Functional invariant natural killer T-cell and CD1d axis in chronic lymphocytic leukemia: implications for immunotherapy

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ABSTRACT

Invariant natural killer T cells recognize glycolipid antigens such as α-galactosylceramide presented by CD1d. In preclinical models of B-cell malignancies, α-galactosylceramide is an adjuvant to tumor vaccination, enhancing tumor-specific T-cell responses and prolonging survival. However, numerical and functional invariant natural killer T-cell defects exist in patients with some cancers. Our aim was to assess this axis in patients with chronic lymphocytic leukemia. The numbers of circulating invariant natural killer T cells and the expression of CD1d on antigen-presenting cells were evaluated in patients with chronic lymphocytic leukemia and age-matched controls. Cytokine profile and *in vitro* proliferative capacity were determined. Patient- and control-derived invariant natural killer T-cell lines were generated and characterized, and allogeneic and autologous responses to α-galactosylceramide-treated leukemia cells were assessed. Absolute numbers and phenotype of invariant natural killer T cells were normal in patients with untreated chronic lymphocytic leukemia, and cytokine profile and proliferative capacity were intact. Chemotherapy-treated patients had reduced numbers of invariant natural killer T cells and myeloid dendritic cells, but α-galactosylceramide-induced proliferation was preserved. Invariant natural killer T-cell lines from patients lysed CD1d-expressing targets. Irradiated α-galactosylceramide-treated leukemic cells elicited allogeneic and autologous invariant natural killer T-cell proliferation, and α-galactosylceramide treatment led to increased proliferation of conventional T cells in response to tumor. In conclusion, the invariant natural killer T-cell and CD1d axis is fundamentally intact in patients with early-stage chronic lymphocytic leukemia and, despite reduced circulating numbers, function is retained in fludarabine-treated patients. Immunotherapies exploiting the adjuvant effect of α-galactosylceramide may be feasible.

Introduction

Invariant natural killer T (iNKT) cells are innate-like T cells expressing a semi-invariant T-cell receptor that recognizes glycolipid antigens presented by the evolutionarily-conserved CD1d molecule. iNKT cell agonists can be used as adjuvants to tumor vaccination for the cellular immunotherapy of cancer.[1] In preclinical models, co-administration of the prototypical iNKT agonist α-galactosylceramide (α-GalCer)[2] with tumor-associated antigens leads to enhanced proliferation of peptide-specific $CD4^+$ and $CD8^+$ T cells,[3] and protective immunity against subsequent tumor challenge.[4-7] The mechanism of this adjuvant activity involves presentation of α-GalCer to iNKT cells by resident dendritic cells (DC),[8] DC maturation,[4] and enhanced DC production of interleukin-12.[9]

Chronic lymphocytic leukemia (CLL) represents an attractive target for cellular immunotherapy: a graft-*versus*-leukemia effect can be observed after allogeneic stem cell transplantation,[10] peptide vaccination can induce leukemia-specific cytotoxic T cells,[11] and remission can be induced using autologous T cells bearing a chimeric antigen receptor directed against CD19.[12] However, significant immunological defects have been documented in patients with CLL, which may disrupt immunotherapeutic approaches. These defects include abnormal T-cell cytokine production[13] and gene expression profiles,[14] disruption of immunological synapse formation,[15] and phenotypic and functional DC defects.[16]

Reduced numbers of iNKT cells have been reported in patients with advanced solid cancers[17,18] and hematologic malignancies,[19,20] and functional iNKT defects have been described in advanced cancer, progressive myeloma and untreated chronic myeloid leukemia.[21-23] The number and function of iNKT cells in patients with CLL has not been previously reported. The development of immunotherapies exploiting the iNKT cell/CD1d axis depends upon the integrity of this system in patients. We, therefore, sought to determine the number, phenotype and function of iNKT cells from patients with CLL, to evaluate CD1d expression, and to determine whether iNKT cells might be employed as adjuvants in the immunotherapy of CLL.

Design and Methods

Patients and control donors

Venous blood was collected from 40 patients who met the diagnostic criteria for CLL (immunophenotypic score ≥ 4)[24] and from 30 healthy, age-matched controls. Exclusion criteria included another

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active malignancy or previous chemotherapy for a disease other than CLL. All donors gave written informed consent. Ethical approval for the study was obtained from the Central Regional Ethics Committee of New Zealand.

Binet and Rai prognostic scores were determined from the clinical examination and absolute differential blood counts (Sysmex XE 2100 analyzer, Roche Diagnostics, Auckland, New Zealand) measured at the time of blood sampling. Details of peripheral blood mononuclear cell (PBMC) isolation and storage, and B-cell depletion and enrichment are given in the *Online Supplementary Design and Methods*.

Flow cytometric analysis

Human CD1d monomer was obtained from the National Institutes of Health (NIH) Tetramer Core Facility, loaded with α-GalCer from Industrial Research Laboratories (Lower Hutt, New Zealand), and tetramerized with streptavidin-PE (BD Biosciences). iNKT cells were identified using sequential gating (*Online Supplementary Figure S1*) and anti-iNKT cell antibody (clone 6B11), which we and others have shown is comparable to the α-GalCer-loaded CD1d tetramer (*data not shown*).[25] Myeloid DC were identified as $Lin1^{-}$, $HLA\text{-}DR^{high}$, $CD11c^{high}$ – in validation experiments, a median of 98.5% of cells gated with this strategy expressed the specific myeloid DC marker, CD1c. ZAP-70 status of CLL cells was determined as previously described.[26] Further details of surface and intracellular staining and flow cytometry are given in the *Online Supplementary Design and Methods*.

Enyzme-linked immunosorbent spot assay

An interferon-γ enzyme-linked immunosorbent spot (ELISpot) assay was performed using a commercial kit (BD Biosciences) according to the manufacturer's instructions: $1x10^6$ B-cell-depleted PBMC were washed, resuspended in complete Iscove's modified Dulbecco's medium (cIMDM) and dispensed into coated ELISpot plates. α-GalCer 200 ng/mL, phosphate-buffered saline/Tween vehicle (as a negative control), or protein phytohemagglutinin (PHA-P) 1 μg/mL (Sigma-Aldrich) (as a positive control) were added, and plates cultured for 18 h at 37°C. After washing and secondary steps, ELISpots were enumerated by an automated reader (Autoimmun Diagnostika, Strassberg, Germany).

Proliferation of invariant natural killer T cells and generation of cell lines

B-cell-depleted PBMC were cultured at 37°C in cIMDM and 100 ng/mL α-GalCer or vehicle. Recombinant human interleukin-2 50 U/mL (Chiron Corporation, Emeryville, CA, USA) was added at 24 h. Cells were harvested after 7 days: live cells were enumerated using trypan blue, while iNKT cells were enumerated by flow cytometry.

Following initial expansion, iNKT cells co-expressing CD3 and Vα24Jα18 T-cell receptor-α chain (6B11 antibody) were purified by fluorescence-activated cell sorting (FACS), then re-stimulated with α-GalCer-pulsed, γ-irradiated (50 Gy) CD1d-transfected C1R cells (C1R-CD1d; a gift from Professor Vincenzo Cerundolo, Oxford, UK). Polyclonal iNKT cell lines were re-stimulated every 2 to 3 weeks, and maintained in media containing 50 U/mL interleukin-2. For six of eight cell lines, FACS purification was repeated after the second re-stimulation to maintain the purity of iNKT cells >95%.

Cytokine production

C1R-CD1d cells ($2x10^4$) were pulsed with 200 ng/mL α-GalCer or vehicle for 2 h, γ-irradiated, then added to $4x10^4$ cultured iNKT cells. After 4 h, supernatant was harvested for analysis by cytokine bead array (Milliplex; Millipore Corporation, Billerica, MA, USA) on a Bio-Plex analyzer (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

Cytotoxicity of invariant natural killer T-cell lines

Cytotoxicity against untransfected or CD1d-transfected C1R cell lines was assessed by a lactate dehydrogenase release assay (Sigma-Aldrich), as described in the manufacturer's instructions, using a spectrophotometric microplate reader (Versamax, Molecular Devices, Surrey Hills, VIC, Australia). Specific lysis was determined as previously reported.[27]

Allogeneic response to chronic lymphocytic leukemia cells treated with α-galactosylceramide

Purified CLL cells were incubated for 18 h with 200 ng/mL α-GalCer or vehicle, γ-irradiated and washed four times before addition at a dose of $4x10^4$ cells/well to wells of a 96-well plate, each well containing $2x10^5$ healthy donor PBMC, and then cultured in cIMDM for 5 days before flow cytometry to assess iNKT cell frequency.

Autologous response to chronic lymphocytic leukemia cells treated with α-galactosylceramide

Immunomagnetically-selected CLL cells were treated with α-GalCer or vehicle, irradiated, washed four times, and cryopreserved. Subsequently, $1x10^5$ to $1x10^6$ CLL cells were thawed, washed, and added back to $1x10^6$ autologous B cell-depleted PBMC cultured with 1 ng/mL interleukin-7. After two further weekly stimulations, iNKT cell frequency was determined by flow cytometry. To assess proliferation of specific T-cell subsets, B-cell-depleted PBMC ($2x10^5$ per well) were labeled with 0.2 μM carboxyfluorescein succinimidyl ester (CFSE) before addition of $4x10^4$/well purified, vehicle- or α-GalCer-treated, irradiated CLL cells as stimulators, in 96-well plates. After culture for 5 days without exogenous cytokines, cultures were harvested and analysed by flow cytometry.

Statistical analysis

Data were analyzed using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Non-parametric tests were used except where stated otherwise. The Mann-Whitney U test was used for unpaired data, a Wilcoxon's matched pairs test for paired data, and Kruskal Wallis one-way analysis of variance with Dunn's post-test for multiple comparisons. P values of < 0.05 were considered statistically significant.

Results

Characteristics of the donors

Thirty untreated patients, ten chemotherapy-treated patients, and 30 healthy, age-matched controls participated in the study. Patient and control groups were closely age-matched (*Online Supplementary Table S1*). Blood samples were taken from the patients a median of 63 months (range, 1 – 302 months) after diagnosis. Treated patients had received a median of two lines of chemotherapy, including fludarabine in all but one case, and had last received chemotherapy a median of 22 months previously (range, 3 – 88 months). The participants' characteristics are listed in *Online Supplementary Table S2*.

Numbers and phenotype of circulating invariant natural killer T cells

The frequency of circulating iNKT cells, as a proportion of all T cells, was determined by flow cytometry.

Compared to controls, a small but significant reduction in iNKT cell frequency was observed in patients (median of 0.02% of all viable T cells in controls; 0.01% in patients) (Figure 1A). Among the patients, no significant relationships were found between iNKT cell frequency and clinical disease stage, absolute lymphocyte count, prior treatment status, or expression of the adverse prognostic marker ZAP-70 (Table 1 and *data not shown*). In keeping with reported findings, we observed a significant inverse correlation between iNKT cell frequency and age (r^2 = -0.28; P<0.05).[28]

Because absolute T-cell numbers are increased in patients with untreated CLL,[13] we determined absolute numbers of T-cell subsets and iNKT cells. Relative to healthy controls, untreated CLL patients had increased absolute numbers of $CD4^+$, $CD8^+$ and double-negative T cells, while declines in all three subsets were seen in treated patients. Absolute numbers of circulating iNKT cells were similar in untreated patients and healthy controls, but were reduced in chemotherapy-treated patients (Figure 1B, *Online Supplementary Table S1*).

The $CD4^+$ subset of iNKT cells has been associated with the production of Th2-type cytokines including interleukins-4 and -13, while the $CD8^+$ subset degranulates in response to allogeneic CLL cells.[29] Trends towards a greater proportion of $CD4^+$, and a lower proportion of $CD8^+$, iNKT cells were observed in patients with CLL, although these did not reach statistical significance (Figure 1C). In both patients and controls, the proportion of $CD4^+$ iNKT cells was inversely correlated with the iNKT cell frequency (r^2 = -0.41; P<0.001) (*data not shown*). Frequencies of iNKT cells expressing the natural killer receptor CD161 and activation marker CD25 were similar.

CD1d expression and myeloid dendritic cell numbers in chronic lymphocytic leukemia

As CD1d is essential for the presentation of glycolipids to iNKT cells,[2] and abnormalities of CD1 expression have been reported in CLL,[16] CD1d expression was investigated. The highest levels of CD1d expression were found on myeloid DC, while leukemic B cells expressed CD1d at a lower level (Figure 2A). Compared with normal B cells, CLL cells had slightly reduced CD1d expression (Figure 2B). CLL cell CD1d expression did not differ between cases of early (Binet stage A) and clinically advanced (Binet stage B or C) CLL (*data not shown*).

Because myeloid DC expressed the highest levels of CD1d, and the frequency of myeloid DC has been reported to be reduced in CLL,[30] the absolute numbers of myeloid DC were determined. The numbers of circulating myeloid DC in patients with untreated CLL were similar to those in controls, but were significantly reduced in treated patients (median $10.7x10^3$/mL in controls; $9.4x10^3$/mL in untreated patients; $3.0x10^3$/mL in treated patients) (Figure 2C, *Online Supplementary Table S1*). Surface expression of CD1d on circulating myeloid DC was similar in patients and healthy donors, although monocyte CD1d expression was reduced in patients (Figure 2D).

Cytokine profile and proliferative capacity of invariant natural killer T cells in chronic lymphocytic leukemia

To investigate iNKT cell function, interferon-γ production in response to α-GalCer was assessed by an ELISpot assay. The addition of α-GalCer to PBMC resulted in an increase in interferon-γ spot-forming units (SFU) in ten out of 11 patients and in all 12 controls tested (Figure 3 A-B). The frequency of α-GalCer-induced interferon-γ SFU was similar in patients and controls (median 69.5 SFU/10^6 PBMC in controls; 104 in patients: difference not significant) (Figure 3B). Interferon-γ responses to α-GalCer were observed in two out of three untreated patients in Binet stage B or C and in all three fludarabine-treated patients tested.

Consistent with prior reports, interferon-γ production by conventional T cells (as shown by intracellular cytokine staining, Figure 3C) was significantly increased in patients with CLL (Figure 3D),[31] although no significant difference was observed between patient and control iNKT cell cytokine production (Figure 3E).

To assess α-GalCer-presenting function and the capacity of iNKT cells to proliferate, B-cell-depleted PBMC from patients and controls were cultured in the presence of α-GalCer and interleukin-2, and iNKT cell numbers were determined on day 7 (Figure 3F). An increase in the number of iNKT cells following culture was observed in 21 of 25 patients (including four of five fludarabine-treated patients and all four patients with Binet stage B or C disease tested) and in 19 of 20 controls (Figure 3G), with a similar fold increase in iNKT cell numbers (median 37.9-fold increase in controls, 25.1-fold increase in patients; P=0.12). In both patients and controls, the proportion of $CD4^+$ iNKT cells increased after *in vitro* proliferation (P<0.01) (*data not shown*).

Characterization of invariant natural killer T-cell lines from patients with chronic lymphocytic leukemia

As direct *ex vivo* assessment of iNKT cell cytotoxic function was not feasible due to the low frequency of circulating iNKT cells, we generated polyclonal iNKT cell lines from four patients and four controls. All iNKT cell lines generated were >98% $CD4^+$ (*data not shown*). iNKT cell lines generated from patients and controls produced similar levels of Th1 and Th2-type cytokines, as determined by cytokine bead array (Figure 4A) or intracellular cytokine staining (Figure 4B). All iNKT cell lines expressed granzyme B (Figure 4C), and cell lines from one patient and one control cell were able to lyse the human lymphoblastoid C1R cell line in a CD1d- and α-GalCer-dependent manner (Figure 4D), indicating that functional iNKT cell lines can be generated from patients with CLL.

Allogeneic and autologous responses to chronic lymphocytic leukemia cells treated with α-galactosylceramide

Preclinical studies showed that the efficacy of irradiated whole tumor vaccines against B-cell malignancies is enhanced by co-administration with α-GalCer to activate iNKT cells.[7,32] To test this principle in CLL, washed, irradiated α-GalCer-treated CLL cells were added to healthy donor PBMC. This resulted in significant enrichment of allogeneic iNKT cells in eight out of nine donor/recipient pairs tested (Figure 5A).

To determine whether α-GalCer-treated leukemic cells could also recruit iNKT cells in the autologous setting, B-cell-depleted PBMC from patients were repeatedly stimulated with α-GalCer-treated autologous leukemic cells. This resulted in enrichment of iNKT cells in three patients tested (Figure 5B). A fourth patient tested did not respond (*data not shown*).

Preclinical data indicate that α-GalCer-treated whole tumor cells provide protective immunity by inducing tumor-specific CD4+ and CD8+ conventional T cells.[3,32] To ascertain whether proliferation of conventional CD4+ and CD8+ T cells in response to CLL was enhanced by α-GalCer treatment, a CFSE dilution assay was employed to track dividing cells. Vehicle- or α-GalCer-treated irradiated CLL cells were added back to B-cell-depleted, CFSE-labeled autologous PBMC. As expected, divided iNKT cells (CD3+, CD1d tetramer+, CFSE^{low}) were detected only

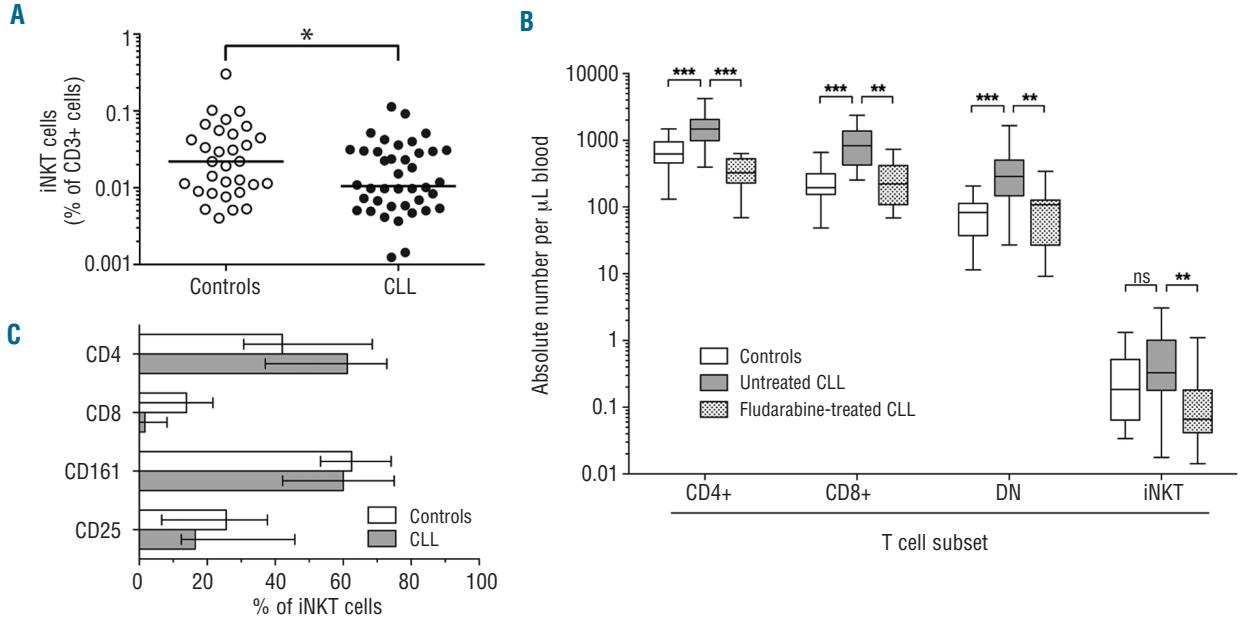

Figure 1. Numbers and phenotype of circulating iNKT cells in CLL. (A) Frequency of iNKT cells (as percent of viable T cells) in PBMC from patients with CLL (n=40) and healthy controls (n = 30) (*P<0.05). (B) Absolute number of circulating CD4+, CD8+, double negative (CD4-CD8-; DN) and iNKT cells from untreated patients (n=30), fludarabine-treated patients (n=9) and healthy controls (n=30) (***P<0.001; **P<0.01; ns: not significant). (C) Proportion of iNKT cells expressing CD4, CD8, CD161 and CD25 from patients (n=28) and healthy controls (n=24) (no significant differences).

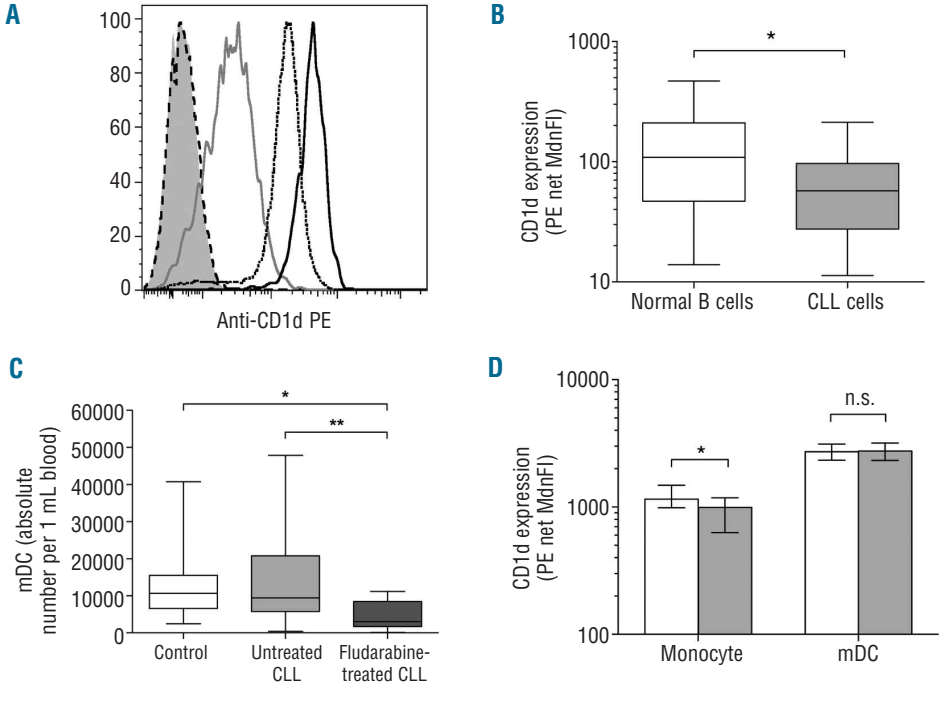

Figure 2. CD1d expression on circulating PBMC in patients with CLL. (A) Representative flow cytometry histogram showing CD1d expression on PBMC subsets from a patient with CLL. T-cell (dashed line), B-cell (gray line), monocyte (dotted line) and myeloid dendritic cell (mDC) (solid line) CD1d expression and fluorescence of matched isotype control-stained cells (solid gray) are shown. (B) CD1d expression on normal B cells (CD19+CD5-) and CLL cells (CD19+CD5+) from patients with CLL (n=20) (*P<0.05). (C) Absolute number of circulating mDC in healthy controls (n=30) and in patients with untreated (n=30) and fludarabine-treated CLL (n=9) (**P<0.01, *P<0.05). (D) CD1d expression levels on monocytes and on mDC from healthy controls (white bars, n=20) and patients with CLL (gray bars, n=20) (*P<0.05, ns: not significant).

when CLL cells had been treated with α-GalCer (Figure 5C). However, as well as inducing iNKT cell proliferation, treatment of CLL cells with α-GalCer resulted in an increased proportion of divided conventional T cells ($CD3^+$, CD1d tetramer$^-$, $CFSE^{low}$) in seven out of ten patients tested ($P<0.05$) (Figure 5D). These divided, non-α-GalCer-specific T cells included both $CD4^+$ and $CD8^+$ subsets (constituting a median of 43% and 24% of divided T cells in the α-GalCer-treated samples, respectively). α-GalCer treatment of CLL cells did not influence the cells' expression of MHC class I or II, or the co-stimulatory markers CD80 and CD86, confirming that this effect was not due to endotoxin contamination of reagents (*data not shown*).

Discussion

This report describes the first study comprehensively investigating the numbers and function of iNKT cells in patients with CLL. We found normal absolute numbers of circulating iNKT cells in patients with untreated CLL, with a reduction in chemotherapy-treated patients. Numbers and CD1d expression of myeloid DC were normal in untreated patients, and the cytokine profile and proliferative capacity of circulating iNKT cells was intact. Polyclonal iNKT cell lines could be derived from patients, and exhibited similar phenotypic and functional characteristics to those from healthy controls, including the ability to lyse a human B-cell line in a CD1d- and α-GalCer-

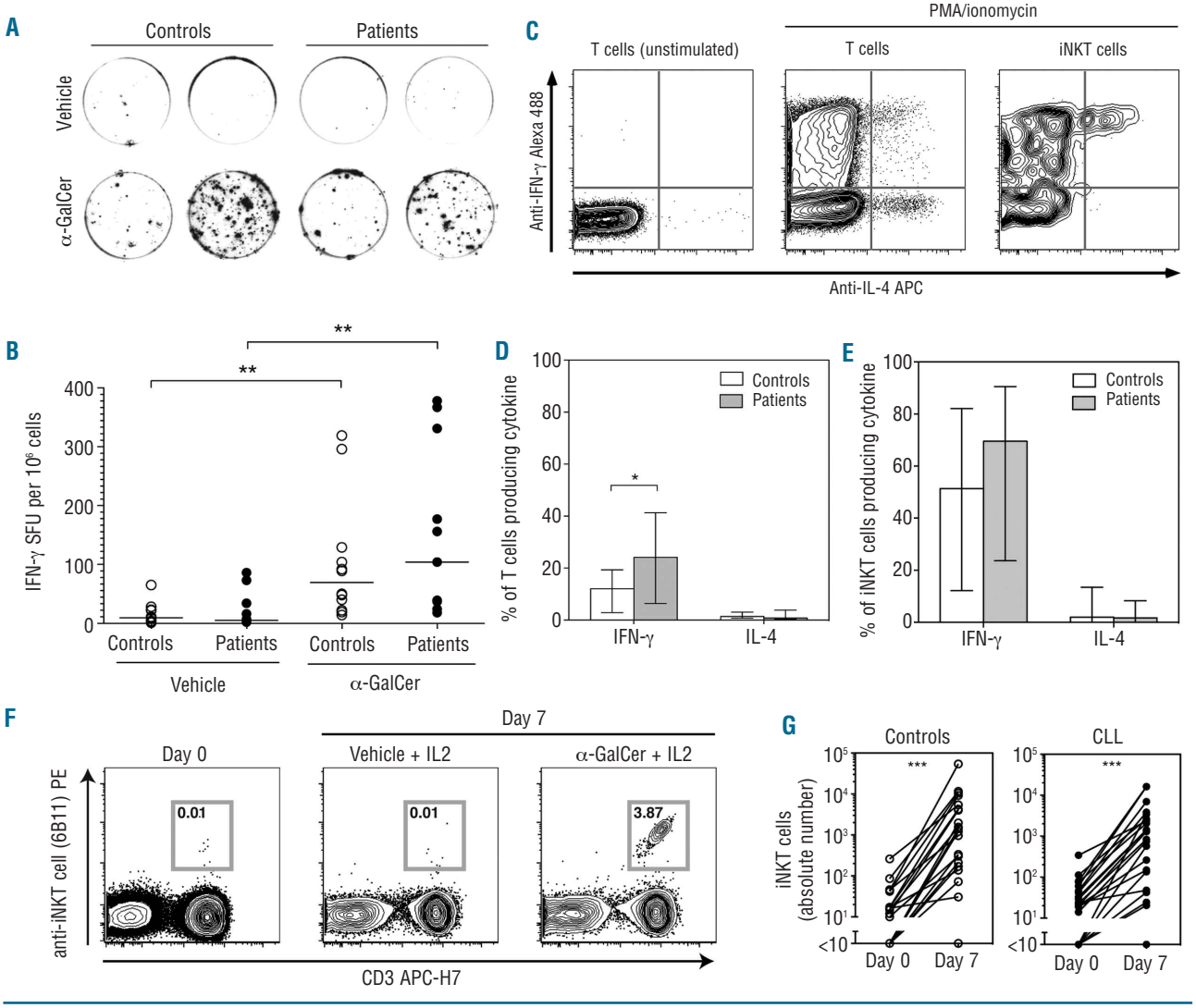

Figure 3. Function of circulating iNKT cells from patients with CLL. (A) Representative interferon (IFN)-γ ELISpot formation from two controls and two patients with CLL, in response to vehicle or α-GalCer. (B) α-GalCer-induced IFN-γ production assessed by ELISpot on B-cell-depleted PBMC from untreated patients (n=11) and healthy controls (n=12). Bars represent medians (**$P<0.01$; differences between controls and patients not significant). (C) Representative intracellular cytokine staining for IFN-γ and interleukin (IL)-4, gated separately on T cells and on iNKT cells. (D) Intracellular cytokine expression of T cells from patients with CLL (n=11) and healthy controls (n=11). Bars represent medians, error bars represent quartiles (*$P<0.05$). (E) Intracellular cytokine expression of iNKT cells from patients with CLL (n=11) and healthy controls (n=11). Bars represent medians, error bars represent quartiles (differences not significant). (F) Representative flow cytometry plots showing iNKT cell proliferation in response to α-GalCer and IL-2. Numbers represent percentage of $CD19^-$ lymphocyte events lying within the iNKT cell gate. (G) *In vitro* proliferation of iNKT cells from healthy controls (n=20) and patients (n=20) (***$P<0.001$).

dependent manner. Finally, α-GalCer-treated leukemic cells led to proliferation of both allogeneic and autologous iNKT cells, and α-GalCer treatment of CLL cells led to enhanced proliferation of conventional T cells.

The low frequency of circulating iNKT cells requires careful flow cytometric identification,[33,34] and age-matching of patient and control groups is essential in view of the known age-related decline in iNKT cell numbers.[28] To overcome methodological issues, the present study made use of a stringent gating strategy and the specific 6B11 antibody clone to identify iNKT cells, the results of which correlated strongly with those using an α-GalCer-loaded CD1d tetramer.

The finding of a normal absolute number, but reduced frequency, of circulating iNKT cells in untreated patients with CLL suggests that iNKT cells do not participate fully in the expansion of other T-cell subsets reported in CLL.[13] In contrast, in chemotherapy-treated patients, absolute numbers of both iNKT cells and myeloid DC were reduced. Treatment of CLL with fludarabine, cyclophosphamide and rituximab results in a prolonged reduction in circulating $CD4^+$, $CD8^+$ and γδ T-cell subsets, with relative preservation of the numbers of natural killer cells.[35] This study suggests that, following chemotherapy, iNKT cells are depleted to an extent similar to other T cells. However, as this was a cross-sectional rather than longitudinal study, the possibility that the lower iNKT cell numbers observed in treated patients were due to characteristics of the disease or patients, rather than chemotherapy, cannot be excluded.

Gene expression profiling has demonstrated lower levels of CD1d transcripts in CLL cells than in normal B cells,[36] and the surface expression of CD1d is lower on CLL cells than on the cells from patients with other indolent B-cell lymphoproliferative disorders.[37] This study confirms that CLL cells express surface CD1d, but at a level modestly lower than on normal B cells.[38] Despite this, we found that α-GalCer-treated CLL cells were able to elicit iNKT cell proliferation, confirming a previous finding.[39] Our demonstration of normal DC numbers in untreated CLL, and intact CD1d expression on myeloid DC, is also important, as DC are essential for the adjuvant effects of α-GalCer in models of cancer immunotherapy,[4,5] and a prior study showed down-regulation of the CD1a isoform on human DC cultured with CLL cells.[16]

Production of interferon-γ by iNKT cells is impaired in progressive myeloma,[22] and *in vitro* iNKT cell proliferation is reduced in untreated chronic myeloid leukemia.[23] In the present study, cytokine production by, and *in vitro* proliferation of, iNKT cells, was intact in CLL compared to age-matched controls.

Although we did not find major differences in iNKT cell number and function in the clinically advanced and chemotherapy-treated cases tested, most patients we studied had indolent CLL. We cannot exclude the possibility that numerical or functional iNKT cell defects would have emerged had a larger cohort of patients with advanced CLL been studied. Because of the limited prog-

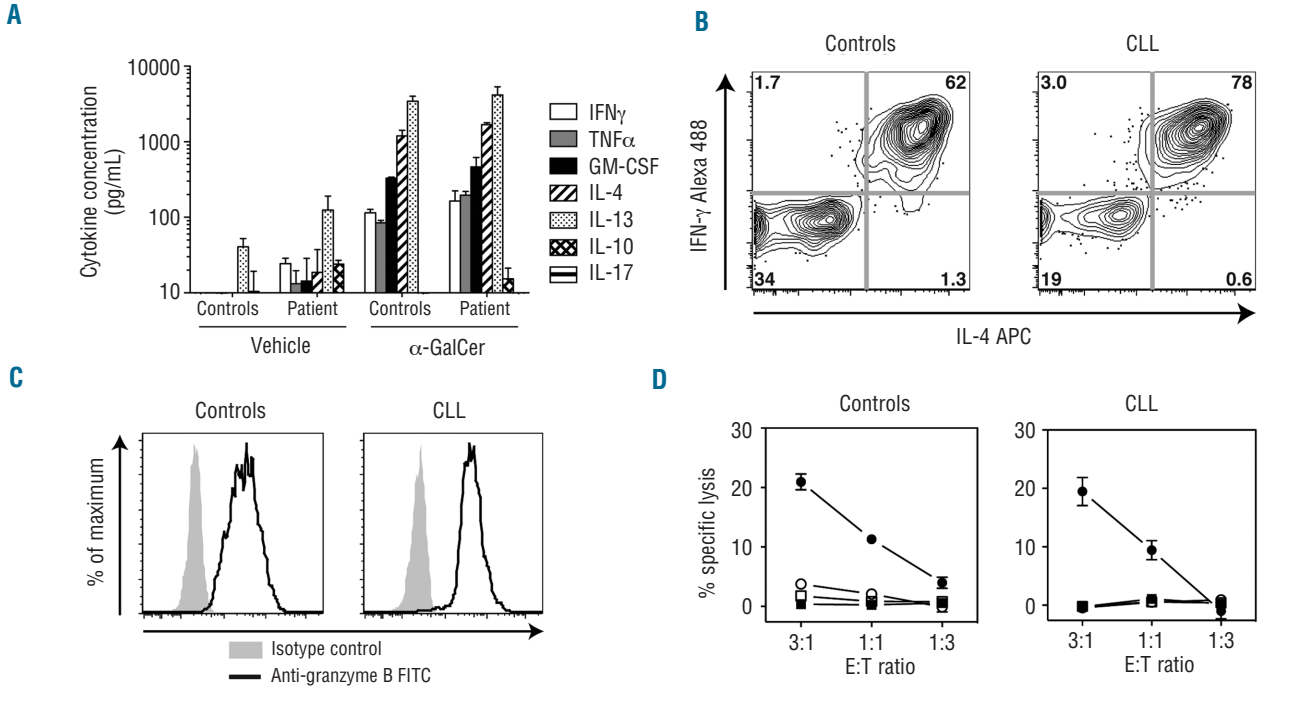

Figure 4. Patient-derived iNKT cell lines exhibit normal cytokine production and cytotoxicity. (A) Supernatant cytokine levels following 24h culture of patient-derived or control-derived iNKT cell lines with CD1d-transfected C1R cells in the presence of vehicle or α-GalCer. (B) Representative flow cytometry plots showing intracellular interferon (IFN)-γ and interleukin (IL)-4 expression of a patient- and a control-derived iNKT cell line. (C) Representative flow cytometry histograms showing granzyme B expression by a patient- and a control-derived iNKT cell line. (D) Cytotoxicity of a patient- and control-derived iNKT cell line against C1R target cells, as determined by lactate dehydrogenase release. Filled circles: CD1d-transfected C1R targets plus α-GalCer. Hollow circles: CD1d-transfected C1R targets plus vehicle. Filled squares: untransfected C1R targets plus α-GalCer. Hollow squares: untransfected C1R targets plus vehicle. Error bars represent the standard error of three replicates.

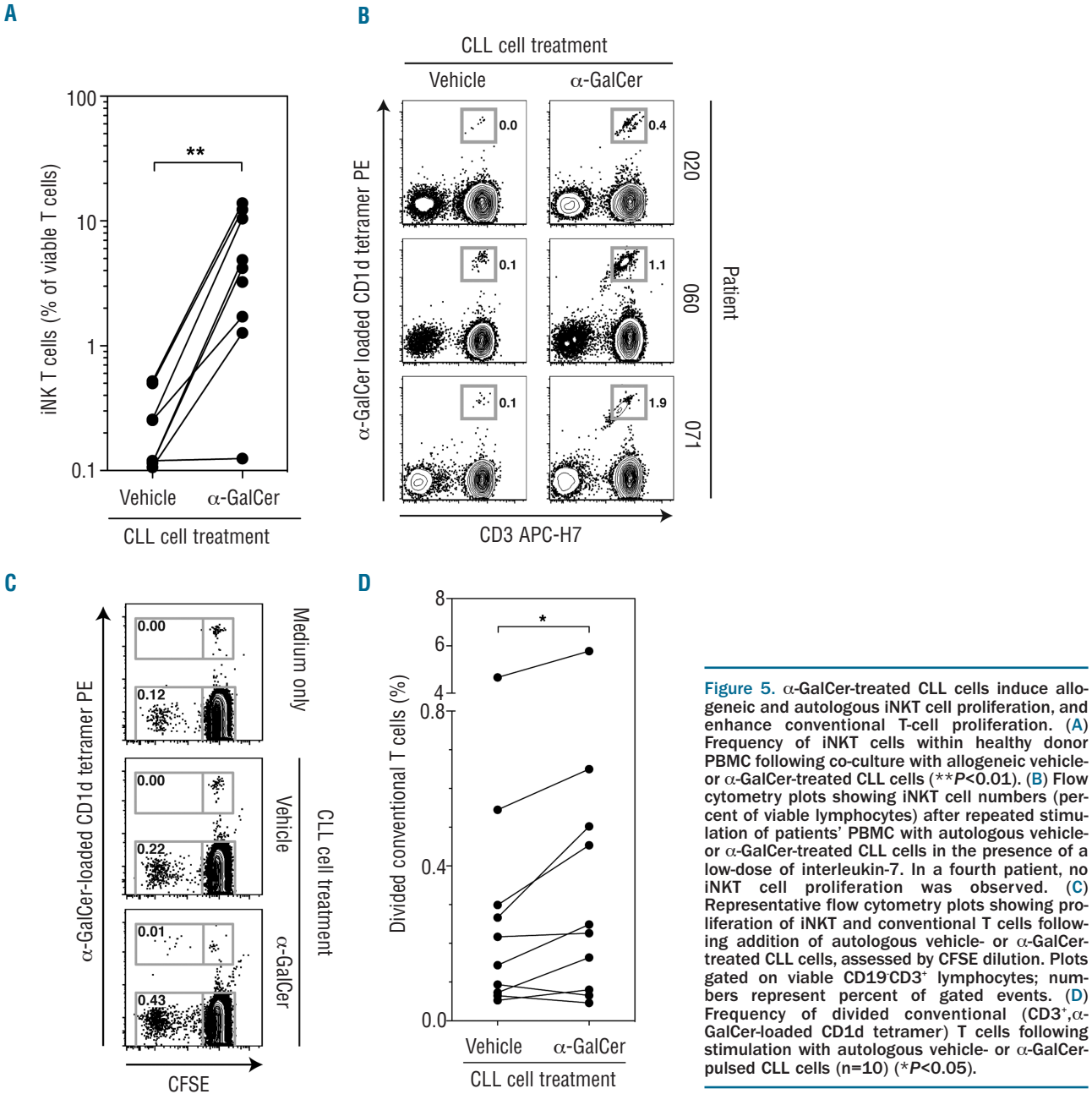

Figure 5. α-GalCer-treated CLL cells induce allogeneic and autologous iNKT cell proliferation, and enhance conventional T-cell proliferation. (**A**) Frequency of iNKT cells within healthy donor PBMC following co-culture with allogeneic vehicle- or α-GalCer-treated CLL cells (***P*<0.01). (**B**) Flow cytometry plots showing iNKT cell numbers (percent of viable lymphocytes) after repeated stimulation of patients' PBMC with autologous vehicle- or α-GalCer-treated CLL cells in the presence of a low-dose of interleukin-7. In a fourth patient, no iNKT cell proliferation was observed. (**C**) Representative flow cytometry plots showing proliferation of iNKT and conventional T cells following addition of autologous vehicle- or α-GalCer-treated CLL cells, assessed by CFSE dilution. Plots gated on viable $CD19^{-}CD3^{+}$ lymphocytes; numbers represent percent of gated events. (**D**) Frequency of divided conventional ($CD3^{+}$,α-GalCer-loaded CD1d tetramer) T cells following stimulation with autologous vehicle- or α-GalCer-pulsed CLL cells (n=10) (**P*<0.05).

nostic information available and the size of this cohort of patients, we cannot determine whether CLL cell cytogenetic changes or immunoglobulin heavy chain variable gene (*IGHV*) mutational status affect the iNKT cell/CD1d axis, although ZAP-70 expression, which is associated with unmutated *IGHV* status,[26] had no obvious impact on iNKT cell frequency.

Healthy donor-derived iNKT cell lines have been reported to lyse α-GalCer-pulsed CLL cells *in vitro*.[39] In this study, we have shown that iNKT cell lines with cytotoxic function can be generated from the peripheral blood of CLL patients. However, the prolonged *in vitro* culture of iNKT cells we employed yielded a $CD4^{+}$ iNKT cell population producing high levels of Th2-type cytokines, a feature associated with tolerance rather than tumor rejection in pre-clinical models.[40] This was observed in iNKT cell lines from both patients and healthy donors, and may be related to culture conditions.[41] Although adoptively-transferred iNKT cells have been shown to retard the growth of a CD1d-expressing lymphoid tumor *in vivo*,[42] we suggest that Th2 skewing of *in vitro*-generated iNKT cell lines generated in the presence of interleukin-2 might compromise this approach.

The immunological adjuvant properties of α-GalCer can be exploited by using α-GalCer-treated irradiated whole tumor cells as a vaccine.[3] In this strategy, tumor-associated antigens are taken up by resident DC and presented alongside α-GalCer. The recognition of α-GalCer by iNKT cells results in DC maturation, and enhanced conventional $CD4^{+}$ and $CD8^{+}$ T-cell responses against the co-presented

tumor-associated antigens, resulting in tumor protection. Vaccines comprising α-GalCer-treated tumor cells have proven effective against B-cell malignancies *in vivo*.[3,7,32] However, whether the low frequency of iNKT cells observed in older adults and in patients with cancer is a barrier to their use for immunotherapy is unknown.

In the present study, α-GalCer-treated, irradiated leukemic cells were readily derived from the peripheral blood of patients with CLL, and induced the proliferation of iNKT cells in both allogeneic and autologous settings. Intriguingly, an increase in the proliferation of non-CD1d restricted T cells was observed in response to autologous α-GalCer-treated CLL cells. Because of the low frequency of proliferating T cells we were not able to demonstrate their anti-tumor specificity. Nonetheless, we suggest that our findings justify clinical studies to determine whether the adjuvant effects of α-GalCer in preclinical immunotherapies for B-cell malignancies can be realized in humans.

In summary, this study demonstrates that iNKT cells are numerically and functionally intact in patients with untreated CLL, and that functional iNKT cell lines can be derived from patients. α-GalCer-treated irradiated CLL cells induce proliferation of both allogeneic and autologous iNKT cells and, compared to untreated CLL cells, lead to enhanced proliferation of conventional T cells. These findings may inform the development of immunotherapies targeting the iNKT cell and CD1d axis.

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Authorship and Disclosures

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