-sectional study, the results could be confounded by other factors.

### 7.2.2 Relationship between iNKT cell numbers and progression-free survival

In section 3.3.7, I examined the association between circulating iNKT cell numbers and progression-free survival among patients with untreated CLL. The low rate of disease progression seen (28% over a three year follow-up period) highlights the fact that this is a cohort of patients with indolent CLL. Only one patient died during follow-up.

I found no correlation between iNKT cell frequency or absolute numbers and CLL disease progression. My findings contrast with studies reporting that iNKT cell frequency correlates with overall survival in patients with head and neck squamous cell carcinoma,<sup>308</sup> and that absolute numbers of iNKT cells correlate with overall survival in patients with acute myeloid leukaemia.<sup>305</sup>

The findings I present should be interpreted with some caution. First, I do not have data on the most established prognostic markers in early CLL, FISH cytogenetic findings (particularly 17p and 11q deletion status) or IgVH rearrangement status. Thus, it is not possible to perform a multivariate analysis that includes these parameters. Second, my cohort is relatively small (29 patients) and fewer than half progressed or died during follow-up, so there is a chance that an association could have been found had a larger cohort, or longer follow-up period, been available (although I do note that the two studies above each found an association between iNKT cell numbers and survival using a similar-sized cohort). Third, as I have sampled the peripheral blood only, I cannot exclude the possibility that changes in iNKT cell tissue distribution take place in high-risk or progressive CLL, and that peripheral blood iNKT cell frequency does not reflect total iNKT cell numbers.

Despite these limitations, in conjunction with the finding that iNKT cell frequency does not correlate with CLL disease stage, lymphocyte count or ZAP-70 status (figure 3.8), the lack of an association between iNKT cell frequency or number and progression-free survival suggests that iNKT cell deficiency does not play a central role in the progression of CLL.

Table 7.3 Reported immunophenotype of circulating iNKT cells in healthy donors

Donor characteristics	n	iNKT cell identification	iNKT cells of given phenotype (%)				Reference
			CD4+	CD8+	CD161+	CD25+	
<b>Median age 64y</b>	<b>28</b>	<b>CD3+6B11+</b>	<b>42</b>	<b>14</b>	<b>63</b>	<b>26</b>	<b>This study</b>
Ages not given	20	CD3+Tetramer+ <sup>*</sup>	51 <sup>†</sup>	51 <sup>†</sup>	72 <sup>†</sup>	—	220
Mean age 24y	31	CD3+Tetramer+ <sup>*</sup>	56 <sup>†</sup>	31 <sup>†</sup>	—	—	286
Age range 15 – 52y	30	CD3+Tetramer+ <sup>*</sup>	41 <sup>†</sup>	25 <sup>†</sup>	85 <sup>†</sup>	3.5 <sup>†</sup>	218
Young, mean age 29y	19	CD3+V $\alpha$ 24+V $\beta$ 11+	17 <sup>†</sup>	29 <sup>†</sup>	—	—	223
Elderly, mean age 74y	30	CD3+V $\alpha$ 24+V $\beta$ 11+	44 <sup>†</sup>	28 <sup>†</sup>	—	—	223

<sup>\*</sup> 'Tetramer' refers to  $\alpha$ -GalCer-loaded CD1d tetramer

<sup>†</sup> Expressed as mean, not median

### 7.2.3 CD1d expression

In section 4.3.1, I investigated CD1d expression on subsets of PBMCs from patients with CLL. I quantified CD1d expression using flow cytometry. Compared to real-time reverse transcription quantitative polymerase chain reaction (RT-PCR), flow cytometry has the advantage of assessing expression at the protein level, and unlike both real-time RT-PCR and Western blotting, allows simultaneous analysis of expression on multiple cell types within mixed PBMCs, without need for cell purification. Flow cytometric quantification of surface and intracellular markers is, however, susceptible to systematic errors. I have outlined common sources of error and their possible solutions in table 7.4.

Having observed the precautions suggested in table 7.4, I have confidence in the findings presented, but would still consider expression quantification by flow cytometry to be a semi-quantitative rather than fully quantitative technique.

#### 7.2.3.1 Expression of CD1d by CLL cells

Downregulation of MHC Class I has long been recognised as a mechanism of immune evasion in cancer,<sup>479,480</sup> and has been reported on leukaemic cells in CLL.<sup>481</sup> CD1d is a MHC Class I-like,  $\beta_2$ M-associated molecule, and may also be downregulated in B cell malignancies: patients with unmutated IgVH CLL exhibited lower CD1d expression than those with mutated IgVH,<sup>417</sup> while in myeloma, progressive disease is associated with loss of CD1d expression on the malignant B cells.<sup>446</sup> I therefore chose to analyse CLL cell CD1d expression.

My observation that CD19+CD5+ leukaemic cells expressed CD1d at a similar level to that of normal B cells (figure 4.1b) was consistent with reported findings.<sup>417</sup> Although I did not observe a progressive decline in CD1d progression among patients with advanced CLL (figure 4.1c), it is possible that a difference would emerge if a larger group of patients with more advanced disease were studied.

#### 7.2.3.2 Number and CD1d expression of mDC

**Numbers of mDC** The reported defects of DC from patients with CLL have been discussed in section 1.1.3.5. One report suggested that mDC are reduced in patients with CLL, but notably, this study expressed mDC as a proportion of total PBMCs, the denominator including the very large numbers of leukaemic cells—absolute numbers of circulating mDC were not given.<sup>78</sup>

I used absolute full blood counts performed on the same day as PBMC cryopreservation to derive figures for the absolute numbers of circulating mDC (figure 4.2b). This showed normal absolute numbers of mDC in patients with untreated CLL, although I found a significant reduction among those who had received fludarabine chemotherapy.

The significance of the reduction among fludarabine-treated patients is unclear: fludarabine has not been previously reported to cause a durable decline in mDC numbers. Indeed, in one longitudinal study of patients with the indolent B cell malignancy hairy

cell leukaemia, mDC numbers were low pre-treatment and *recovered* after chemotherapy with cladribine, a purine analogue similar to fludarabine.<sup>482</sup> As this was a cross-sectional rather than longitudinal study, the observed reduction in mDC among fludarabine-treated patients could be due to disease or immunological characteristics rather than an effect of the treatment *per se*.

**CD1d expression on mDC** The expression of CD1d on *in vitro* generated human moDC is affected by culture conditions, the presence of TGF- $\beta$ <sup>483</sup> and the absence of IgG<sup>448</sup> being associated with reduced CD1d levels. Raised serum TGF- $\beta$ <sup>102</sup> and reduced serum IgG levels<sup>65</sup> have both been described in patients with CLL. In view of this, I had hypothesised that CD1d expression would be reduced on circulating mDC from patients with CLL.

Contrary to my hypothesis, I found normal CD1d expression levels on patient mDC, with no evidence of a relationship between mDC CD1d expression and serum IgG levels (figure 4.3). Thus, at least in patients with untreated CLL, mDC are normal in number and express CD1d at normal levels, suggesting that they may retain the capacity to present  $\alpha$ -GalCer to iNKT cells.

### 7.2.3.3 Number and CD1d expression of monocytes

Monocytes may be important for the maintenance of the leukaemic clone in CLL: the addition of monocytes supports the survival of CLL cells *in vitro*, an effect which may be mediated by soluble CD14,<sup>331</sup> while monocytes can inhibit rituximab-induced antibody-dependent cellular cytotoxicity (ADCC) against CLL cells.<sup>484</sup>

Data on the numbers of circulating monocytes in CLL are conflicting, one study indicating higher monocyte numbers among patients with CLL and high circulating leukocyte counts,<sup>449</sup> while another describes normal monocyte numbers.<sup>485</sup>

My data indicate that CD14hi monocytes are normal in number in CLL, but there was a non-significant trend towards an increase in the number of 'inflammatory' CD16+ monocytes in patients, especially in those who had received a fludarabine-based chemotherapy (figure 4.4). If confirmed with a larger cohort, this would be in keeping with reports of increased CD16+ monocytes in patients with other malignancies, such as gastric cancer, breast cancer and cholangiocarcinoma.<sup>453,454,457</sup>

Examining CD1d expression levels, the CD16+ monocytes expressed CD1d at a significantly lower level than their CD14hi counterparts (figure 4.5). However, even among patients with untreated CLL, the majority of monocytes were CD14hi, so the overall level of monocyte CD1d expression was similar to that in controls. The finding of differential CD1d expression of these monocyte subsets has not previously been reported, and it may be of interest to determine whether the capacity of 'inflammatory' CD16+ monocytes to activate iNKT cells is altered relative to that of the major CD14hi subset.

The CD16+ monocyte subset constitutes around 10% of monocytes in healthy individuals, and is expanded in infections and in patients with cancer.<sup>486</sup> The functional role of CD16+ monocytes has not been well defined, in part because mice lack a CD16 (Fc $\gamma$ RIII) expressing monocyte population. However, human CD14loCD16+ monocytes

express high levels of the fractalkine receptor CX3CR1, in contrast to CD14<sup>hi</sup>CD16<sup>-</sup> monocytes, which have the opposite phenotype.<sup>487,488</sup> In mice, CX3CR1<sup>+</sup> monocytes infiltrate the peritoneum during ovarian carcinoma progression, and suppress naive CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses.<sup>489</sup> It is not clear whether or not the CX3CR1-expressing population of circulating monocytes (CD16<sup>+</sup> in humans) generates myeloid-derived suppressor cells.

These results have some potential limitations. The flow cytometric identification of CD16<sup>+</sup> monocytes can be complicated by contamination with NK cells or granulocytes. Addition of an anti-MHC Class II antibody to the monocyte identification panel can overcome this (class II being highly expressed by monocytes, but not by NK cells).<sup>490</sup> In my study, granulocytes are excluded by the density centrifugation step prior to PBMC storage, and should additionally be excluded by the forward and side scatter gate. My preliminary experiments indicated that monocytes identified by forward and side scatter were > 95% MHC Class II positive. I excluded CD14 low events from the CD16<sup>+</sup> gate (figure 4.5a), which should exclude any NK cells which have entered the monocyte gate. Together, these measures should have limited NK cell or granulocyte contamination. However, MHC Class II was not routinely included in my monocyte identification panel, so I cannot exclude a small number of contaminating events. Finally, as I have reported CD1d expression levels as median, rather than mean, fluorescence intensities, the presence of a minor population of contaminating cells should not affect reported expression levels.

The effect of fludarabine on monocyte subpopulations has not been reported. However, total monocytes are modestly reduced after fludarabine-containing chemotherapies, as might be expected for a myelosuppressive chemotherapy.<sup>175</sup> The monocytic cell line U937 undergoes apoptosis upon exposure to fludarabine, but in response to lower fludarabine concentrations, upregulates ICAM-1 and produces IL-8.<sup>491</sup>

Other factors influence CD16<sup>+</sup> monocyte numbers, including exercise (which temporarily raises CD16<sup>+</sup> monocyte numbers), exogenous corticosteroids (which reduce CD16<sup>+</sup> monocyte numbers) and gender (women exhibiting lower CD16<sup>+</sup> monocyte numbers).<sup>490</sup> In this study, all subjects were at rest immediately before phlebotomy and donors taking systemic corticosteroids were excluded. However, there was a greater proportion of men in the fludarabine-treated CLL patient group (table 3.1), which could have contributed to the finding of higher CD16<sup>+</sup> monocyte numbers in this group.

Finally, it is not possible to attribute any of the observed changes in monocyte subsets in fludarabine-treated patients to immunological or disease characteristics or to the treatment itself; this would require a longitudinal study.

These limitations notwithstanding, I have demonstrated that monocyte numbers are retained in CLL, that overall monocyte CD1d expression is intact, and have made the interesting observation that CD1d expression differs between CD14<sup>hi</sup> and CD16<sup>+</sup> monocyte subsets, a finding which has not previously been reported.

### 7.2.4 Function of iNKT cells

In chapter 5, I used a number of assays to evaluate the function of iNKT cells from patients with CLL. The cytokine production and proliferative capacity of iNKT cells within whole

Table 7.4 Sources of error in quantitative flow cytometry

Source of error	Solution(s)
Autofluorescence	Avoid fluorophores which emit in autofluorescence-prone channels (e.g. FITC) Measure FMO fluorescence in identically treated and gated cells, and subtract from result (i.e. report net fluorescence)
Inaccurate compensation	Rigorous use of FMO controls Measure FMO fluorescence in identically treated and gated cells, and subtract from result (i.e. report net fluorescence)
Variable staining conditions	Handle all samples identically Stain and analyse all samples within the same session
Variations in cell number	Count cells and stain identical cell numbers for each sample Use non-limiting dilution of antibody
Variable background staining	Use blocking reagents (e.g. polyclonal IgG) to minimise non-specific binding
Skewing of results by contaminating cells	Report fluorescence intensities as median rather than mean fluorescent intensity Analyse pure cell populations, or gate carefully on cell of interest



PBMCs were assessed. I went on to generate iNKT cell lines from patients and controls, and characterised their immunophenotype, cytokine profile and cytotoxicity.

### 7.2.4.1 Cytokine production by iNKT cells

A reduction in IFN- $\gamma$  production by iNKT cells has been documented in patients with advanced prostate cancer and myeloma.<sup>304,307</sup> I hypothesised that a similar defect may exist in patients with CLL.

**$\alpha$ -GalCer induced IFN- $\gamma$  ELISpot assay** Figure 5.2 demonstrates intact IFN- $\gamma$ -induced ELISpot formation in patients with CLL. In patients with myeloma,  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpots were reduced in patients with progressive disease.<sup>307</sup> Few of the patients I studied by ELISpot had advanced or treated CLL, although ELISpots remained detectable in three patients who had clinically advanced CLL (Binet clinical stage B or C) at the time of analysis, and in three separate patients who had previously received a fludarabine-based chemotherapy.

Preliminary experiments had indicated that rigorous titration, and the use of a 'resting period' after B-cell depletion of PBMCs, were necessary to obtain ELISpot results within a measurable range and to reduce background spot formation. Compared with the measurement of intracellular cytokines, the ELISpot assay has a relatively narrow 'dynamic range', with the capacity to reliably count between around 20 and 400 ELISpots per well. This may have the effect of 'clipping' the results: some donors with negative results might have had detectable numbers of ELISpots had higher cell numbers of cells per well been used, and some donors may have generated more ELISpots than the automated counter could reliably detect. In these cases, titrations with lower numbers of cells per well would have resolved further differences. I did not have sufficient PBMCs to perform ELISpot assays at numerous titration levels for each donor, so selected a single number of cells per well for all donors. As such, ELISpot could be considered a semi-quantitative rather than fully quantitative assay.

The ELISpot results indicated that  $\alpha$ -GalCer-induced IFN- $\gamma$  production is intact in the PBMCs of patients with CLL. This result could be considered at odds with the data presented in figure 3.7, which indicate that iNKT cell numbers are reduced as a proportion of T cells in patients. However, as discussed above, ELISpot can be considered semi- rather than fully quantitative. Moreover, the identity of the IFN- $\gamma$ -producing cell types is not determined by ELISpot. Thus, iNKT cells may be activated by  $\alpha$ -GalCer, and induce neighbouring cells (such as 'conventional' T cells or NK cells) to produce IFN- $\gamma$ . As will be discussed in the next section, IFN- $\gamma$  expression of T cells is *increased* in CLL, thus the higher number of ELISpots than expected may in part be due to enhanced secondary production of IFN- $\gamma$  by admixed T cells in this group.

Despite its limitations, I consider the demonstration of  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpot production important, because it implies that the processes of  $\alpha$ -GalCer presentation by APCs, recognition by iNKT cells and downstream production (or induction) of cytokine release are all intact in patients with CLL. Moreover, ELISpot provides an indication of

$\alpha$ -GalCer-induced cytokine production in mixed PBMCs which is independent of the flow cytometric identification of iNKT cells.

**Intracellular cytokine staining** Unlike ELISpot, intracellular cytokine staining (ICS) allows immunophenotypic characterisation of individual cytokine-producing cells. I used a seven colour flow cytometry panel to simultaneously characterise IFN- $\gamma$  and IL-4 production of iNKT and T cell subsets following stimulation with PMA and ionomycin.

Whereas cytokine-producing T cells predominantly produced either IFN- $\gamma$  or IL-4, but not both, the majority of IL-4 producing iNKT cells also produced IFN- $\gamma$  (figure 5.3a). Dual production of cytokines by iNKT cells has been demonstrated in both mice<sup>492,493</sup> and humans,<sup>461,494</sup> and contrasts with the Th1 and Th2 paradigm of ‘conventional’ T helper cells, in which Th1 and Th2 differentiation are regarded as mutually exclusive pathways. Unlike T helper cells, murine NKT cells activate the transcription of IL-4 and IFN- $\gamma$  early during thymic development, and harbour high levels of mRNA transcripts for both cytokines.<sup>495</sup> The molecular mechanisms underlying this have been discussed elsewhere.<sup>221,240</sup>

Overall, the proportion of iNKT cells which produced IFN- $\gamma$  was higher than the proportion of T cells producing this cytokine (figure 5.3b). This is in keeping with published reports.<sup>220</sup> Considering the CD4+ and CD4- subsets of iNKT cells separately, my observation of increased IL-4 production in CD4+ iNKT cells was also in keeping with published findings.<sup>218,220</sup>

The observation that in patients with CLL a larger proportion of T cells expressed IFN- $\gamma$  on ICS than in controls (figure 5.5a), is consistent with prior reports.<sup>458,460</sup> In contrast, I found no difference between patient and control T cell expression of IL-4. This appears to be at odds with some reports which describe increased T cell IL-4 production in CLL.<sup>95,496</sup> Possible explanations for this include the degree of leukaemic cell depletion, which was more thorough in my study than in those showing increased T cell IL-4 production: IL-6 produced by residual CLL cells may induce T cell IL-4 production.<sup>334</sup> Another factor may be disease stage: one study suggested that IL-4 production by T cells is greater in patients with more advanced disease,<sup>458</sup> and the majority of patients in this study had early stage CLL (table 3.1).

In contrast with the differences observed in T cell cytokine production, the expression of both IFN- $\gamma$  and IL-4 by patient iNKT cells was similar to that of controls (figure 5.5b).

One disadvantage of evaluating cytokine profile by ICS on PMA and ionomycin-stimulated PBMCs is that the T cell stimulation is far from physiological—the cytokine profile elicited may not reflect that observed on stimulation with a peptide (or glycolipid) ligand. Nonetheless, my findings by ICS are consistent with those observed by ELISpot, and together these two techniques provide evidence that the capacity of iNKT cells to produce IFN- $\gamma$  and IL-4 is grossly intact in the peripheral blood of patients with CLL.

### 7.2.4.2 Proliferation of iNKT cells

In section 5.3.3, I evaluated the proliferative capacity of iNKT cells from patients with CLL. The *in vitro* proliferation of iNKT cells is reduced in healthy elderly individuals<sup>441</sup> and in patients with advanced prostate cancer,<sup>304</sup> while it appears to be intact in patients with melanoma<sup>497</sup> and myeloma.<sup>307</sup>

To assess iNKT cell proliferation, I added  $\alpha$ -GalCer followed by IL-2 to B-cell depleted PBMCs. The rationale of using PBMCs rather than purified T cells was to simultaneously assess the integrity of  $\alpha$ -GalCer presentation by patient APCs, recognition by iNKT cells, and the downstream proliferative response. The choice of added cytokine, a high concentration of IL-2, was made because preliminary experiments had indicated that this resulted in optimal early iNKT cell proliferation (figure 5.7).

Proliferation of iNKT cells was similar in patients and controls, with nearly all patients, and all controls, exhibiting some degree of iNKT cell expansion (figure 5.8). Fold proliferation of iNKT cells was similar in the two groups. The *in vitro* culture conditions employed favoured proliferation of the CD4+ subset of iNKT cells in both patients and controls (figure 5.9), a finding which has previously been observed in healthy donor PBMCs.<sup>463</sup>

The presence of CLL cells in *in vitro* culture systems is typically associated with inhibition of T cell proliferation,<sup>94</sup> an effect which may be mediated by CLL cell expression of CD200,<sup>498</sup> production of IL-6,<sup>91</sup> or secretion of soluble IL-2 receptor.<sup>499</sup> I hypothesised that the addition of CLL leukaemic cells would inhibit iNKT cell proliferation.

In contrast, my findings showed that patient iNKT cells were able to proliferate in whole PBMC cultures, without prior B cell depletion (figure 5.11). Moreover, the addition of CLL cells appeared to enhance rather than reduce iNKT cell proliferation in these *in vitro* conditions (figure 5.12a). This is in keeping with a finding that depletion of CD19+ B cells from human PBMCs resulted in impaired *in vitro* iNKT cell proliferation, and that  $\alpha$ -GalCer-loaded B cells are able to induce iNKT cell proliferation.<sup>269</sup>

The presentation of  $\alpha$ -GalCer by B cells rather than by mDCs ('professional' APCs) has been associated with increased production of IL-4 by iNKT cells.<sup>246</sup> Given that CD4+ iNKT cells are associated with IL-4 production (figure 5.4), I analysed the CD4 status of iNKT cells after proliferation in the presence of various numbers of added CLL cells, and did not find any difference (figure 5.12b).

Together, these findings demonstrate that iNKT cells within the PBMCs of patients with CLL retain their capacity to proliferate *in vitro*, and indicate that iNKT cell proliferation is not impaired by the presence of CLL cells.

### 7.2.4.3 Generation of iNKT cell lines

Having demonstrated the capacity of iNKT cells from patients with CLL to proliferate, I wished to generate iNKT cell lines to facilitate further investigation of iNKT cell function.

Table 7.5 lists published methods for the generation of polyclonal iNKT cell lines. Some protocols begin with initial iNKT cell expansion within PBMCs, using  $\alpha$ -GalCer together with one or more gamma chain cytokines. This is followed by iNKT cell selection using immunomagnetic bead or FACS, followed by a series of restimulations to expand iNKT cell numbers further. Other reported methods omit the initial expansion of iNKT cells within whole PBMCs: iNKT cells are instead enriched from PBMCs directly using immunomagnetic selection or FACS,<sup>276,500</sup> or are specifically stimulated by the addition of autologous or artificial APCs to PBMCs or anti-CD3 mAb.<sup>477,500,501</sup>

I chose to employ initial iNKT cell expansion within whole PBMC, because the starting proportion of iNKT cells was very low in both patient and control groups (figure 3.7), and because I had already demonstrated that iNKT cell proliferation could be successfully achieved in PBMCs in this cohort (figure 5.8). Following iNKT cell expansion, FACS was used to purify the iNKT cell population to a relatively high purity (figure 5.14).

I evaluated several potential feeder cells for their capacity to support iNKT cell proliferation, and found that the C1R cell line was as effective as autologous PBMC (figure 5.13). I chose to use the C1R cell line because the availability of patient autologous PBMCs was limited. Interestingly, CD1d expression by the C1R cell line was not required to support iNKT cell expansion, a finding which might be accounted for by the presence of alternative iNKT cell stimuli, such as KIR mismatches between the C1R cell line and the donor iNKT cells, or by low levels of CD1d expression on the untransfected C1R cell line. Nonetheless, I chose to use the CD1d-transfected C1R cell line as a feeder cell for iNKT cell expansion.

Maintenance of the polyclonal iNKT cell lines was hampered by declining iNKT cell purity after two restimulations (figure 5.15). The contaminating cells were a mixture of ‘conventional’ CD4+ and CD8+ T cells and NK cells, suggesting that the feeder cell line used, CD1d-transfected C1R, was stimulating these subsets. Although characterised as a HLA class I-low cell line, the C1R cell line does express the HLA-Cw4 protein and low levels of HLA-B35,<sup>502</sup> as well as expressing MHC class II. It is not surprising, therefore, that the C1R cell line can stimulate ‘conventional’ CD4+ and CD8+ T cells, as well as stimulating NK alloreactivity.

Purity of the polyclonal iNKT cell lines was readily re-established by repeat FACS. However, the number of iNKT cells in culture began to decline after a fourth restimulation (figure 5.15). From the start of PBMC culture to the end of the fourth restimulation, iNKT cells expanded a median of 9352 times, corresponding to just over 13 iNKT cell population doublings:  $\log_2(9352) \approx 13$ . Under optimal *in vitro* conditions, human T cells would be expected to achieve a much higher number of population doublings. The older age of the donors in this study is not likely to be the explanation for this, as T cells from elderly individuals have been shown to be capable of achieving > 30 population doublings *in vitro*.<sup>503</sup> Rather, the failure of iNKT cells to maintain expansion in my cultures may have been due IL-2 induced apoptosis, a phenomenon which has long been described in ‘conventional’ T cells,<sup>504</sup> and which may be due to upregulation of Fas ligand on antigen- and IL-2-stimulated T cells.<sup>505</sup>

Improved viability, and therefore larger iNKT cell numbers, may have been obtained by selecting alternative cytokines: The viability of cultured T cells has been reported to be higher in the presence of IL-7 rather than IL-2.<sup>506</sup> As outlined in table 7.5, and as my

data confirm (figure 5.7), both IL-7 and IL-15 can support iNKT cell proliferation *in vitro*. Compared with IL-2, IL-7 and IL-15 may also favour the expansion of CD4<sup>-</sup> iNKT cells, a subset associated with anti-tumour activity.<sup>433</sup>

The experiments presented demonstrate the feasibility of generating iNKT cell lines from the peripheral blood of patients with CLL. Although the optimisation of purification, re-stimulation and expansion methods could lead to higher iNKT cell line purity and numbers, and to cell lines with a different cytokine profile, such comparisons were beyond the immediate scope of this project. I proceeded to characterise the iNKT cell lines I had generated with the method outlined above.

### 7.2.4.4 Phenotype and cytokine profile of iNKT cell lines

The iNKT cell lines generated were overwhelmingly CD4<sup>+</sup> (figure 5.16), a finding which has previously been reported.<sup>463,472</sup> The selective expansion of CD4<sup>+</sup> iNKT cells in the presence of IL-2 may be explained by published observations that expression of the IL-2 receptor  $\alpha$  chain, CD25, is higher on the CD4<sup>+</sup> than the CD4<sup>-</sup> subset of iNKT cells.<sup>219,220</sup>

In contrast with the cytokine profile of circulating iNKT cells (figure 5.3), the iNKT cell lines generated from both patients and controls exhibited dual expression of both IFN- $\gamma$  and IL-4 (figure 5.17) on ICS. This property is consistent with the CD4<sup>+</sup> immunophenotype of the iNKT cell lines, and has previously been described.<sup>463,516</sup>

In order to demonstrate  $\alpha$ -GalCer reactivity of the iNKT cell lines generated, and to further assess their cytokine profile, I added the CD1d-transfected C1R cell line to iNKT cell lines in the presence or absence of  $\alpha$ -GalCer, and determined cytokine levels in the supernatants (figure 5.18).

Although the treatment of C1R-CD1d cells with  $\alpha$ -GalCer led to a marked enhancement of cytokine production in co-cultures with iNKT cells, a baseline level of cytokine production was seen in the presence of vehicle-treated C1R-CD1d cells. Several explanations could account for this. First, vehicle-treated C1R-CD1d cells could have presented weak endogenous glycolipids to iNKT cells, resulting in low-level iNKT cell activation. Second, vehicle-treated C1R-CD1d cells could have partially activated iNKT cells via KIR mismatches, as iNKT cells are known to express functional NK receptors.<sup>228</sup> Third, the polyclonal iNKT cell lines could be contaminated with 'conventional' T cells able to recognize peptide antigens presented by MHC on the C1R cells (as discussed in section 7.2.4.3, C1R cells express MHC Class II and some Class I antigens). Fourth, the polyclonal iNKT cell lines could be contaminated with NK cells able to recognize mismatching KIR ligands on the C1R cells. Finally, contaminants within the cultures such as bacterial endotoxins could have induced spontaneous cytokine production. As the increase in cytokine production in the presence of  $\alpha$ -GalCer was unequivocal, I did not explore the background levels of production further. However, if investigation were critical, repetition of the experiment with the following additional controls could be informative: iNKT cells alone (in the absence of C1R cells), untransfected C1R cells and  $\alpha$ -GalCer-treated C1R-CD1d cells in the presence of a blocking anti-CD1d antibody.

The finding of the Th2-type cytokines IL-4 and IL-13 in the supernatants of the  $\alpha$ -GalCer-

Table 7.5 Methods of polyclonal iNKT cell line generation

Step	Method	Advantages and disadvantages	Reference(s)
Initial stimulation	$\alpha$ -GalCer addition to whole PBMCs	Enriches iNKT cells prior to purification step, below	507-510
	IL-2	Favours CD4+ iNKT cells	463
Cytokine addition	IL-7	Favours CD4- iNKT cells	463,511
	IL-15	Enhanced cytotoxicity, favours CD4- iNKT cells	463,511
	FACS	High purity of sorting, requires specialised equipment, infection control issues	509,510,512,513
iNKT purification	Immunomagnetic separation	Little specialised equipment required, can be performed in sterile hood, purity lower than FACS	509
	Selective restimulation only	Requires no purification steps, significant contamination with non-iNKT cells possible	514
Restimulation	Autologous APC	No stimulation of NK or T cells, availability of cells may be limited	307,476,477,509,515
	CD1d-transfected cell line	Readily available, may also stimulate NK and T cells	276,509
	Anti-CD3 mAb	Readily available, non-selective (will also stimulate T cells)	509,512
Restimulation	Plate-bound anti-iNKT cell mAb (6B11 clone)	Readily available, no requirement for donor APCs	516
	CD1d-Ig coated artificial APCs	Readily available, no requirement for donor APCs	501

treated co-cultures alongside the Th1-type cytokines IFN- $\gamma$  and TNF- $\alpha$  was consistent with the intracellular cytokine findings.

In contrast with the high levels of IL-4 and IL-13 production, production of the immunosuppressive cytokine IL-10 by the iNKT cell lines was not observed. Although murine iNKT cells have been shown to produce IL-10 directly,<sup>241</sup> IL-10 production by human iNKT cells has not been demonstrated. However, human iNKT cells are able to induce the secondary production of IL-10 by APCs.<sup>262</sup>

Production of GM-CSF by human iNKT cells has been demonstrated, and may be of importance in normal haematopoiesis: a proportion of haematopoietic progenitor cells express CD1d, and the addition of human iNKT cells results in a dose-dependent increase in myeloid colony formation *in vitro*.<sup>513</sup> My data indicate that both patient- and control-derived iNKT cell lines produced similar amounts of GM-CSF in an  $\alpha$ -GalCer-dependent manner (figure 5.18).

Although a subpopulation of murine iNKT cells produce IL-17,<sup>241</sup> production of IL-17 by human iNKT cells has never been conclusively demonstrated. Rather, human IL-17 producing cells appear to be non-invariant CD161+ NK-like T cells.<sup>517</sup> My data showing the absence of IL-17 production by both patient and control-derived CLL cell lines are consistent with this.

In conclusion, the surface immunophenotypic and intracellular cytokine properties of *in vitro* generated iNKT cell lines appear to differ from those of iNKT cells analysed directly *ex vivo*. Nonetheless, the finding that iNKT lines can be generated from patients with CLL, and have similar characteristics to those generated from healthy controls, suggests that critical impairments of iNKT cell proliferative capacity and cytokine production are not present in patients with CLL.

### 7.2.4.5 Cytotoxicity of iNKT cells

The expression of granzyme B by *in vitro* generated iNKT cell lines (figure 5.19) is consistent with the known effects of IL-2, which leads to upregulation of granzyme expression by NK and by T cells.<sup>518</sup>

Using a LDH release assay, I was able to demonstrate  $\alpha$ -GalCer and CD1d-dependent lysis of a C1R cell line (figure 5.20). This demonstrates that the iNKT cell lines generated exhibit antigen specificity, and that their cytotoxic function is intact.

Although specific, degree of cytotoxicity seen was relatively modest in this experiment. A number of reasons may account for this. First, the effector:target ratios I used were modest, in part because of restrictions on the number of effector cells available from preceding iNKT cell line expansion. Second, the LDH release assay may not be the most sensitive assay available—alternative cytotoxicity assays will be discussed in section 7.2.5.1. Finally, the iNKT cell lines, which I derived in the presence of IL-2, were overwhelmingly CD4+, and may not be the optimal cytotoxic effectors—CD4- iNKT cells are reported to exhibit greater cytotoxicity than their CD4+ counterparts.

Several molecular mechanisms have been implicated in cytotoxicity by iNKT cells, as previously discussed in section 1.2.5. The LDH release assay measures completed lysis of tar-

get cells,<sup>519</sup> and does not therefore distinguish between apoptosis or necrosis. Therefore, any or all of the described mechanisms of iNKT cell cytotoxicity may be operating.

The calculation method I employed takes account of spontaneous LDH release from both effector and target cells, and as such should provide a robust measure of cytotoxicity.<sup>438</sup> However, the LDH release assay carries the disadvantage that relatively high numbers of viable target (and therefore effector) cells were required to yield detectable supernatant LDH concentrations. In conjunction with the fall in iNKT cell line numbers after repeated restimulation (figure 5.15b), this prevented the assessment of cytotoxicity by all cell lines generated. Alternative methods of detecting target cell killing may have overcome this limitation, and will be discussed in section 7.2.5.1.

As a consequence of their prolonged culture in the presence of IL-2, the cytokine production and cytotoxicity of iNKT cell lines may not reflect those of *ex vivo* iNKT cells. The scarcity of iNKT cells in patient and control PBMCs prohibited direct assessment of *ex vivo* cytotoxicity. However, the expression of IFN- $\gamma$ , IL-4 and granzyme B by *ex vivo* patient iNKT cells appeared to be intact (figures 5.5b and 5.6), and my data do offer a proof of principle that the generation of iNKT cell lines of high purity and with specific cytotoxicity against CD1d positive targets is possible from CLL patient PBMCs.

### 7.2.5 Potential roles of iNKT cells in immunotherapy

In chapter 6, I used a series of *in vitro* assays to assess various methods of exploiting iNKT cells for the adoptive immunotherapy of CLL. In this section, I shall discuss my experimental findings, and relate them to findings in preclinical and clinical studies.

#### 7.2.5.1 iNKT cells as effectors

**Cytokine production** One proposed method of utilising iNKT cells for immunotherapy is to administer  $\alpha$ -GalCer systemically, and to rely on iNKT cell-derived cytokines to trans-activate other effectors, such as NK cells, as discussed in section 1.2.6.4.

In a preliminary experiment,  $\alpha$ -GalCer induced the production of IFN- $\gamma$  and IL-13 when added to the PBMCs of donors with high frequencies of circulating iNKT cells (figure 6.1).

In a larger group of controls, but not in patients,  $\alpha$ -GalCer addition to cultured PBMCs led to a significant increase in IFN- $\gamma$  production. There was a non-significant trend towards increased IL-10 and IL-4 levels in the supernatant of  $\alpha$ -GalCer-treated PBMCs from both controls and patients (figure 6.2). This experiment showed no evidence of  $\alpha$ -GalCer-induced production of GM-CSF, TNF- $\alpha$  or IL-13.

The failure to demonstrate  $\alpha$ -GalCer-induced IFN- $\gamma$  production in patients may be explained by the observation that iNKT cells constitute a smaller percentage of T cells in patients than controls (figure 3.7). Indeed, in a study using PBMCs from healthy human donors, the addition of previously cultured, activated iNKT cells to a level of at least 0.1% of PBMCs was necessary to elicit a significant effect of  $\alpha$ -GalCer on supernatant IFN- $\gamma$  levels or NK cell activity.<sup>402</sup>



This experimental finding may appear at odds with the  $\alpha$ -GalCer-induced ELISpot data presented in figure 5.2, in which significant  $\alpha$ -GalCer-induced IFN- $\gamma$  production could be observed in both patients and controls. The difference between the findings in the two assays may be statistical chance. However, the two assays do differ in an important manner: ELISpot aims to quantify the number of IFN- $\gamma$ -producing (or -inducing) iNKT cells, whereas the measurement of IFN- $\gamma$  in culture supernatants is also dependent on the amount of IFN- $\gamma$  produced by each cell. Thus, transactivated T or NK cells, for example, might produce higher levels of IFN- $\gamma$  in healthy donor than in patient PBMCs.

Despite the lack of an IFN- $\gamma$  response, a trend towards increased concentrations of the potentially immunosuppressive cytokines IL-4 and IL-10 was seen when  $\alpha$ -GalCer was added to both patient and control PBMCs. The iNKT cell lines I generated did not produce IL-10 upon stimulation, but  $\alpha$ -GalCer-activated iNKT cells have been shown to induce IL-10 production by APCs.<sup>262</sup>

Together, these data suggest that the addition of  $\alpha$ -GalCer to whole PBMCs from patients with CLL is not likely to result in a cytokine milieu favouring activation of innate cells and cytotoxicity, and may instead result in the production of immunosuppressive cytokines.

Clinical data do not lend support to the concept of systemic  $\alpha$ -GalCer administration for the immunotherapy of cancer: In a phase 1 clinical trial of intravenous  $\alpha$ -GalCer administration to patients with solid tumours, only one of 24 patients had a persistent rise in serum IFN- $\gamma$  and IL-12 levels. The one responding patient had a relatively high number of circulating iNKT cells before  $\alpha$ -GalCer administration, at around 1000 iNKT cells/mL blood.<sup>303</sup>

**Maturation of APCs** Next, I looked for evidence that  $\alpha$ -GalCer could induce APC activation in cultures of whole human PBMCs. A preliminary experiment demonstrated  $\alpha$ -GalCer-induced upregulation of CD86 and HLA-DR on monocytes in PBMCs from a donor with a high proportion of iNKT cells. However, this effect was not consistently observed in healthy donors with more typical proportions of iNKT cells (figure 6.3). I concluded that I would be unlikely to be able to demonstrate  $\alpha$ -GalCer-induced APC activation in PBMCs from patients with CLL.

B cells are APCs in their own right, and the administration of systemic  $\alpha$ -GalCer to mice results in early B cell activation.<sup>520</sup> I briefly explored this, by examining B cell activation markers after  $\alpha$ -GalCer treatment of PBMCs from a donor with a high proportion of iNKT cells. This experiment (figure 6.4) demonstrated slight upregulation of B cell CD54 and CD86 expression 60 hours after  $\alpha$ -GalCer administration. The finding that significant B cell upregulation of activation markers was not observed at the earlier eight and 24 hour timepoints, and that class II expression was not upregulated, suggested that any direct effect of iNKT cells on B cell activation within human PBMCs was weak, even when studied using PBMCs from a donor with a high frequency of iNKT cells. I therefore chose not to explore this experimental line further in patients, in whom iNKT cells are typically found at a much lower frequency in PBMCs.

In contrast to the weak effects on  $\alpha$ -GalCer on B cell activation in mixed PBMC cultures, the co-culture of purified iNKT cells with autologous CLL cells at a 1:1 to 4:1 ratio led

to upregulation of CD80 and CD86 on the leukemic cells (figure 6.5). Interestingly, this effect was not  $\alpha$ -GalCer-dependent.

The purified iNKT cell lines used had been generated in the presence of IL-2 and using repeated TCR stimulation, conditions which have been associated with surface CD40 ligand (also referred to as CD40L or CD154) expression by CD4<sup>+</sup> T cells.<sup>464</sup> CD154 expression by T cells can mediate CLL cell activation via CD40.<sup>521</sup> Flow cytometry confirmed that the purified patient iNKT cell lines expressed surface CD154 and that CLL cells expressed CD40 (figure 6.6). The degree of CD154 expression was dim, although I did not assess intracellular CD154 in the iNKT cells, which may have been present at a higher level than surface CD154. My data do not prove that a CD40/CD154 interaction is occurring, but this is one possible explanation for the observed  $\alpha$ -GalCer-independent CLL cell activation by iNKT cells. CD40L (CD154) expression on iNKT cells, and  $\alpha$ -GalCer-independent activation of B cells by iNKT cells have been reported elsewhere—my findings are consistent with this.<sup>522</sup>

**Cytotoxicity** As discussed in section 1.3.3.2, the  $\alpha$ -GalCer-dependent lysis of human CD1d-expressing target cells, including myelomonocytic leukaemia and CLL cells, by iNKT cells has been demonstrated *in vitro*.<sup>229,417</sup> Moreover, pre-clinical studies demonstrate the *in vivo* potential of iNKT cells to eradicate CD1d-expressing lymphoid malignancies.<sup>309,310</sup>

Having demonstrated that a patient-derived iNKT cell line could lyse a CD1d-transfected target cell line (figure 5.20), I performed experiments aimed at demonstrating lysis of leukaemic CLL cells by patient-derived iNKT cells. Using an LDH release assay, I found some evidence of lysis of CLL cells by an autologous iNKT cell line (figure 6.8a). However, lysis was inconsistent when tested in a larger series of effector:target pairs (figure 6.8b). This contrasts with the effective lysis of CD1d-transfected C1R cells by iNKT cell lines (figure 5.20).

A number of reasons may account for the apparent resistance of CLL cells to killing by iNKT cells. Compared to CD1d-transfected C1R target cells, CLL cells express CD1d at a very low level (figure 4.1b and data not shown). As discussed in section 1.2.2.3, iNKT cells express NK inhibitory and activating receptors, and CLL cells are characteristically resistant to NK cell lysis.<sup>523</sup> Unlike C1R cells, CLL cells would be expected to express the inhibitory NK receptor ligand HLA-C. CLL cells have also been reported to express other ligands for inhibitory NK receptors such as HLA-E<sup>523</sup> and HLA-G.<sup>337</sup>

Methodological difficulties may have contributed to my inability to conclusively demonstrate killing of CLL cells by iNKT cells. The cytotoxicity experiments were limited by the inefficiency of lysis of CLL cells by iNKT cells, the limited numbers of iNKT effector cells available from preceding cultures, and the requirement for large numbers of targets (and therefore effectors) using the LDH release assay. I therefore considered ways to enhance the susceptibility of CLL cells to lysis by iNKT cells.

Expression of CD1d on target cells is necessary for *in vivo* lysis by iNKT cells in the presence of  $\alpha$ -GalCer,<sup>310</sup> the level of CD1d expression determining the susceptibility of the target cell to lysis.<sup>309</sup> As CLL cells express CD1d at a relatively low level, I wished to determine whether CD1d could be upregulated, to enhance their susceptibility to lysis.

Upregulation of MHC Class I by IFN- $\gamma$  is well-described, an effect which I was able to confirm in primary CLL cells (figure 6.9b). I hypothesised that IFN- $\gamma$  would also result in upregulation of CD1d on CLL cells, potentially enhancing their susceptibility to lysis by iNKT cells. However, treatment of CLL cells with IFN- $\gamma$  did not result in upregulation of CD1d (figure 6.9c).

A number of other methods for assessing cytotoxicity against CLL cells have been reported, some of which are listed in table 7.6. I performed preliminary experiments using dihexyloxacarbocyanine iodide (DiOC6) and Annexin V staining to detect CLL cell killing by iNKT cells, but these killing assays proved difficult to interpret, the results being highly dependent upon the precise flow cytometric gating strategy used. Flow cytometric detection of apoptotic cells demands careful titration of incubation times, and highly consistent flow cytometric thresholding and gating, as cells which have been reduced to debris would no longer be detectable within a typical lymphocyte forward and side scatter gate by flow cytometry. This could lead to under- or overestimation of cell lysis, depending on the flow cytometric gates or thresholds used.

A more sensitive release assay, such as  $^{51}\text{Cr}$  or calcein AM release, may be more suitable assays for the detection of killing of CLL cells. Like LDH release, these techniques detect completed target cell lysis (irrespective of the mechanism of target cell death), but unlike LDH release, require relatively small numbers of target (and therefore effector) cells. Neither assay relies on flow cytometric gating.

Although I might have been able to demonstrate killing of CLL cells by autologous iNKT cells had I used an alternative killing assay, I do not believe that iNKT cells would prove useful effector cells against CLL cells in clinical practice for several reasons. First, iNKT cells do not specifically lyse leukaemic cells—CLL cells express CD1d at a lower level than normal monocytes and mDCs, and at the same level as normal B cells, suggesting that these non-malignant cells would be at least as susceptible to killing by iNKT cells. Second, lysis of CLL cells by iNKT cells is inefficient, requiring high effector:target ratios to achieve modest degrees of specific lysis. Third, iNKT cells are present at very low frequencies in patient PBMCs, suggesting that their effective use as effector cells would require their expansion, potentially demanding prolonged *ex vivo* culture.

For these reasons, I opted to assess other potential roles of iNKT cells in the cellular immunotherapy of CLL.

### 7.2.5.2 iNKT cells as adjuvants

As outlined in section 1.2.6 and illustrated in figure 1.6,  $\alpha$ -GalCer-activated iNKT cells can act as adjuvants to peptide antigens. The co-administration of a peptide antigen with  $\alpha$ -GalCer results in presentation of both antigens on the same DC. Recognition of  $\alpha$ -GalCer on DC-expressed CD1d by iNKT cells leads to a reciprocal interaction between the DC and iNKT cell that leads to upregulation of costimulatory markers on the DC and production of IL-12.<sup>242,258</sup> This DC activation (or ‘licensing’) enhances ‘conventional’ T cell responses against the peptide antigen co-presented on MHC.<sup>258–260</sup> This adjuvant activity is critical in models of  $\alpha$ -GalCer-based immunotherapy for B cell malignancies, where the administration of  $\alpha$ -GalCer-treated whole tumour cells leads to enhanced CD4+ and

Table 7.6 Methods of assessing cytotoxicity against CLL cells

Method	Comments	Reference(s)
$^{51}\text{Cr}$ release	Gold standard assay Few target and effector cells required Detects completed lysis of targets Utilises toxic radioisotope	337,360,361,524
LDH release	Many target and effector cells required Requires careful controls, as effectors as well as targets can release LDH Detects completed lysis of targets	372
Calcein AM release	Few target and effector cells required Detects completed lysis of targets	337,477
Annexin V staining	Flow cytometric method Few target and effector cells required	525
DiOC6 staining	Flow cytometric method Few target and effector cells required	417,526
Fluorescent caspase substrates	Flow cytometric method Few target and effector cells required Specific for apoptosis induction	337
CD107a upregulation	Measures effector cell degranulation, not killing of target cells	337,527
Granzyme B ELISpot	Measures effector cell granzyme B release, not killing of target cells Sensitive assay	325,366

CD8+ protection against the malignancy.<sup>424,425</sup>

I therefore sought to assess the potential for  $\alpha$ -GalCer as an adjuvant in patients with CLL.

Vaccine recall responses present a simple and potentially clinically-relevant method of assessing responses to a peptide antigen. Initially I used the incorporation of radiolabelled thymidine to assess antigen-specific T cell proliferation.<sup>528</sup> This assesses proliferation in response to antigen, but does not identify the dividing cells within whole PBMCs. An initial experiment suggested that the addition of  $\alpha$ -GalCer can augment recall responses to both influenza and diphtheria/tetanus vaccines, at least in a donor with a high proportion of iNKT cells (figure 6.10).

The diphtheria/tetanus vaccine used (ADT<sup>TM</sup> Booster) contains an aluminium hydroxide adjuvant. To avoid the possibility of enhancement of iNKT cell proliferation by aluminium hydroxide, potentially confounding the analysis, I chose to perform further assays using the influenza vaccine (Vaxigrip<sup>TM</sup>), which comprises formaldehyde-inactivated influenza virus particles, without an added adjuvant. Donors who had been previously vaccinated with the relevant year's influenza vaccine were selected for this analysis.

The use of CFSE to assess lymphocyte proliferation allows simultaneous assessment of the immunophenotype of dividing cells.<sup>529,530</sup> CFSE dilution indicated that the proliferative response of conventional T cells to influenza vaccine was indeed increased by  $\alpha$ -GalCer, but that a reciprocal interaction also occurred: influenza vaccine increased the proliferative response of iNKT cells to  $\alpha$ -GalCer (figure 6.11).

When a larger number of controls and patients were studied, the effect of  $\alpha$ -GalCer on the influenza vaccine recall response was much more mixed, with a statistically significant reduction in <sup>3</sup>H thymidine incorporation after  $\alpha$ -GalCer addition in one patient (figure 6.12). Methodological factors may have affected this result, however: it is notable that the two donors with the most marked proliferative responses in response to vaccine alone showed a trend towards reduced proliferation when  $\alpha$ -GalCer was added. It is possible that the addition of  $\alpha$ -GalCer augmented early T and/or iNKT cell proliferation sufficiently to result in exhaustion of the culture media, and that by the time <sup>3</sup>H thymidine was added to the cultures at day five, the cells were already dying. Further experiments using limiting dilutions of the vaccine, or shorter culture periods, would have to be performed to investigate this possibility.

Possible mechanisms for the enhancement of 'conventional' T cell proliferation in response to  $\alpha$ -GalCer addition include cytokine production in response to  $\alpha$ -GalCer or  $\alpha$ -GalCer-induced maturation of APCs presenting vaccine-derived peptides. The experiments presented here cannot distinguish these two mechanisms. As discussed in section 1.2.4, iNKT cells can produce cytokines such as IL-2 and IL-21, which may directly support the proliferation of 'conventional' T or NK cells, cytokines such as GM-CSF and TNF- $\alpha$  that may activate DCs, and cytokines such as IFN- $\gamma$  and IL-4 that may polarize responding 'conventional' T cells. iNKT cells can also interact with DCs directly, via CD40/CD40L, to activate DCs and enhance immunological responses to co-presented peptide agonists (see section 1.2.6.1). Had the adjuvant effect of  $\alpha$ -GalCer been more consistent, I could have designed Transwell studies to determine the relative contribution of cytokine-mediated and contact-dependent pathways.

Together, the experiments I performed suggest that although  $\alpha$ -GalCer has the potential to augment vaccine recall responses in whole human PBMCs, this effect is small or insignificant in the majority of the older individuals in this study, in whom iNKT cells constitute a very small proportion of T cells.

### 7.2.5.3 CLL cells as a whole tumour vaccine

**Proliferation of iNKT cells in response to  $\alpha$ -GalCer-pulsed CLL cells** Potential sources of TAAs include whole tumour cells, tumour lysates, extracted or synthetic proteins or peptides, and tumour RNA. I chose to employ whole tumour (leukaemic) cells, as these were readily available, incorporate numerous potential antigens, and, as demonstrated in section 4.3.1, express CD1d.

First, I sought to assess whether CLL cells pulsed with  $\alpha$ -GalCer, could induce iNKT cell proliferation. These experiments confirmed that  $\alpha$ -GalCer-pulsed CLL cells led to proliferation of iNKT cells within whole PBMC cultures, both in the autologous and allogeneic settings (figures 6.13a and 6.14). The use of the autologous setting is important, as iNKT cells can express activating KIRs, which contribute to alloreactivity:<sup>228</sup> The finding that autologous  $\alpha$ -GalCer-pulsed CLL cells induce iNKT cell proliferation demonstrates that such KIR signals are not essential for CLL-induced iNKT cell proliferation.

Several potential mechanisms might account for the proliferation of iNKT cells induced by irradiated,  $\alpha$ -GalCer-pulsed CLL cells. First, irradiated CLL cells, which express CD1d, may present  $\alpha$ -GalCer directly to iNKT cells. Second, the irradiated CLL cells may be phagocytosed by other APCs within the PBMC cultures, resulting in re-presentation of the CLL-cell associated  $\alpha$ -GalCer by CD1d-expressing APCs such as mDCs or monocytes. Third, the  $\alpha$ -GalCer may dissociate from the irradiated CLL cells—free  $\alpha$ -GalCer may then be taken up and presented by other APCs within the PBMC cultures.

The data presented in this thesis do not allow differentiation of these mechanisms. Experiments using defined cell populations, for instance assessing proliferation after adding  $\alpha$ -GalCer-pulsed CLL cells to purified autologous iNKT cells, would demonstrate whether irradiated CLL cells are capable of direct  $\alpha$ -GalCer presentation to iNKT cells. The dissociation of  $\alpha$ -GalCer from the CLL cells in culture could be excluded using transwell experiments.

**Effect of  $\alpha$ -GalCer on proliferation of PBMCs in response to allogeneic CLL cells** Having demonstrated that iNKT cell proliferation occurs in response to  $\alpha$ -GalCer-pulsed CLL cells, it was not surprising that  $\alpha$ -GalCer treatment led to increased proliferation of allogeneic PBMCs in response to CLL cells, as assessed by <sup>3</sup>H thymidine incorporation in responding PBMCs (figure 6.15).

In a preclinical model, the combination of  $\alpha$ -GalCer and a TLR ligand results in a substantial potentiation of the immune response.<sup>311</sup> In human PBMCs, TLR ligands have been shown to enhance presentation of  $\alpha$ -GalCer by DCs, and enhance iNKT cell IFN- $\gamma$  production.<sup>434</sup>

CLL cells express a range of TLRs, including TLR7.<sup>370</sup> TLR7 agonists such as loxoribine and the imidazoquinoline S28690 result in upregulation of activation markers on CLL cells.<sup>470</sup> To explore the effect of combining a TLR ligand and  $\alpha$ -GalCer, I used the TLR7/8 agonist, resiquimod. Flow cytometric analyses confirmed that resiquimod leads to CLL cell upregulation of CD80 and CD86, and demonstrated that, while resiquimod led to a significant and consistent increase in MHC Class I and II expression on CLL cells,  $\alpha$ -GalCer had no effect on expression of these markers (figures 6.16 and 6.17).

Resiquimod- and  $\alpha$ -GalCer-treatment of CLL cells appeared to augment proliferation of allogeneic PBMCs in an independent fashion (figure 6.18). Analysing the numbers of T cells in CLL-stimulated PBMCs,  $\alpha$ -GalCer treatment resulted in iNKT cell proliferation (as would be expected), while resiquimod treatment resulted in increased numbers of ‘conventional’ CD4 and CD8 T cells. There was no consistent effect of  $\alpha$ -GalCer treatment on the number of CD4 and CD8 ‘conventional’ T cell numbers in this experiment (figure 6.19).

**$\alpha$ -GalCer treatment of CLL cells leads to increased proliferation of autologous ‘conventional’ T cells** In a subsequent experiment, I examined the effect of  $\alpha$ -GalCer treatment of CLL cells on the *autologous* T cell response using CFSE dilution (figure 6.20). This confirmed that  $\alpha$ -GalCer-treated CLL cells led to proliferation of autologous iNKT cells.

A modest increase in proliferation of ‘conventional’ T cells was also seen in the  $\alpha$ -GalCer-treated group. One explanation for this observation is that the dividing ‘conventional’ T cells represent leukemia-reactive T cells, and that their proliferation is enhanced by  $\alpha$ -GalCer treatment of the leukemic cells. This would be consistent with the findings of preclinical studies of  $\alpha$ -GalCer-treated tumour cells for the immunotherapy of B cell malignancies.<sup>424,425</sup> However, the enhancement of proliferation observed in my experiment was modest, and I was not able to demonstrate anti-tumour activity of the divided T cells due to the very small number of cells available.

An alternative explanation would be that iNKT and CLL cells both enhance background T cell proliferation in the cultures. A modest degree of spontaneous T cell proliferation is seen in the medium-only control (figure 6.20a), and could represent T cell proliferation in response to culture contaminants or to contents of the media, such as human serum. Irradiated CLL cells might enhance this background proliferation through release of stimulatory cytokines, and this effect could be enhanced further by iNKT cell-produced cytokines, such as IL-2, in the  $\alpha$ -GalCer-treated CLL cell group.

The increased proliferation of ‘conventional’ T cells in response to  $\alpha$ -GalCer treatment of CLL cells in the autologous but not the allogeneic setting was unexpected. The impact of NK alloreactivity may provide a possible explanation for the failure to demonstrate an effect of  $\alpha$ -GalCer in the allogeneic setting: In the allogeneic setting, NK alloreactivity would be expected to provide strong early cytokine signals even in the absence of  $\alpha$ -GalCer—iNKT cells, which are far less abundant in PBMCs than NK cells, are redundant. In the autologous setting, however, no NK alloreactivity occurs, so the impact of iNKT cell recruitment by  $\alpha$ -GalCer becomes significant.

### 7.2.6 Strengths and limitations of this study

This is the first study, of which I am aware, to characterise iNKT cells from patients with CLL. The previous section reviewed the findings of this study, discussing the methodology, and placing the findings in the context of the published literature. In this section, I shall consider the design and methodological strengths and weaknesses of this study.

**Participant characteristics and numbers** The frequency of circulating iNKT cells declines with age,<sup>210,223,441</sup> hence the importance of the close age-matching between patient and controls in this study. By excluding patients and controls with active malignancy or who are taking systemic immunosuppressive medications, these potential confounders were avoided. Nonetheless, other confounders may exist: both patients and controls were volunteers, and as such represent self-selecting groups of individuals who had both the capacity and motivation to attend a research institute to provide blood samples.

There is a wide variation in iNKT cell numbers in healthy humans. This requires the analysis of large numbers of individuals to detect statistically significant differences in numbers of iNKT cells between different groups. I recruited sufficient participants in this study to demonstrate statistical significance in the difference in the proportion of iNKT cells (as a percentage of T cells), but did not find significant differences in absolute iNKT cell numbers or in iNKT cell immunophenotype. It is possible that additional differences would have emerged had a larger number of patients and/or controls been studied.

The numbers of patients within the smaller subgroups who had clinically-advanced or fludarabine-treated CLL were small. As such, differences in iNKT cell numbers or function in these groups may have been missed. Nonetheless, this study may be considered to provide a 'proof of principle' that iNKT cells are present and can proliferate in such patients.

Limitations on the numbers of cryopreserved PBMCs, availability of reagents, and time precluded performance of the full suite of functional iNKT cell analyses in all patient and control samples. Had I performed functional analyses on all samples, it is possible that differences in iNKT cell function would have emerged. For example, although I observed no statistically-significant differences in *ex vivo* iNKT cell proliferation between patients and controls, a trend towards reduced proliferation in patients was observed. Had more samples been included in this analysis, it is possible that a statistically-significant difference would have been demonstrated. Nonetheless, although I cannot exclude small differences, the studies of iNKT cell function performed effectively rule out severe defects in iNKT cell function in patients with CLL.

**Cross-sectional design** CLL is an indolent malignancy, the progression from early CLL to advanced disease requiring therapy typically taking many years. As such, time limitations demanded a cross-sectional rather than longitudinal design for this study, taking individual samples from patients at a fixed point in time, rather than serial samples from patients before and after disease progression and treatment. Performing longitudinal studies in such an indolent malignancy would likely require a tissue bank containing large numbers of cryopreserved PBMCs, which was not available.



A longitudinal study would be better suited to the exploration of relationships between CLL progression and iNKT cell numbers, phenotype and function, by allowing the use of more powerful paired or repeated-measures statistical tests to compare iNKT cell parameters before and after disease progression or treatment.

The cross-sectional design of this study does carry an advantage: as a single individual was responsible for the collection of all clinical data, and for the collection, separation and cryopreservation of both patient and control samples, variability in sample handling was minimised.

**Peripheral blood analysis** This study has examined iNKT cells within the peripheral blood. In common with most other translational studies examining T cell responses, I chose to study peripheral blood because of its ready availability, and because it simultaneously provided a source of both viable immune system cells and leukaemic cells suitable for phenotypic and functional analyses.

As discussed in section 1.2.2.1, iNKT cells can be detected within organs, and are particularly enriched in the liver.<sup>212</sup> It is plausible that differential migration of iNKT cells may occur in disease states such as CLL: an apparent reduction of iNKT cells observed in the peripheral blood may actually represent migration of the cells into solid tissues. Published reports offer potential mechanisms for such iNKT cell migration: in mice, iNKT cells express CCR4, which is required for their migration into the lung,<sup>531</sup> while human CLL cells can express CCL22, a ligand for CCR4.<sup>532</sup> Thus, a limitation of this study is that the peripheral blood iNKT cells analysed may not be numerically, immunophenotypically or functionally representative of tissue iNKT cells.

The results of clinical trials fail to clarify the relevance of peripheral blood iNKT cell numbers to the efficacy of iNKT cell-directed immunotherapy: in a phase I study of intravenous  $\alpha$ -GalCer in patients with cancer, cytokine responses to  $\alpha$ -GalCer were restricted to those with the highest pre-treatment numbers of circulating iNKT cells.<sup>303</sup> In contrast, in a phase I/II clinical study of vaccination with  $\alpha$ -GalCer-pulsed APCs, the number of circulating iNKT cells before treatment bore little relation to the number of  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpots after vaccination.<sup>411</sup>

Thus, although peripheral blood provided a convenient source of iNKT cells for analysis, the relevance of the circulating iNKT cells I characterised to the efficacy of any iNKT cell-based immunotherapy remains unclear.

**Applicability of findings from iNKT cell lines** The methodological challenges experienced in the generation of iNKT cell lines have been discussed in section 7.2.4.3. In comparison to circulating iNKT cells, the cell lines generated were biased towards CD4 expression and IL-4 production, suggesting that they are not fully representative of *ex vivo* iNKT cells. However, iNKT cell numbers were too small to allow direct *ex vivo* isolation and functional assessment, and the data on iNKT cell lines does provide a proof of principle that functional lines can be derived from patients with CLL.

As discussed in section 7.2.4.3, is possible that iNKT lines generated in the presence of cytokines other than IL-2, such as IL-7 or IL-15, may have exhibited a different cytokine

profile, or may have exhibited better long-term viability during prolonged culture. However, time and the limited numbers of patient PBMCs available prohibited an exhaustive exploration of iNKT cell line generation.

**Use of PBMCs for functional assays** Many of the functional assays performed used mixed PBMCs, the rationale of which was to model the impact of iNKT cell engagement *in vivo*, where iNKT cells represent a very small proportion of lymphocytes, and where the effects of iNKT cell activation may be modulated by interactions with other cell types.

These assays failed to demonstrate significant cytokine production, APC maturation or vaccine recall response augmentation in response to  $\alpha$ -GalCer. It seems likely that had iNKT cells been present at a sufficiently high frequency, these effects would have been demonstrable, as they were observed in a donor with a high proportion of iNKT cells. However, the aim of these experiments was to help select the most promising means to exploit the iNKT cell/CD1d axis in patients, in whom circulating iNKT cells are rare.

Although mixed PBMCs include a mixed population of lymphocytes and APCs, they exclude a number of cells which may interact with iNKT cells *in vivo*: interactions between iNKT cells and granulocytes,<sup>277,279</sup> hepatocytes,<sup>533</sup> and even atherosclerotic plaques<sup>534</sup> have been described. In addition, the microenvironment of a cell culture well differs substantially from any condition found *in vivo*, in terms of cytokine, hormone and metabolite concentrations, cell motility and concentration, and physical shear. The *in vitro* effect of iNKT cell activation may not, therefore, be representative of that which would be observed *in vivo*. Where possible, I have sought to correlate my *in vitro* findings in human PBMCs with reported *in vivo* findings in mice, and with findings in human clinical studies.

**Effects of  $\alpha$ -GalCer-pulsed CLL cells** I have demonstrated that CLL cells are able to retain  $\alpha$ -GalCer and to induce the proliferation of iNKT cells. It is possible that monocytes or mDCs contaminating the purified CLL cells were responsible for the carriage of  $\alpha$ -GalCer in these experiments. However, the effect was still readily detectable when highly-purified (FACS-selected) CLL cells were used.

That  $\alpha$ -GalCer-pulsed CLL cells can result in iNKT cell proliferation in the autologous as well as allogeneic setting is important, as it suggests a potential immunotherapeutic strategy. Moreover, this effect could be detected after irradiation, washing and cryopreservation of the CLL cells. The rationale of irradiating and cryopreserving the CLL cells in this experiment was to mimic the preparation of autologous tumour cells as a whole tumour vaccine in a putative future phase I study.

Although the enhanced proliferation of 'conventional' T cells in response to  $\alpha$ -GalCer-treated leukaemic cells shown in figure 6.20 could indicate enhanced antitumour responses, it is possible that the proliferating T cells are not tumour-specific, or even that they are tumour-specific but are immunosuppressive rather than cytotoxic. I was unable to functionally characterise the T cells that had proliferated in response to  $\alpha$ -GalCer-treated CLL cells because of the small numbers of cells available, and methodological difficulties with the chosen cytotoxicity assay (LDH release). The limited sensitivity of the

LDH release assay for the assessment of cytotoxicity against CLL cells has already been discussed, and alternative assays discussed in section 7.2.5.1. Nonetheless, the efficacy of  $\alpha$ -GalCer-pulsed tumour cells as a vaccine against B cell and myeloid malignancies has been demonstrated in pre-clinical studies (section 1.3.3.3).

Despite the limitations of this study, this is the first study, of which the author is aware, to assess the number and function of iNKT cells from patients with CLL. It establishes that iNKT cells are detectable in nearly all patients with CLL, including most of those who have previously received fludarabine-containing chemotherapies, that iNKT cell immunophenotype and cytokine profile is intact, and that patient iNKT cells are capable of proliferation. Patient APCs express CD1d at normal levels. This study provides 'proof of principle' that functional iNKT cell lines can be derived from patients with CLL. Through a series of *in vitro* experiments, this study suggests that the low frequency of iNKT cells in patients may limit responses to systemic  $\alpha$ -GalCer, but that iNKT proliferation can be induced by  $\alpha$ -GalCer-treated autologous leukaemic cells. Finally, this study suggests that  $\alpha$ -GalCer treatment of autologous leukaemic cells may enhance the proliferation of non-CD1d restricted T cells in response to tumour.

## 7.3 Cellular immunotherapy of CLL

### 7.3.1 Factors associated with successful cellular immunotherapy

In this section, I shall outline factors which have been associated with successful immunotherapy of cancer in humans.

Clinical trials of therapeutic cancer vaccination and adoptive immunotherapies can offer useful insights. For example, a review of patients who received adoptive transfer of *in vitro*-expanded tumour-infiltrating lymphocytes for the treatment of metastatic melanoma offers some insight into the role of prior lymphodepleting chemotherapy.<sup>535</sup> Adoptive transfer of autologous T cells bearing a chimeric antigen receptor (CAR) directed against CD19 has been used for the treatment of B cell malignancies in phase 1 trials, and although clinical experience to date is limited, a systematic review has identified factors associated with successful treatment.<sup>536</sup>

Allogeneic stem cell transplantation (allo-SCT) has been in routine clinical use for the treatment of haematological malignancies since the 1970s, and been progressively refined as factors associated with long-term disease-free survival have been identified. The large numbers of patients included in registry studies, clinical trials and case series may provide useful indications of the factors associated with successful immunotherapy.

**Susceptible malignancy** Early experience with *in vivo* and *in vitro* use of IL-2 suggested that melanoma and renal carcinoma were the most immunogenic solid malignancies,<sup>537</sup> and the impressive response rates following adoptive immunotherapy of melanoma would seem to validate this.<sup>535</sup> However, some of the most convincing outcome data for therapeutic cancer vaccination are for prostate cancer,<sup>538</sup> and transgenic T cells have shown

promising activity against tumour types that were not traditionally regarded as highly immunogenic, such as ovarian and synovial cell carcinoma.<sup>385,539</sup>

Clinical experience of allo-SCT suggests that some haematological malignancies are more readily eradicated by this procedure than others. Chronic myeloid leukaemia has a particularly high disease-free survival rates after allo-SCT.<sup>540</sup> In contrast, the benefit of allo-SCT for myeloma is more controversial.<sup>541</sup> CLL, like most other indolent B cell malignancies (with the possible exception of myeloma) appears to be susceptible to allo-SCT.<sup>542</sup>

The experience of allo-SCT for the treatment of non-haematologic malignancies, in contrast, has proved disappointing, with response rates to reduced intensity conditioning allogeneic stem cell transplantation (RIC-allo) generally lower than for haematologic malignancies,<sup>543</sup> with the possible exception of metastatic renal cell carcinoma.<sup>544</sup> Possible reasons for the failure of most non-haematologic malignancies to respond to allo-SCT include:

- **Lack of disease remission:** The outcome of allo-SCT for haematopoietic malignancies is superior if the patient is transplanted when their disease is in remission.<sup>545</sup> Whether because of inherent chemoresistance, the inability of chemotherapies to penetrate solid tumours, or other microenvironmental factors, the complete remission rates after chemotherapy for most metastatic solid carcinomas are lower than for most haematological malignancies.
- **Unfavourable cytogenetic features:** the outcome of patients receiving allo-SCT for refractory leukaemia not in remission is particularly poor in those with poor-risk cytogenetic changes.<sup>546</sup> Unfavourable cytogenetic changes may be more frequent in solid tumours: whole genome sequencing suggests that the numbers of mutations in protein-coding regions are greater in solid cancers than in acute myeloid leukaemia.<sup>547</sup>
- **Lack of MHC class II expression:** Most haematopoietic tumours express MHC class II: B cells and myeloid APCs express MHC class II constitutively, while T cells express class II upon activation.<sup>548</sup> In contrast, most non-haematopoietic cells do not express MHC class II (although class II expression has been demonstrated on astrocytes,<sup>549</sup> endothelial cells,<sup>550</sup> activated hepatocytes,<sup>551</sup> melanomas<sup>552</sup> and adipocytes<sup>553</sup>). It is tempting to speculate that following allo-SCT, malignant tumours of haematopoietic origin present tumour-associated antigens on MHC class II as well as on MHC class I, and that this contributes to tumour eradication by donor T cells. Consistent with this notion, the transfection of human carcinoma cells with MHC class II and CD80 allows them to present MHC class II-restricted endogenous antigens to tumour-specific CD4+ T lymphocytes.<sup>554</sup>

Whatever the reasons, some tumours appear more susceptible to eradication by certain immunotherapies than others. It is likely that, as for chemotherapeutic agents, different tumours will respond best to different immunotherapeutic strategies.

**Disease remission status** Many tumour immunotherapy studies are undertaken in patients with active, often bulky, malignancies. However, experience of allo-SCT suggests that disease remission at the time of this adoptive immunotherapy is associated with

markedly improved survival allo-SCT.<sup>545,555</sup> There are numerous potential explanations for this observation:

- Chemosensitivity of the malignancy: malignancies which are resistant to pre-transplant chemotherapies are likely to be resistant to transplant conditioning regimens
- Inherent aggressive behaviour of the malignancy: remission status may be a proxy for poor-risk features, such as adverse cytogenetic changes within the tumour
- Kinetics of tumour growth: rapidly-growing malignancies may be over-represented among cases not in remission, and may progress before donor T cells engraft
- Patient frailty: frail patients, those with co-morbidities or with a history of severe infections may be over-represented among those with refractory disease as they receive less intensive chemotherapy, and may also be at higher risk of transplant-related mortality
- Resistance to immunological eradication: refractory malignancies may be those which lack susceptibility to immunological control, due either to tumour factors (such as MHC Class I downregulation or HLA-G expression) or to features of the tumour microenvironment (such as the presence of intratumoural myeloid-derived suppressor cells)

Whether achieving remission truly makes a subsequent immunotherapy more effective, or whether it is simply a proxy for a treatment-sensitive tumour or a 'fit' patient, is moot. Nonetheless, experience from allo-SCT would suggest that cellular immunotherapies might be most effective in patients who are in remission prior to treatment. The fact that early clinical trials tend to be performed in patients with active malignancy may contribute to the disappointing outcomes reported in clinical trials of therapeutic cancer vaccination to date.<sup>538</sup>

**Sufficient T cell repertoire** The T cell-mediated eradication of malignant cells presumably requires a T cell repertoire capable of recognising at least one TAA. However, with advancing age, human CD4+ and CD8+ T cell populations become skewed and develop oligoclonal expansions.<sup>556</sup> In aging mice, similar changes occur, and result in demonstrable gaps in the T cell repertoire.<sup>557</sup>

The presence of T cell specific for a single tumour-associated antigen is not sufficient for disease control: among patients vaccinated against a melanoma-associated antigen, disease progression was documented even in patients with antigen-specific CD8+ T cells accounting for > 10% of the CD8+ T cell repertoire.<sup>558</sup>

The presence of 'helper' CD4+ T cells as well as 'cytotoxic' CD8+ T cells recognising TAAs may contribute to successful immunotherapy: clinical responses were higher among patients vaccinated with DCs bearing MHC Class I and II epitopes than among patients vaccinated with DCs bearing Class I epitopes alone.<sup>559</sup> One clinical case of dramatic regression of metastatic melanoma following adoptive transfer of MHC Class II-restricted tumour-infiltrating lymphocytes provides proof of principle that CD4+ T cells can have a role in tumour eradication.<sup>560</sup>

Prolonged disease-free survival after allo-SCT requires T cells: in registry studies of allo-SCT, depletion of T cells from the haematopoietic stem cell graft results in significantly higher leukaemia relapse rates after allo-SCT.<sup>561</sup>

Consistent with the notion that a broad T cell repertoire is required for optimal tumour control, the age of human haematopoietic stem cell donors has a significant impact on disease-free survival after allo-SCT: in registry studies, the use of older donors is associated with higher relapse rates in the recipient.<sup>562</sup> One explanation for this would be that haematopoietic stem cell grafts from older donors have relatively skewed and deficient T cell repertoires, and as such are incapable of mounting broad T cell responses against recipient tumours.

One potential pitfall of any *autologous* cellular immunotherapy is the possibility that patients with cancer have deficient T cell repertoires which are incapable of mounting effective antitumour T cell responses. This might be overcome by using allogeneic (including umbilical cord or haploidentical) rather than autologous T cells, by transfecting patient T cells with modified or chimeric T cell receptors, or by redirecting T cells towards the tumour using bispecific antibodies. The adoptive transfer of T cells transfected with either a TCR specific for a TAA, or with a CAR directed against a TAA, can result in clinical remissions, even among heavily pretreated patients with cancer.<sup>385,536</sup>

Serial clinical trials of adoptive transfer of *in vitro*-expanded tumour infiltrating lymphocytes, without a modified TCR, have shown clinical response rates of up to 50% in patients with metastatic melanoma.<sup>535,563</sup> This suggests that some patients with advanced cancer *do* possess a T cell repertoire capable of eradicating their own malignancy following the reversal of an immunosuppressive tumour environment using lymphodepleting chemotherapy and *in vitro* expansion and activation of tumour-infiltrating T cells.

**Lymphodepletion** Lymphodepleting chemotherapy, such as cyclophosphamide and/or fludarabine, may contribute to the efficacy of the subsequent immune response against the malignancy.

In preclinical models, lymphodepletion before adoptive transfer of T cells or vaccination results in enhanced T cell proliferation. Lymphodepletion is associated with homeostatic increases in serum IL-7 and IL-15 levels, which drive proliferation and persistence of the adoptively transferred or antigen-stimulated T cells.<sup>564</sup> As in mice, lymphodepletion of human patients results in raised serum levels of IL-7 and IL-15.<sup>565</sup>

Clinical trials of adoptive T cell therapy for melanoma suggest that response rates are higher if the patient receives lymphodepleting chemo- and radiotherapy before adoptive T cell transfer.<sup>535,536,565</sup> Mechanisms for this effect may include the depletion of immunosuppressive T<sub>reg</sub> cells as well as the induction of homeostatic T cell proliferation. A systematic review of clinical trials of adoptive transfer of autologous T cells transfected with an anti-CD19 CAR for the treatment of B cell malignancies found better clinical responses in studies that used pre-transfer lymphodepletion.<sup>536</sup>

Host lymphodepletion before haematopoietic stem cell administration is a critical component of allo-SCT, being required to prevent host rejection of the allogeneic donor stem cells, so whether this is an important contributor to the subsequent immune response

against the recipient malignancy is uncertain.

A theoretical concern of lymphodepletion prior to therapeutic cancer vaccination is that it may deplete elements of the patient's T cell repertoire necessary for subsequent tumour eradication. Alternatives to lymphodepletion could include the administration of exogenous cytokines to mimic IL-7 and IL-15-driven 'homeostatic' T cell proliferation.

**Disruption of immunological tolerance** Immunological tolerance of malignancies can be mediated by T<sub>reg</sub> cells, MDSCs, by metabolic changes within the tumour microenvironment, or by upregulation of T cell inhibitory molecules, such as CTLA-4 or PD-1.

Several of these mechanisms of tolerance may be amenable to specific disruption in the context of cellular immunotherapy. Depletion of T<sub>reg</sub>s with the anti-CD25 mAb daclizumab enhanced T cell responses to a subsequently-administered TAA in patients with metastatic breast cancer.<sup>566</sup> The chemotherapy drug 5-fluorouracil, routinely used for the treatment of colorectal carcinoma, results in specific depletion of MDSCs in mice.<sup>567</sup> Finally, anti-CTLA-4 mAbs have demonstrated single-agent activity against melanoma, prostate and renal cell carcinoma in clinical trials.<sup>568</sup>

Allo-SCT may disrupt this tolerance through lymphodepletion, chemo- or radiotherapy-induced modification of the tumour microenvironment, and by reconstitution of the recipient immune system with naïve lymphocytes.

**Engagement of innate lymphocytes** T cell signals alone may be insufficient to eradicate tumour: the engagement of innate lymphocyte populations such as NK cells (or perhaps iNKT cells) may be important for effective tumour eradication.

The potential efficacy of NK cells as effectors in tumour immunotherapy has been demonstrated: in a phase I clinical trial, activated alloreactive NK cells administered after host lymphodepletion expanded *in vivo*, and in some cases eradicated refractory acute myeloid leukaemia.<sup>569</sup>

Registry data comparing outcomes of allo-SCT from identical twin (syngeneic) with HLA-identical sibling donors shows that the use of a syngeneic donor is associated with a substantially increased relapse risk.<sup>570</sup> As both types of donor are HLA identical, the implication is that mismatches in other genes, such as minor histocompatibility antigens and/or NK receptors are responsible for the differences in relapse risk. Similarly, in CLL, relapse risk following allo-SCT is lower if an HLA-identical unrelated donor rather than a sibling donor is used.<sup>157</sup> Again, this suggests that factors other than the HLA complex affect the immunological control of haematological malignancies.

The choice of conditioning chemotherapy before allo-SCT may affect subsequent NK cell function: the anti-CD52 mAb, alemtuzumab targets NK cells, whereas polyclonal anti-thymocyte globulin (ATG) does not. Alemtuzumab results in more profound early depletion of NK cells following allo-SCT than does ATG.<sup>571</sup> In myeloma, the use of alemtuzumab is associated with higher subsequent disease relapse rates than ATG).<sup>572</sup>

Together, these empirical observations suggest that the efficacy of an immunotherapy might be enhanced if innate lymphocytes, such as NK cells, are activated.

**Activation of antigen-presenting cells** Activated or mature antigen-presenting cells (APCs) are required for the optimal generation of T cell responses. The use of adjuvants that can activate and mature dendritic cells, such as TLR ligands, is common practice in vaccination against infectious diseases.<sup>573</sup> Ensuring activation of APCs may also assist the generation of antitumour immunity—in a meta-analysis of clinical trials of DC vaccination for the treatment of prostate cancer, the use of mature DCs was associated with a significantly higher response rate than immature DCs.<sup>574</sup>

Clinical reports suggest that TLR signals can augment anti-tumour immunity. Clinical regression of tumours after administration of bacteria or bacterial toxins has been reported since the nineteenth century.<sup>575</sup> The intralesional administration of mycobacterial cell wall products is associated with local regression of lesions in patients with sarcoma and melanoma,<sup>576</sup> while intravesical administration of BCG remains in routine use as an effective therapy for bladder cancer.<sup>577</sup> Topical imiquimod (a TLR7/8 agonist) induces local remissions in cutaneous carcinomas.<sup>577</sup> In CLL, lymphomatous skin deposits have also been reported to regress after topical imiquimod administration.<sup>357</sup> Finally, rare spontaneous remissions of acute myeloid leukaemia have been associated with episodes of sepsis, an effect which might be TLR-mediated.<sup>578</sup>

TLR ligands appear to be of value when used in conjunction with therapeutic cancer vaccines. The addition of a synthetic deoxycytidyl-deoxyguanosin oligodeoxynucleotide (CpG ODN) to a tumour-associated peptide antigen resulted in improved immunological responses in patients with melanoma.<sup>579</sup> A change in formulation of incomplete Freund's adjuvant was associated with a decline in immunological responses to a therapeutic peptide vaccine against melanoma.<sup>580</sup>

Dendritic cells treated with the cytokine GM-CSF develop an inflammatory phenotype and induce stronger T cell responses than those generated in the presence of fms-related tyrosine kinase 3 ligand (Flt3L).<sup>581</sup> In men with castration-resistant metastatic prostate cancer, the infusion of PBMCs treated with a fusion protein combining prostatic acid phosphatase (a TAA) with GM-CSF *ex-vivo* led to prolongation of overall survival, compared with infusion of untreated PBMCs.<sup>582</sup>

Incorporation of the transgenes B7.1, ICAM-1 and LFA-3, which encode three APC-expressed costimulatory molecules, led to enhanced *in vitro* responses to a poxviral gene vaccine for prostate cancer. The combined vaccine improves overall survival in men with hormone-refractory metastatic prostate cancer.<sup>583</sup>

DCs express CD40, and can be matured through provision of CD40 ligand or agonistic CD40 antibodies. In a phase I study of patients with myeloma, intranodal administration of DCs pulsed with idiotype protein and matured with CD40 ligand led to immunologic response in all nine patients and stable disease at five years in four patients.<sup>584</sup>

In allo-SCT, the combination of conditioning chemotherapy and/or radiotherapy, and the consequent neutropenia with associated infective episodes, are likely to provide 'danger' signals, resulting in DC activation. As well as potentially stimulating anti-tumour immunity, this may also provoke GVHD: in a xenograft model, depletion of activated DCs by targeting the DC surface activation marker CD83 prevented GVHD.<sup>585</sup>

Together, these clinical observations suggest that the generation of mature activated (or 'licensed') DCs, for example through the co-administration of a vaccine with a TLR ligand,



with GM-CSF or with a CD40-stimulating agent, may support the successful generation of antitumour immunity.

**Summary** In this section, I have considered how clinical experience of cellular immunotherapies, including allo-SCT, can provide insights into the factors associated with successful tumour immunotherapy. Although many of these clinical studies are uncontrolled, and most deal with malignancies other than CLL, I would tentatively suggest that in broad terms, an immunotherapeutic strategy is more likely to be successful if it:

- Targets a malignancy which is shown to be susceptible to the immunotherapy
- Is employed while the malignancy is in remission
- Is used in recipients who retain a broad T cell repertoire, employs healthy allogeneic T cells, or utilises T cell redirection
- Is preceded by host lymphodepletion
- Is preceded by therapies to disrupt immunological tolerance
- Involves engagement of innate lymphocytes (such as NK cells) as well as T cells
- Activates antigen presenting cells (for example by providing a TLR signal)

Table 7.7 (page 204) summaries these suggestions, and proposes means by which each of these factors might be addressed in practice.

In the next section I shall consider how iNKT cell engagement might help (or hinder) the development of a cellular immunotherapy which meets these requirements.

#### 7.3.2 Potential role of iNKT cell engagement in CLL immunotherapy

In section 7.3.1, I proposed a series of factors associated with successful cellular immunotherapy in clinical practice. In this section, I shall consider how the engagement of iNKT cells might help to address these factors.

Experimental evidence that the engagement of iNKT cells can augment antitumour immunity has been discussed in section 1.3.3 (page 55). Briefly, preclinical models suggest that iNKT cells can enhance antitumour responses through three mechanisms: the trans-activation of NK cells leading to enhanced NK cell cytotoxicity against tumour cells, the direct lysis of CD1d-expressing target tumour cells by iNKT cells in the presence of  $\alpha$ -GalCer, and the activation (or 'licensing') of  $\alpha$ -GalCer-presenting DCs leading to enhanced 'conventional' CD4+ and CD8+ T cell responses against co-presented TAAs.

The data presented in section 6.3.1 indicate that  $\alpha$ -GalCer-induced cytokine production in whole PBMC cultures is limited. Moreover, even if administration of  $\alpha$ -GalCer to patients could elicit sufficient cytokine production to activate NK cells, NK cytotoxicity against autologous CLL cells is likely to be constrained by CLL cell expression of inhibitory MHC Class I molecules, and by HLA-G expression on CLL cells.<sup>337</sup>

In section 5.3.6, I show that iNKT cell lines can be generated from the peripheral blood of patients with CLL. However, these cell lines had a CD4+ phenotype, produced large

**Table 7.7 Factors associated with successful cellular immunotherapy**

<b>Requirement</b>	<b>Potential solution(s)</b>
Susceptible malignancy	Select appropriate immunotherapy strategy for the disease
Disease in remission	Chemotherapy, radiotherapy or surgery before immunotherapy
T cell repertoire sufficient to recognise tumour	Verify presence of tumour-reactive autologous T cells Use allogeneic T cell source Use transgenic T cells Use redirected T cells
Expansion of tumour-reactive T cells	Lymphodeplete before immunotherapy Administer exogenous cytokines to patient <i>Ex vivo</i> T cell expansion
Disruption of tolerance	Chemotherapy or radiotherapy Anti-CD25, anti-CTLA4, or anti-PD1 mAb
Engagement of innate lymphocytes	Opsinise tumour with mAb Elicit IFN- $\gamma$ release Inhibit HLA-G
Activation of APCs	Chemotherapy or radiotherapy TLR ligand GM-CSF Irradiated tumour as vaccine

amounts of Th2-type cytokines such as IL-13, and exhibited only modest cytotoxicity against CD1d-transfected C1R cells, and only in the presence of  $\alpha$ -GalCer (section 5.3.8). Cytotoxicity against allogeneic and autologous CLL cells was even more limited (section 6.3.4). Thus, I suggest that the notion of using iNKT cells to directly lyse CLL cells is unlikely to prove effective, due to the relatively insensitivity of CLL cells to iNKT cell lysis (perhaps related to the relatively low level of CD1d expression by CLL cells), the low frequency of circulating effector iNKT cells in patients with CLL, and the requirement for CD1d-presented  $\alpha$ -GalCer for cell lysis.

I suggest that the capacity of iNKT cells to enhance proliferation of antigen-specific CD4+ and CD8+ T cells appears the most promising role for these cells in cellular immunotherapy. As discussed in section 1.3.3 (page 55), preclinical models have shown that  $\alpha$ -GalCer-pulsed tumour cells can induce 'conventional' CD4+ and CD8+ T cell responses against CD1d-expressing haematologic malignancies.<sup>422,424,425</sup>

The data presented in this thesis lend some support to the latter approach in CLL. The data presented in chapter 3 confirm that iNKT cells can be detected in the peripheral blood of patients with CLL, and are of normal phenotype. In chapter 4, I confirm that CLL cells express CD1d, and that CD1d expression on mDCs is normal in patients with CLL. The data presented in chapter 5 show that iNKT cells from patients with CLL exhibit normal cytokine production, and retain proliferative capacity. Finally, in chapter 6, I demonstrate that  $\alpha$ -GalCer-pulsed CLL cells are able to recruit iNKT cells in both the allogeneic and autologous settings, and present data suggesting that  $\alpha$ -GalCer pulsing of tumour cells may enhance proliferation of non-CD1d restricted 'conventional' T cells.

#### 7.3.3 Limitations of iNKT cell-directed immunotherapies

Two major factors may limit the application of iNKT cells to tumour immunotherapy against CLL: the very low frequency of circulating iNKT cells in humans, and the potential for iNKT cells to exhibit immunosuppressive activity.

**Numbers of iNKT cells** Both in healthy controls and in patients with CLL, the number of iNKT cells varies widely between individuals (figure 3.7). However, in general, iNKT cells constitute a small proportion of T cells in patients with cancer (table 7.2).

During the experiments presented in this thesis, I found that the low frequency of iNKT cells in PBMCs from patients with CLL hindered *in vitro* analysis of their function. It is possible that the low frequency of iNKT cells in older patients, and in those with cancer in particular, may also severely limit the efficacy of any iNKT cell-based immunotherapy *in vivo*. On the other hand, it is by no means clear that the low frequency of circulating iNKT cells is a barrier to their exploitation.

*In vivo*, trafficking of T cells through lymphoid organs means that even T cells at a very low frequency are able to come into contact with their cognate antigen, to expand and to promote antibody production—the *in vitro* setting cannot emulate this. Moreover, as discussed in section 1.2.2.1, iNKT cells are enriched in certain organs, including the liver, spleen and thymus, which I was not able to sample. In keeping with this, some clinical data suggest that a low frequency of circulating iNKT cells does not necessarily

preclude a response to an iNKT cell-directed therapy: In a phase I/II clinical study of vaccination with  $\alpha$ -GalCer-pulsed APCs, the number of circulating iNKT cells prior to treatment bore little relation to the number of  $\alpha$ -GalCer-induced IFN- $\gamma$  ELISpots following vaccination.<sup>411</sup>

If iNKT cell numbers do prove limiting, immunotherapy strategies might be designed to induce iNKT cell proliferation. Although the *in vitro* expansion protocol I used in sections 5.3.6 to 5.3.8 was suboptimal, generating CD4<sup>+</sup> Th2-type cytokine-producing iNKT cells with limited cytotoxicity, it provides proof of principle that functional iNKT cell lines can be expanded from the peripheral blood of patients with CLL. As discussed in section 7.2.4.3, alternative *in vitro* iNKT cell expansion strategies could be explored. There is also evidence that *in vivo* expansion of human iNKT cells is feasible: the administration of  $\alpha$ -GalCer-pulsed moDCs resulted in increased circulating iNKT cell numbers in patients with cancer.<sup>412,413</sup>

Thus, low iNKT cell frequencies in humans may hinder their successful exploitation for cancer immunotherapy, although this will not be certain until clinical trials are undertaken. *In vitro* or *in vivo* expansion of iNKT cells might help to overcome this limitation.

**Immunosuppression by iNKT cells** As well as showing the capacity to enhance anti-tumour responses in some preclinical models, iNKT cells appear to suppress anti-tumour immunity in certain scenarios. Wild-type mice were more susceptible to a CD1d-transfected T cell lymphoma than iNKT cell-deficient strains. In the wild-type (iNKT cell-replete) mice, inoculation with the CD1d-transfected lymphoma was associated with production of the immunosuppressive cytokine, IL-13.<sup>313</sup> In another model, mice treated with radiotherapy for a 4T1 mammary carcinoma had a better response in the absence of iNKT cells.<sup>312</sup>

A number of effector mechanisms may account for the immunosuppressive activity of iNKT cells observed in some settings:

- The CD4<sup>+</sup> subpopulation of iNKT cells can produce large quantities of the immunosuppressive Th2 cytokines IL-4, IL-10 and IL-13. Unlike their CD4<sup>-</sup> counterparts, CD4<sup>+</sup> iNKT cells fail to reject a sarcoma in mice.<sup>222</sup> Presentation of  $\alpha$ -GalCer by non-professional APCs such as B cells also results in Th2-skewed iNKT cell cytokine production.<sup>246</sup>
- A population of Foxp3<sup>+</sup> iNKT cells can be induced in the presence of TGF- $\beta$ , and can directly suppress T cell proliferation through a contact-dependent mechanism.<sup>264</sup> This may be of relevance in CLL, as the leukaemic cells are known to produce TGF- $\beta$  in culture, and patients with active CLL have raised plasma TGF- $\beta$  levels.<sup>102</sup>
- Human iNKT cells can induce monocytes to differentiate into IL-10-producing immunosuppressive APCs.<sup>262</sup>
- Type II NKT cells, which are CD1d restricted, but which unlike iNKT cells do not recognise  $\alpha$ -GalCer, can suppress antitumour immunity via production of IL-13.<sup>314</sup>

The factors influencing the relative production of Th1 and Th2 cytokines by iNKT cells

have already been discussed in section 1.2.4. Briefly, the activation of iNKT cells in the presence of weak or endogenous ligands, non-professional APCs, high doses of IL-2, or inhibitory NK receptors, tends to favour the production of Th2 cytokines and tolerance.

Consideration of the mode of iNKT cell activation, and of the cytokine and cellular milieu in which it occurs, may help to avoid the unintended induction of immunosuppressive iNKT cell activity. Selectivity for type I NKT cells (iNKT cells) over immunosuppressive type II NKT cells can be achieved by using  $\alpha$ -GalCer for NKT cell activation (table 1.9, figure 1.3). Targeting iNKT cells to professional APCs might be achieved by administering  $\alpha$ -GalCer on *in vitro*-generated mature moDCs, or on dying tumour cells (which are then taken up by native DCs). The production of immunosuppressive TGF- $\beta$  by leukaemic cells may be minimised by the application of iNKT cell-based immunotherapy after induction of disease remission. Finally, as discussed in section 7.2.4.3, if iNKT cells are to be expanded *in vitro*, using cytokines other than IL-2 might avoid the selection of CD4+ Th2 cytokine-producing iNKT cells.<sup>433</sup>

Thus, iNKT cells clearly have the potential to be immunosuppressive. Any tumour immunotherapy strategy using iNKT cells should be designed to avoid the induction of immunosuppressive iNKT cells, and clinical trials of iNKT cell-based immunotherapies should include secondary endpoints that detect the induction of tumour tolerance.

**Summary** Preclinical data suggest that recruitment of iNKT cells with  $\alpha$ -GalCer can lead to enhancement of CD4+ and CD8+ ‘conventional’ T cell responses against tumours, including CD1d-expressing haematologic malignancies. I have established that the iNKT cell axis is functional in patients with CLL, and that  $\alpha$ -GalCer-pulsed CLL cells can elicit iNKT cell proliferation and may enhance ‘conventional’ T cell responses.

Although preclinical data suggest that iNKT cell recruitment may enhance antitumour responses, two factors may limit their use in clinical practice: the very low iNKT cell frequency in human PBMCs, and the immunosuppressive potential of iNKT cells.

The low frequency of iNKT cells is not necessarily a barrier to their effective exploitation in clinical immunotherapy, as the iNKT cell frequency within tissues may be higher, and some immunotherapeutic strategies incorporate *in vitro* or *in vivo* iNKT cell expansion. Immunosuppression by iNKT cells might be minimised by measures to disrupt any immunosuppressive cytokine or cellular environments, and by avoiding iNKT cell expansion strategies which favour Th2 cytokine production.

In the next section, I shall attempt to incorporate these ideas into a proposal for a potential cellular immunotherapy of CLL.

#### 7.3.4 Proposal for a cellular immunotherapy of CLL

As discussed in section 1.3.1, clinical experience indicates that CLL is amenable to immunologic control and eradication. In this section, I propose a strategy for cellular immunotherapy of CLL, which aims to incorporate some of the factors associated with successful immunotherapy suggested in section 7.3.1.

Experience from clinical trials of immunotherapy for melanoma, and from allo-SCT, suggests that application of a tumour immunotherapy while the disease is in remission enhances the chance of long-term disease control. The current ‘gold standard’ of treatment for CLL, at least in patients without the 17p cytogenetic deletion, is chemotherapy with fludarabine, cyclophosphamide and rituximab (FCR), both as initial treatment,<sup>5,140</sup> and at the time of relapse.<sup>586,587</sup>

Following FCR chemotherapy, T cell depletion lasts at least a year, although NK cell numbers and function are relatively preserved.<sup>175</sup> The combination of fludarabine and cyclophosphamide is commonly used for conditioning prior to allogeneic stem cell transplantation, and has also been used for lymphodepletion prior to T cell transfer in clinical trials of adoptive immunotherapy for cancer.<sup>588</sup> Fludarabine treatment of patients with CLL leads to a depletion of T<sub>reg</sub> cell numbers and function,<sup>182</sup> while low-dose cyclophosphamide has also been associated with T<sub>reg</sub> depletion.

Standard chemoimmunotherapy of CLL with FCR, then, results in a high rate of disease remission, induces lymphodepletion, and may disrupt T<sub>reg</sub>-mediated immune tolerance. The period following FCR chemotherapy may represent a good opportunity to introduce a cellular immunotherapy.

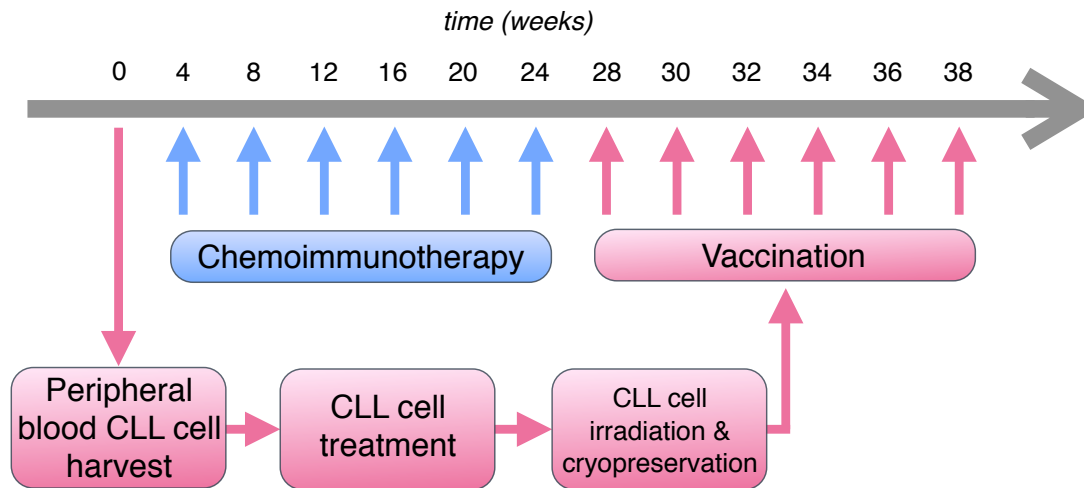
As discussed in section 1.3.3.3, preclinical data indicate that  $\alpha$ -GalCer-pulsed tumour cells can act as a powerful whole-tumour vaccine against experimental B cell malignancies.<sup>424,425</sup> The translational experiments presented in this thesis indicate that  $\alpha$ -GalCer-pulsed CLL cells can lead to proliferation of patient iNKT cells and may enhance the proliferation of autologous non-CD1d restricted T cells.

Leukaemic cells are abundant in the peripheral blood of untreated patients with CLL, and can be readily purified and stored. I propose that treated irradiated CLL cells may provide a useful whole tumour vaccine. An intravenous route of vaccination may be favoured, as preclinical studies indicate that intravenous, but not subcutaneous, injection of  $\alpha$ -GalCer-loaded plasmacytoma cells, resulted in CD4+ and CD8+ T cell responses and tumour protection.<sup>425</sup> Unlike solid tumours, CLL is always a systemic malignancy, and FCR chemoimmunotherapy is not curative, so intravenous administration does not carry the risk of metastasis.

A variety of strategies for the treatment of CLL cells prior to irradiation and infusion as a whole tumour vaccine could be used:

- **$\alpha$ -GalCer treatment:** Treatment of leukaemic cells with  $\alpha$ -GalCer can recruit autologous iNKT cells, which may lead to activation of endogenous DCs presenting CLL tumour-associated antigens, and may enhance subsequent ‘conventional’ T cell responses against CLL. The experiments in this thesis lend some support to this concept, by demonstrating that patients with CLL have detectable iNKT cells, that CLL cells express CD1d, that patient iNKT cells are able to proliferate and produce cytokines, and that autologous  $\alpha$ -GalCer-treated CLL cells can lead to iNKT cell proliferation. Expression of CD1d on CLL cells is low compared to mDCs (figure 4.1), and higher levels of CD1d expression may support the strategy of vaccination with whole  $\alpha$ -GalCer-treated tumour cells.<sup>422</sup> Although the treatment of CLL cells with IFN- $\gamma$  did not increase their CD1d expression (figure 6.9), other strategies such as CD1d transfection might enhance the ability of CLL cells to carry and to present

**Figure 7.1 A whole tumour cell vaccination strategy for CLL.** Leukaemic cells are isolated from the peripheral blood of patients with CLL requiring treatment, are treated to enhance immunogenicity, irradiated and cryopreserved as a whole tumour cell vaccine. The patient is treated with standard fludarabine, cyclophosphamide and rituximab chemoimmunotherapy. After treatment, repeated doses of the tumour vaccine are administered during T cell reconstitution.

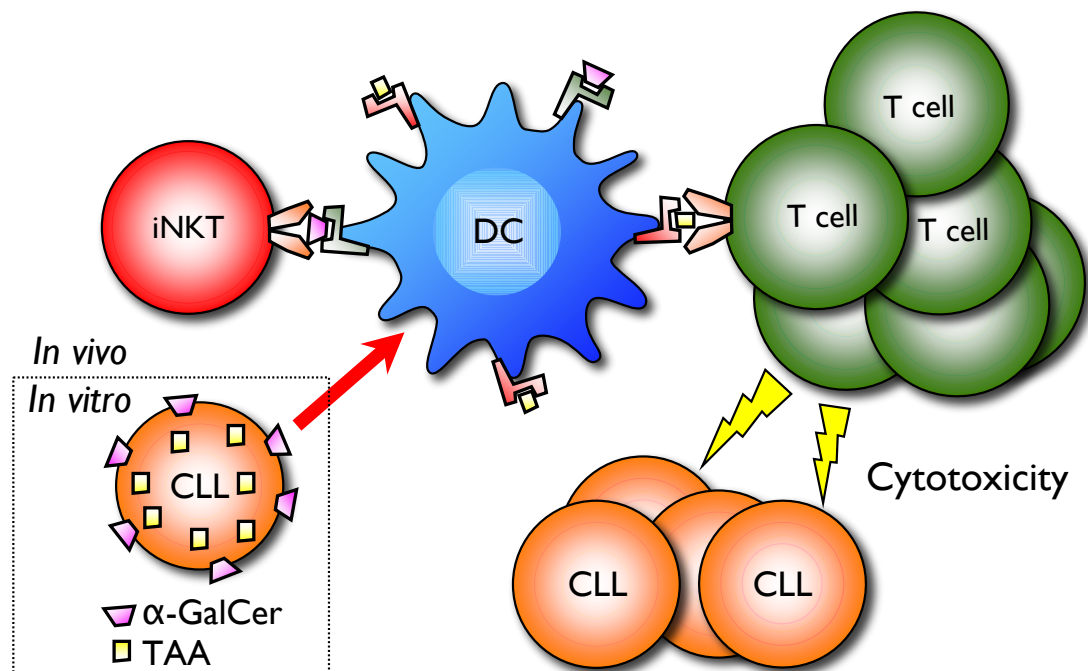


$\alpha$ -GalCer. Finally, I have not been able to conclusively demonstrate that  $\alpha$ -GalCer treatment of leukaemic B cells enhances proliferation of tumour-reactive ‘conventional’ T cells, although this has been shown in pre-clinical models of B cell malignancies.<sup>424,425</sup>

- **TLR ligand treatment:** As discussed in section 1.3.2.2, CLL cells express TLR ligands, and treatment of CLL cells with TLR7 and TLR9 ligands leads to upregulation of costimulatory markers and enhancement of immunologic responses against leukaemic cells.<sup>369</sup> Experiments presented in this thesis confirm this (figures 6.16 and 6.18). In pre-clinical models, TLR ligands and  $\alpha$ -GalCer can be used together to enhance immunological responses to malignancies,<sup>311</sup> an effect which has also been shown using human cells *in vitro*.<sup>434</sup>
- **CD40 ligation:** As discussed in section 1.3.2.2, CLL cells express CD40, and treatment with CD40 ligand leads to CLL cell upregulation of costimulatory molecules and enhancement of allogeneic CD4 and CD8 T cell (and autologous CD4 T cell) responses to tumour.<sup>359,360</sup>

Figure 7.1 illustrates a proposed strategy, in which leukaemic cells would be isolated from the peripheral blood of patients with CLL, possibly including immunomagnetic selection. Isolated leukaemic cells would be treated using one or more of the above strategies, irradiated, and cryopreserved in batches for later use as a whole tumour vaccine. Patients would then receive standard chemoimmunotherapy (FCR) to a maximal response. Following completion of chemotherapy, a series of intravenous vaccinations with treated irradiated tumour cells would be administered.

**Figure 7.2 Rationale of tumour vaccination strategy.** Patients are immunised with  $\alpha$ -GalCer-bearing, irradiated leukaemic cells. These are taken up by resident DCs, which present both TAAs on MHC class I and II, and  $\alpha$ -GalCer on CD1d. Recognition of  $\alpha$ -GalCer results in iNKT cell proliferation and cytokine production, which in turn leads to DC activation. This enhances T cell responses to the co-presented TAAs, resulting in augmented cytotoxicity against residual, unmodified CLL cells.



The rationale for treatment of CLL cells with  $\alpha$ -GalCer before whole tumour vaccination is outlined in figure 7.2.  $\alpha$ -GalCer-pulsed whole irradiated tumour cells would be taken up by patients' resident DCs. The DCs would present TAAs alongside  $\alpha$ -GalCer. Recruitment of iNKT cells by  $\alpha$ -GalCer on the DCs would augment DC expression of costimulatory molecules, and enhance proliferation of non-CD1d restricted T cells in response to the co-presented peptide TAAs. These conventional T cells then provide ongoing tumour immunity. The use of repeated doses may be important: Following early vaccinations, iNKT cell numbers might expand, enhancing their adjuvant activity for subsequent vaccine doses. Alternative strategies for enhancing immunogenicity of CLL cells, such as treatment with TLR or CD40 ligands could be used alone or in addition to this  $\alpha$ -GalCer treatment.

In addition to clinical outcome measures such as minimal residual disease eradication, laboratory assessments of response to immunotherapy might include analysis of serum cytokines, CLL cell-induced T cell proliferation, CLL cell-induced IFN- $\gamma$  ELISpot formation, and cytotoxicity against CLL cells, all before and after vaccination.

This tumour vaccination approach has several potential limitations. First, the restricted T cell repertoire of fludarabine-treated patients with CLL may not include sufficient potentially tumour-reactive T cells to eradicate the malignancy. Second, production of



potentially-immunosuppressive cytokines in response to treated tumour cells might result in suppression rather than enhancement of the antitumour response. Third, in relation to the use of  $\alpha$ -GalCer, an adjuvant effect of iNKT cells has not been reported in humans, and I have not been able to conclusively demonstrate it in this thesis. Fourth, because median overall and progression-free survival are long after first treatment with FCR alone, a very long follow-up period, or the use of a surrogate endpoint, would be required for any study. I shall address each of these limitations in turn.

First, despite their restricted T cell repertoire, translational studies have suggested that tumour-reactive T cells are present in the peripheral blood of patients with CLL, including some of those who have been previously treated with fludarabine-based chemotherapies.<sup>323,325</sup> Although fludarabine treatment of patients with CLL results in relative preservation of memory T cells compared to naïve T cells,<sup>589</sup> T cell receptor gene rearrangement excision circles (TRECs) could be detected in ten of thirteen older adults (median age 58 years) after fludarabine chemotherapy,<sup>590</sup> indicating that thymic production of naïve T cells does occur after fludarabine. Together, these findings suggest that, while the numbers of naïve T cells are reduced after fludarabine-containing chemotherapies, mature T cells, which may include CLL-reactive T cells, are retained, and thymic reconstitution of the naïve T cell pool can occur. Finally, indirect evidence suggesting immunological control of CLL by autologous T cells after fludarabine comes from long-term follow-up data for FCR-treated patients with CLL, which show a plateau in progression-free survival curves beyond eight years.<sup>591,592</sup>

Second, the application of a tumour vaccination strategy after FCR chemotherapy should avoid promoting immunosuppression, as the frequency of TGF- $\beta$ -producing leukaemic B cells and Tregs would be reduced by pre-vaccination chemotherapy. Although my experiments show that *in vitro* cultured iNKT cells are capable of producing immunosuppressive cytokines, this may be related to the use of high doses of IL-2 during cell culture, as it was not as evident in iNKT cells tested *ex vivo*. By avoiding prolonged *in vitro* iNKT cell culture and high doses of IL-2, a study using a design similar to this should avoid induction of an immunosuppressive phenotype.

Third, the experiments in this thesis show that although at a low frequency, iNKT cells can be detected in the peripheral blood of nearly all patients with CLL, and that they are capable of proliferating *in vitro*, including in response to autologous  $\alpha$ -GalCer-pulsed irradiated leukaemic cells. Clinical trials have demonstrated the capacity of immunotherapeutic strategies to induce *in vivo* iNKT cell expansion in patients with malignancies (table 1.17). Conclusive evidence for an adjuvant effect of iNKT cell agonists in humans is lacking, but this has been shown in preclinical models of immunotherapy for B cell malignancies.

Fourth, phase II or phase III trials examining the efficacy of an immunotherapeutic approach such as that suggested here could use eradication of MRD as a surrogate endpoint to overcome the very long follow-up periods required to detect differences in progression free survival after first line chemotherapy. Very low levels of residual CLL cells can be detected in the blood or bone marrow of most FCR-treated patients using polychromatic flow cytometry or molecular techniques,<sup>593,594</sup> and levels of MRD correlate with clinical outcome after FCR chemotherapy.<sup>595</sup> Alternatively, higher-risk groups of patients could be selected, such as those with the 11q or 17p deletions, or unmutated IGVH regions, as

earlier relapses would be expected in these groups after standard first-line chemoimmunotherapy.

To summarize, the proposed immunotherapeutic strategy combines standard chemoimmunotherapy, to induce both disease remission and lymphodepletion, with subsequent tumour vaccination incorporating iNKT cell engagement and/or upregulation of CLL cell costimulatory markers with TLR or CD40 ligands. Such an immunotherapy strategy addresses many of the factors associated with successful immunotherapy proposed in table 7.7.

### 7.4 Conclusions and future directions

The experiments presented in this thesis provide the first data characterising iNKT cell numbers and function in patients with CLL. The findings indicate that although iNKT cells constitute a reduced proportion of T cells, they are present in normal absolute numbers in the peripheral blood of patients with CLL. The immunophenotype of circulating iNKT cells in patients is normal in terms of CD4, CD8 and CD25 expression. The frequency of circulating iNKT cells is not associated with CLL disease stage or ZAP-70 status, and does not predict disease progression, suggesting that iNKT cells are not central to the pathogenesis of CLL progression.

The expression of the glycolipid-presenting molecule CD1d on circulating APCs is intact in CLL, although patients have a higher proportion of CD16+ monocytes, which express CD1d at lower levels than their CD14high counterparts.

In terms of function, intracellular cytokine profile and granzyme B expression are normal in iNKT cells of patients with CLL, and functional iNKT cell lines can be derived from patients with CLL. These cell lines produce both Th1- and Th2-type cytokines, and can lyse CD1d-transfected target cells in an  $\alpha$ -GalCer-dependent manner.

Despite these findings, the addition of  $\alpha$ -GalCer to whole PBMCs from patients with CLL does not result in substantial cytokine production, a finding attributable to the low iNKT cell frequencies in most individuals. The lysis of CLL cells by patient-derived iNKT cells is inefficient, requiring high effector to target ratios. However, the addition of  $\alpha$ -GalCer-pulsed CLL cells to B cell-depleted PBMCs induces proliferation of iNKT cells in both allogeneic and autologous settings, and  $\alpha$ -GalCer-treatment enhances the proliferation of non-CD1d restricted T cells in response to irradiated autologous leukaemic cells.

The results presented in chapters 3 to 5 suggest that despite a relative reduction in iNKT cell numbers, absolute numbers are normal and iNKT cell cytokine production and proliferative capacity is retained. This suggests that the iNKT cell/CD1d axis is essentially intact in patients with CLL. However, the results presented in chapter 6 highlight two key challenges to any iNKT cell-based tumour immunotherapy.

First, the scarcity of circulating iNKT cells may limit the efficacy of an  $\alpha$ -GalCer-based immunotherapy—immunologic effects of  $\alpha$ -GalCer which are readily detectable in mice, or in human donors with high numbers of iNKT cells, are difficult to demonstrate in those with iNKT cell numbers more typical of older adults, including CLL patients

Second, iNKT cells, particularly those which have undergone prolonged *in vitro* culture in the presence of IL-2, are capable of producing high concentrations of Th2-type cytokines, which could hinder rather than help the generation of antitumour responses.

To overcome the small numbers of iNKT cells, patients with higher numbers of circulating cells could be selected for inclusion of any clinical studies. However, this would limit the applicability of any immunotherapy, and does not take account of the possibility that iNKT cells may be more numerous in tissues other than peripheral blood. A more attractive solution may be to design an immunotherapeutic strategy which incorporates *ex vivo* or *in vivo* expansion of iNKT cells.

Although I was able to demonstrate *ex vivo* generation of iNKT cell lines from patients with CLL, the process was laborious, requiring several purification steps and prolonged culture periods, and yielding limited numbers of iNKT cells. Moreover, the resultant iNKT cells produced high levels of IL-4 and IL-13, which may not be desirable characteristics for application in antitumour immunotherapy. Refining iNKT cell expansion methods may overcome a number of these limitations. However, if expansion of iNKT cells could be achieved *in vivo*, prolonged *in vitro* culture might be avoided altogether.

Experience in phase I clinical trials suggests that  $\alpha$ -GalCer-pulsed APCs can lead to *in vivo* iNKT cell proliferation in humans (table 1.17). My *in vitro* data indicate that  $\alpha$ -GalCer-pulsed CLL cells can induce iNKT cell proliferation. Compared to mDCs, CLL cells are very readily obtained and purified, and would intrinsically express the full range of patient-specific TAAs.

The use of  $\alpha$ -GalCer-pulsed tumour cells represents an attractive and simple means of delivering TAAs alongside  $\alpha$ -GalCer adjuvant. As discussed in section 1.3.3.3, preclinical data lends support to the approach of using whole tumour cells pulsed with  $\alpha$ -GalCer as a vaccine in B cell malignancies.

Further experiments could be performed to elucidate the mechanisms by which  $\alpha$ -GalCer-pulsed CLL cells result in iNKT cell proliferation (either by directly presenting  $\alpha$ -GalCer to iNKT cells, or through re-presentation by other APCs). *In vitro* experiments could explore the co-operation between various TLR ligands and  $\alpha$ -GalCer in the enhancement of T cell responses against autologous CLL cells. Demonstrating that the responding T cells have cytotoxic potential against autologous CLL cells would also be important, and might be achieved with granzyme B ELISpot assays, or by using an alternative cytotoxicity assay.

Application of the immunotherapeutic strategy of  $\alpha$ -GalCer-pulsed tumour cells in one or more of the murine models of CLL discussed in section 1.1.3.6 could provide additional support for future clinical trials of  $\alpha$ -GalCer-pulsed CLL cells as a tumour vaccine.

Should translational or early phase clinical studies fail to demonstrate an effect of  $\alpha$ -GalCer in the enhancement of anti-tumour responses in humans, the impact of engaging other unconventional T cells could be explored in future. Humans express group 1 CD1 molecules, including CD1a, CD1b and CD1c. Group 1 CD1-restricted T cells can be isolated from human peripheral blood, respond to distinct lipid agonists (table 1.8), and appear to have a role in the immunological response to tuberculosis.<sup>195</sup> They are capable of Th1-type cytokine production, and their frequency in human peripheral blood is at least order of magnitude greater than that of iNKT cells.<sup>596</sup> The availability of CD1

tetramers and the development of a transgenic mouse expressing human group 1 CD1 molecules will facilitate future study of this field.<sup>196,596</sup> As described in section 1.2.2.2, phosphoantigen-reactive V $\gamma$ 9V $\delta$ 2 T cells and MR1-restricted MAIT cells represent other human invariant T cell populations that share functional characteristics with, but are more prevalent than, iNKT cells. It is not yet known whether tumour vaccination strategies incorporating ligands for these cells are effective.

In conclusion, the experiments presented in this thesis provide the first evidence that the iNKT cell and CD1d axis is largely phenotypically and functionally intact in patients with CLL, that functional iNKT cell lines can be generated from the peripheral blood of patients with CLL, and that autologous  $\alpha$ -GalCer-treated irradiated leukaemic cells can induce the proliferation of patient iNKT cells. On the basis of *in vitro* functional assays of patient PBMCs, I suggest that the most promising means of employing iNKT cells for the cellular immunotherapy of CLL may be to exploit their adjuvant function to enhance 'conventional' CD4+ and CD8+ T cell responses against tumour-associated peptide antigens. Finally, I propose a feasible whole tumour vaccination strategy using  $\alpha$ -GalCer-treated irradiated leukaemic cells for the treatment of CLL.

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## **Appendix A**

# **Study recruitment materials**

26 January 2009

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Dear \*\* \*\*\*\*\*,

**Re: Invitation to participate in a study of chronic lymphocytic leukaemia**

We are writing to invite you to take part in a study of chronic lymphocytic leukaemia. This study is taking place at the Malaghan Institute of Medical Research, in conjunction with Wellington Blood & Cancer Centre. Our hospital clinic records indicated that you might be eligible for this study, and we are contacting you with the permission of your GP.

The aim of this study is to look at immune system function in people with and without chronic lymphocytic leukaemia. Taking part in the study involves completing a questionnaire, having a brief examination for enlarged lymph nodes, and giving a blood sample at the Malaghan Institute of Medical Research at Victoria University in Kelburn. The whole process takes about 30 minutes. The study involves one visit only, and it does not involve any treatments. The Central Regional Ethics Committee has approved this study.

If you are interested in taking part in the study and would like more information, please complete the attached form and send it back to us in the envelope provided. Taking part in the study is entirely voluntary (your choice), and whether you participate or not, the study does not affect your medical care.

Yours faithfully,

Dr Robert Weinkove  
Haematology Registrar, Malaghan Institute

Asst Professor John Carter  
Consultant Haematologist, CCDHB

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*Please indicate your response by ticking one of the boxes below.*

- Yes**, I am interested in the chronic lymphocytic leukaemia study. Please contact me with further details.

My telephone number is: \_\_\_\_\_

or: My email address is: \_\_\_\_\_

- No**, I would rather not take part in this study.

*Please post this form to:*

Dr Robert Weinkove, Clinical Research Fellow,  
Malaghan Institute of Medical Research,  
PO Box 7060, Wellington

*Or email:*

[rweinkove@malaghan.org.nz](mailto:rweinkove@malaghan.org.nz)

*Telephone:*

04 499 6914 extension 851 (24h answerphone)

# Could you help with leukaemia research?

Researchers at the Malaghan Institute of Medical Research are conducting a study comparing the immune system in people with and without chronic lymphocytic leukaemia (CLL). This study may help us to design new treatments for CLL and similar conditions.

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We are looking for:

**healthy volunteers aged 50 and over, and  
people with chronic lymphocytic leukaemia**

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The study involves:

- A short questionnaire
- A blood sample
- A brief examination to look for lymph nodes (for people with CLL only)

If you are interested in participating, please contact:

Dr Robert Weinkove  
Clinical Research Fellow, Malaghan Institute of Medical Research  
Telephone: **04 499 6914 extension 851** (24h answerphone)  
Email: **[rweinkove@malaghan.org.nz](mailto:rweinkove@malaghan.org.nz)**





## **Appendix B**

# **Participant information sheets**

## **iNKT cells in Chronic Lymphocytic Leukaemia Information Sheet for Patients**

You are invited to take part a study looking at the immune system in chronic lymphocytic leukaemia (CLL). This study is voluntary (your choice). You do not have to take part in this study. You may withdraw from the study at any time without affecting to your health care now or in the future. You will be given as much time as you need to decide if you wish to take part in this study. You may have a friend, family or whānau support to help you understand this study and any other explanation you may require.

CLL is the most common leukaemia in New Zealand. Many people with CLL do not need treatment for many years, and some never need treatment at all. Sometimes CLL is more aggressive and requires treatment with chemotherapy or other methods.

CLL causes changes to the body's immune system that make it difficult for the immune system to fight the leukaemia. We want to study a particular part of the immune system in people with and without CLL. The type of blood cell we shall study is called the 'invariant natural killer T cell' (iNKT cell). We think that iNKT cells are able to direct the immune system.

### **ABOUT THE STUDY**

#### **What are the aims of the study?**

- To find out if people with CLL have different numbers of iNKT cells in their blood to people without CLL, and to see if the cells behave differently in the laboratory.
- Using tests within the laboratory, to find out whether substances which stimulate the iNKT cells can help the immune system to fight leukaemia

We hope that the knowledge we gain will help us to develop new ways to treat CLL and similar diseases.

#### **Who can be in the study?**

People with CLL and healthy volunteers of a similar age. This study is for adults (over 18 years) only.

If you have ever had chemotherapy for a condition other than CLL, if you have ever been given medicines that affect the immune system (such as azathioprine or ciclosporin), or if you have an infection at the moment, you should not enter the study.

#### **Where is the study held?**

Blood samples will be collected at the Malaghan Institute of Medical Research in Kelburn or at the Blood and Cancer Centre at Wellington Hospital. The blood samples will be tested in the laboratories of the Malaghan Institute of Medical Research in Kelburn.

#### **How long is the study?**

The study will take about 45 minutes of your time. The study will be completed in December 2010.

#### **What does the study involve?**

- Your permission. After explaining the study, we shall ask you to complete and sign a consent form.
- A short questionnaire (10 minutes long) for you to complete. The study investigator can help you to complete this.
- A brief medical examination by a doctor to check the size of any lymph glands in your neck, armpits and groin, and to see if your liver and spleen are enlarged. You may request a chaperone if you wish.
- A sample of blood (up to 120ml) from a vein in the arm.
- Your paper and electronic medical records will be reviewed to check information relevant to the study and to check for health problems that might prevent you from participating in the study.

**iNKT cells in CLL study. Information sheet for patients version 6, 05/08/2009**

**This study is voluntary (your choice). You may withdraw from the study at any time without affecting your health care now or in the future.**

### **What will happen to my blood sample?**

Most of your blood sample will be analysed at the research laboratory in the Malaghan Institute of Medical Research where the numbers and activity of iNKT cells and other immune system cells will be measured.

An anonymous sample of your blood will be sent to Capital and Coast District Health Board to check the blood count (the number of white and red blood cells and platelets) and to measure markers of immune function (immune globulin levels).

Anonymous samples of your blood will be sent to Capital and Coast District Health Board and to Canterbury Health Laboratories to analyse genetic changes inside the leukaemia cells. This is important because other researchers have shown that CLL can behave differently depending on which genetic changes the leukaemia cells have. We shall **not** test for any genetic traits or for any genes that can be inherited.

### **What if my blood test results are abnormal?**

We expect to see some abnormalities of your blood count because you have chronic lymphocytic leukaemia. However, if the blood tests we do as part of the study show unexpected abnormalities (such as significant anaemia, very low platelets or an very high white blood cell count), the study investigators will contact you and ask you to visit your family doctor or a hospital doctor for a repeat of the blood test.

### **How will my blood sample be disposed of?**

Your blood samples will be disposed of by incineration in accordance with standard safe practice.

You may choose to have your samples disposed of using appropriate karakia at the end of the study. If you would like this, please tell a study investigator and circle this choice on the consent form.

### **Can my blood sample be returned to me?**

Unfortunately we cannot return any blood samples to participants. This is because the samples will have been treated with chemicals and substances that might be dangerous.

### **Can I change my mind and withdraw from the study?**

Yes. You are free to withdraw from the study at any time, without giving a reason and without affecting your future healthcare. You can ask for your blood sample and for any data to be destroyed. If you would like to do this, please contact one of the study investigators (see page 3 of this leaflet for contact details).

## **BENEFITS AND RISKS**

### **What are the benefits of the study?**

The study will help researchers understand how CLL affects the immune system. The study might help researchers to develop new ways of treating CLL which make use of patients' own immune systems.

### **What are the risks and inconveniences?**

You will need to visit the Malaghan Institute of Medical Research, on the campus of Victoria University in Kelburn, or the Wellington Blood and Cancer Centre at Wellington Hospital to have the blood sample taken. Free parking will be available at the Malaghan Institute of Medical Research and near Wellington Blood and Cancer Centre (at the Cancer Society). There is no payment or reimbursement for participation. If travel costs might prevent you from taking part please let the researchers know, as reimbursement of your travel costs could be provided.

A blood test can be uncomfortable. A temporary bruise may develop. Rarely, people faint after blood drawing. Very rarely, the vein in which the needle has been inserted may become inflamed or infected, which can be treated.

**Will I have to give any more samples?**

We would like each participant to attend and give a blood sample once only. However, if there is a technical problem with analysing your blood sample, or if your leukaemia progresses while the study is still under way, we might ask you to give a second sample of blood. You do not have to do so, and any further involvement in the study is entirely your choice.

**What will the study cost me?**

There is no cost to you apart from the time that you give.

**Confidentiality**

No material that could personally identify you will be used in any reports of this study. Your blood samples will be labelled with a study code number only. Study records will be stored on a password-protected computer and in a locked filing cabinet. Computers and files are kept in a restricted-entry facility, and will only be used by the study researchers. Records from the study will be kept for 10 years by the investigators, and then destroyed in a confidential manner.

**Results of the study**

The overall results of the study (from which you cannot be identified) will be published in scientific journals and presented at research meetings. You can request a copy of the study results by indicating your choice on the consent form. If you need further information please contact the Principal Investigator.

**Ethical approval**

This study has received ethical approval from the Central Regional Ethics Committee.

*The study team thank you for participating.*

**If you have any questions or concerns about your rights as a participant in this study you may wish to contact a Health and Disability Advocate, telephone:**

**0800 42 36 38 (0800 4 ADNETH)**

**You may contact the researchers to ask about anything you do not understand, or with questions regarding this study:**

**Principal Investigator**

**Dr Robert Weinkove**

Clinical Research Fellow

Malaghan Institute of Medical Research

PO Box 7060

Wellington

Phone: (04) 499 6914 ext 851

Mobile: 021 2160135

Email: [rweinkove@malaghan.org.nz](mailto:rweinkove@malaghan.org.nz)

**or Co-Investigator**

Professor John Carter

Consultant Haematologist

Blood & Cancer Centre

Wellington Hospital

Wellington

Phone: (04) 385 5999 ext 6756

**Compensation**

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

**INKT cells in CLL study. Information sheet for patients version 6, 05/08/2009**

**This study is voluntary (your choice). You may withdraw from the study at any time without affecting your health care now or in the future.**



## iNKT cells in CLL study Information Sheet for Volunteers

You are invited to take part a study comparing the immune system of healthy volunteers with that of patients with chronic lymphocytic leukaemia (CLL). This study is voluntary (your choice). You do not have to take part in this study. You may withdraw from the study at any time without affecting to your health care now or in the future. You will be given as much time as you need to decide if you wish to take part in this study. You may have a friend, family or whanau support to help you understand this study and any other explanation you may require.

CLL is the most common leukaemia in New Zealand. CLL causes changes to the body's immune system that make it difficult for the immune system to fight the leukaemia. We want to study a particular part of the immune system in people with and without CLL. The type of blood cell we shall study is called the 'invariant natural killer T cell' (iNKT cell). We think that iNKT cells are able to direct the immune system.

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### ABOUT THE STUDY

#### What are the aims of the study?

- To find out if people with CLL have different numbers of iNKT cells in their blood to healthy volunteers, and to see if these cells behave differently in the laboratory.
- Using tests within the laboratory, to find out whether substances which stimulate the iNKT cells can help the immune system to fight leukaemia

We hope that the knowledge we gain will help us to develop new ways to treat CLL and similar diseases.

**Who can be in the study?**  
Healthy volunteers and people with CLL. This study is for adults (over 18 years) only.

If you have ever had chemotherapy before, if you have ever been given medicines that affect the immune system (such as azathioprine or ciclosporin), or if you have an infection at the moment, you should not enter the study.

#### Where is the study held?

Blood samples will be collected at the Malaghan Institute of Medical Research in Wellington. The blood samples will be tested in the laboratories of the Malaghan Institute of Medical Research and at Capital and Coast District Health Board.

#### How long is the study?

The study will take about 45 minutes of your time. The whole study will be completed in December 2010.

#### What does the study involve?

- Your permission. After explaining the study, we shall ask you to complete and sign a consent form.
- A short questionnaire (10 minutes long) for you to complete. The study investigator can help you to complete this.
- A sample of blood (up to 120ml) from a vein in the arm.

#### What will happen to my blood sample?

Most of your blood sample will remain at the Malaghan Institute of Medical Research for special tests to measure the numbers and activity of iNKT cells and other immune system cells. Anonymous samples of your blood will be sent to Capital and Coast District Health Board to check your blood count (the numbers of white and red blood cells and platelets) and to measure markers of immune function (immune globulin levels).

**How will my blood sample be disposed of?**

Your blood samples will be disposed of by incineration in accordance with standard safe practice.

You may choose to have your samples disposed of using appropriate karakia at the end of the study. If you would like this, please tell a study investigator and circle this choice on the consent form.

**Can my blood sample be returned to me?**

Unfortunately we cannot return any blood samples to participants. This is because the samples will have been treated with chemicals and substances that might be dangerous.

**Can I change my mind and withdraw from the study?**

Yes. You are free to withdraw from the study at any time, without giving a reason and without affecting your future healthcare. You can ask for your blood sample and for any data to be destroyed. If you would like to do this, please contact one of the study investigators (see page 3 of this leaflet for contact details).

**BENEFITS AND RISKS****What are the benefits of the study?**

The study will help researchers understand how CLL affects the immune system. The study might help researchers to develop new ways of treating CLL which make use of patients' own immune systems. This study will not be of direct benefit to you.

**What are the risks and inconveniences?**

You will need to visit the Malaghan Institute of Medical Research, on the campus of Victoria University in Kelburn to have the blood sample taken. Free parking will be available. There is no payment or reimbursement for participation. If travel costs might prevent you from taking part please let the researchers know, as reimbursement of your travel costs could be provided.

A blood test can be uncomfortable. A temporary bruise may develop. Rarely, people faint after blood drawing. Very rarely, the vein in which the needle has been inserted may become inflamed or infected, which can be treated.

**Will I have to give any more samples?**

We would like each participant to attend and give a blood sample once only. However, if there is a technical problem with analysing your blood sample, we might ask you to give a second sample of blood. You do not have to do so, and any further involvement in the study is entirely your choice.

**What if my blood results are abnormal?**

We have asked you to enter the study as a healthy volunteer. However, there is a small chance that the blood count we perform shows an abnormal result. For instance, it might show that you have anaemia (low number of red blood cells), or that you have an abnormal number of white blood cells or platelets. If this happens we shall contact you and ask you to visit your family doctor or an emergency doctor for a repeat blood test.

**What will the study cost me?**

There is no cost to you apart from the time that you give.

## **Confidentiality**

No material that could personally identify you will be used in any reports of this study. Your blood samples will be labelled with a study code number only. Study records will be stored on a password-protected computer and in a locked filing cabinet. Computers and files are kept in a restricted-entry facility, and will only be used by the study researchers. Records from the study will be kept for 10 years by the investigators, and then destroyed in a confidential manner.

## **Results of the study**

The overall results of the study (from which you cannot be identified) will be published in scientific journals and presented at research meetings. You can request a copy of the study results by indicating your choice on the consent form. If you need further information please contact the Principal Investigator.

## **Ethical approval**

This study has received ethical approval from the Central Regional Ethics Committee.

*The study team thank you for participating.*

**If you have any questions or concerns about your rights as a participant in this study you may wish to contact a Health and Disability Advocate, telephone:**

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### **Principal Investigator**

**Dr Robert Weinkove**

Clinical Research Fellow

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Email: [rweinkove@malaghan.org.nz](mailto:rweinkove@malaghan.org.nz)

### **Co-Investigator**

Professor John Carter

Consultant Haematologist

Blood & Cancer Centre

Wellington Hospital

Wellington

Phone: (04) 385 5999 ext 6756

## **Compensation**

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

**iNKT cells in CLL study. Information sheet for Volunteers version 5, 08/06/2009**

**This study is voluntary (your choice). You may withdraw from the study at any time without affecting your health care now or in the future.**





## **Appendix C**

# **Participant questionnaire**

## Questionnaire

### iNKT cells in Chronic Lymphocytic Leukaemia (CLL) study

Name:	_____
Address:	_____ _____ _____
Telephone:	H: _____ M: _____
Date of birth:	_____
Email:	_____

*Please circle your answer to each question.*

**Q1. Do you have CLL (chronic lymphocytic leukaemia)?** Yes / No

**Q2. Do you have or have you ever had cancer (except CLL)?** Yes / No

**Q3. Have you ever received chemotherapy or radiotherapy?** Yes / No

**Q4. Are you taking or have you ever taken any of the following medicines?**

Steroid tablets or injections (e.g. prednisolone, dexamethasone, hydrocortisone) Yes / No

Other immune suppression medicines (e.g. azathioprine, ciclosporin, tacrolimus, mycophenolate, cyclophosphamide) Yes / No

**Q5. Do you have an infection at the moment?** Yes / No

*This section of the questionnaire is for participants with CLL only:*

**Q6. When was your CLL diagnosed?** \_\_\_\_\_

**Q7. Do you experience drenching night sweats?** Yes / No

**Q8. Have you had unexplained weight loss in the past 6 months?** Yes / No

**Q9. Do you experience unexplained fevers?** Yes / No

*Investigator use only:*

**Nodes:** \_\_\_\_\_ **Hepatomegaly:** YES/NO **Splenomegaly:** YES/NO

## **Appendix D**

### **Consent forms**

## CONSENT FORM (patients)

### iNKT cells in Chronic Lymphocytic Leukaemia (CLL) study

<b>Request for interpreter: Please circle your response below</b>			
English	I wish to have an interpreter.	<b>Yes</b>	<b>No</b>
Maori	E hiahia ana ahau ki tetahi kaiwhakamaori/kaiwhaka pakeha korero.	<b>Ae</b>	<b>Kao</b>
Cook Island	Ka inangaro au i tetai tangata uri reo.	<b>Ae</b>	<b>Kare</b>
Fijian	Au gadreva me dua e vakadewa vosa vei au.	<b>Io</b>	<b>Sega</b>
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu.	<b>E</b>	<b>Nakai</b>
Samoan	Ou te mana'o ia i ai se fa'amatala upu.	<b>loe</b>	<b>Leai</b>
Tokelaun	Ko au e fofou ki he tino ke fakaliliu te gagana Peletania ki na gagana o na motu o te Pahefika	<b>loe</b>	<b>Leai</b>
Tongan	Oku ou fiema'u ha fakatonulea.	<b>Io</b>	<b>Ikai</b>

<b>Consent: Please circle your responses below</b>		
I have read and understand the information sheet dated 08/06/2009 for patients taking part in the iNKT cells in CLL study. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.	<b>Yes</b>	<b>No</b>
I have had the opportunity to use whanau support or a friend to help me ask questions and understand the study.	<b>Yes</b>	<b>No</b>
I have had time to consider whether I will take part in this study.	<b>Yes</b>	<b>No</b>
I understand that taking part in this study is voluntary, that I may withdraw from the study at any time and that this would not affect my future healthcare.	<b>Yes</b>	<b>No</b>
I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.	<b>Yes</b>	<b>No</b>
I know who to contact if I have any side effects after having my blood taken or have any questions about the study.	<b>Yes</b>	<b>No</b>
I agree to the researchers storing a specimen of my blood for its use in this study, or for extensions to this study approved by the Central Regional Ethics Committee.	<b>Yes</b>	<b>No</b>
I agree to any remaining samples being destroyed at the end of the study.	<b>Yes</b>	<b>No</b>
I understand that I should tell the researcher if I would like any remaining samples disposed with appropriate karakia at the end of the study.	<b>Yes</b>	<b>No</b>
I agree to the researchers sending an anonymous sample of my blood to Capital & Coast District Health Board to check my blood count	<b>Yes</b>	<b>No</b>
I agree to the researchers contacting me if my blood count result is unexpectedly abnormal.	<b>Yes</b>	<b>No</b>
I agree to the researchers sending anonymous samples of my blood to Capital & Coast District Health Board and Canterbury Health Laboratories to measure markers of immune function and test for genetic changes in the leukaemia cells.	<b>Yes</b>	<b>No</b>
I agree to the researchers reviewing relevant medical records to check that I am suitable for the study and to record information about the stage of my CLL.	<b>Yes</b>	<b>No</b>
I wish to receive a copy of the results of the study.	<b>Yes</b>	<b>No</b>



## CONSENT FORM (volunteers)

### iNKT cells in Chronic Lymphocytic Leukaemia (CLL) study

<b>Request for interpreter: Please circle your response below</b>			
English	I wish to have an interpreter.	<b>Yes</b>	<b>No</b>
Maori	E hiahia ana ahau ki tetahi kaiwhakamaori/kaiwhaka pakeha korero.	<b>Ae</b>	<b>Kao</b>
Cook Island	Ka inangaro au i tetai tangata uri reo.	<b>Ae</b>	<b>Kare</b>
Fijian	Au gadreva me dua e vakadewa vosa vei au.	<b>Io</b>	<b>Sega</b>
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu.	<b>E</b>	<b>Nakai</b>
Samoan	Ou te mana'o ia i ai se fa'amatala upu.	<b>loe</b>	<b>Leai</b>
Tokelaun	Ko au e fofou ki he tino ke fakaliliu te gagana Peletania ki na gagana o na motu o te Pahefika	<b>loe</b>	<b>Leai</b>
Tongan	Oku ou fiema'u ha fakatonulea.	<b>Io</b>	<b>Ikai</b>

<b>Consent: Please circle your responses below</b>		
I have read and understand the information sheet dated 08/06/2009 for volunteers taking part in the iNKT cells in CLL study. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.	<b>Yes</b>	<b>No</b>
I have had the opportunity to use whanau support or a friend to help me ask questions and understand the study.	<b>Yes</b>	<b>No</b>
I have had time to consider whether I will take part in this study.	<b>Yes</b>	<b>No</b>
I understand that taking part in this study is voluntary, that I may withdraw from the study at any time and that this would not affect my future healthcare.	<b>Yes</b>	<b>No</b>
I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.	<b>Yes</b>	<b>No</b>
I know who to contact if I have any side effects after having my blood taken or have any questions about the study.	<b>Yes</b>	<b>No</b>
I agree to the researchers storing a specimen of my blood for its use as a part of this study or extensions to this study approved by the Regional Ethics Committee.	<b>Yes</b>	<b>No</b>
I agree to any remaining samples being destroyed at the end of the study.	<b>Yes</b>	<b>No</b>
I understand that I should tell the researcher if I would like any remaining samples disposed with appropriate karakia at the end of the study.	<b>Yes</b>	<b>No</b>
I agree to the researchers sending an anonymous sample of my blood to Capital & Coast District Health Board to check my blood count and to measure markers of immune function.	<b>Yes</b>	<b>No</b>
I agree to the researchers contacting me if my blood count result is unexpectedly abnormal.	<b>Yes</b>	<b>No</b>
I wish to receive a copy of the results of the study.	<b>Yes</b>	<b>No</b>







## Appendix E

# Publications

### Publications resulting from work presented in this thesis:

Weinkove R, Brooks CR, Carter JM, Hermans IF, Ronchese F. Functional invariant natural killer T cell and CD1d axis in chronic lymphocytic leukemia: implications for immunotherapy. *Haematologica* 2012 **97**:xxx [Epub ahead of print]  
doi: 10.3224/haematol.2012.072835

### Publications contributed to during the PhD studies:

Hunn MK, Farrand KJ, Broadley KWR, Weinkove R, Ferguson P, Miller RJ, Field CS, Petersen T, McConnell MJ, Hermans IF. Vaccination with irradiated tumor cells pulsed with an adjuvant that stimulates NKT cells is an effective treatment for glioma. *Clinical Cancer Research* 2012; **18(23)**: 1-14.

Dangerfield EM, Cheng JM, Knight DA, Weinkove R, Dunbar PR, Hermans IF, Timmer MS, Stocker BL. Species-specific activity of glycolipid ligands for invariant NKT cells. *Chem-biochem* 2012; **13(9)**:1359-56.

Brooks CR, Weinkove R, Hermans IF, van Dalen CJ, Douwes J. Invariant natural killer T cells and asthma: immunologic reality or methodologic artifact? *Journal of Allergy and Clinical Immunology* 2010; **126(4)**:882-885.