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Improving CLL Vγ9Vδ2-T cell fitness for cellular therapy by ex vivo activation and ibrutinib

Running title: Vγ9Vδ2-T cells in chronic lymphocytic leukemia

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Key points

- Healthy Vγ9Vδ2-T cells recognize and lyse CLL cells, but CLL-derived Vγ9Vδ2-T cells have impaired cytotoxicity and cytokine production.
- Vγ9Vδ2-T cell dysfunction is reversible upon *ex vivo* activation with autologous moDCs, and ibrutinib promotes an anti-tumor T_H1 phenotype.

Abstract

The efficacy of autologous (αβ)-T cell-based treatment strategies in chronic lymphocytic leukemia (CLL) has been modest. The Vγ9Vδ2T-cell subset comprises of cytotoxic T lymphocytes with potent anti-lymphoma activity via a major histocompatibility complex (MHC)-independent mechanism. We studied whether Vγ9Vδ2-T cells can be exploited as autologous effector lymphocytes in CLL.

Healthy control (HC) Vγ9Vδ2-T cells were activated by and had potent cytolytic activity against CLL cells. However, CLL-derived Vγ9Vδ2-T cells proved dysfunctional with respect to effector cytokine production and degranulation, despite an increased frequency of the effector-type subset. Consequently, cytotoxicity against malignant B cells was hampered. A comparable dysfunctional phenotype was observed in healthy Vγ9Vδ2-T cells after coculture with CLL cells, indicating a leukemia-induced mechanism. Gene expression profiling implicated alterations in synapse formation as a conceivable contributor to compromised Vγ9Vδ2-T cell function in CLL patients.

Dysfunction of Vγ9Vδ2-T cells was fully reversible upon activation with autologous monocyte-derived dendritic cells (moDCs). moDC-activation resulted in efficient expansion and predominantly yielded Vγ9Vδ2-T cells with a memory phenotype. Furthermore, ibrutinib treatment promoted an anti-tumor T_H1 phenotype in Vγ9Vδ2-T cells and we demonstrated binding of ibrutinib to IL-2-inducible kinase (ITK) in Vγ9Vδ2-T cells.

Taken together, CLL-mediated dysfunction of autologous Vγ9Vδ2-T cells is fully reversible, resulting in potent cytotoxicity towards CLL cells. Our data support the potential use of Vγ9Vδ2-T cells as effector T cells in CLL immunotherapy and favor further exploration of combining Vγ9Vδ2-T cell-based therapy with ibrutinib.

Introduction

Although novel drugs that inhibit key kinases of the B cell receptor (BCR) signaling pathway are valuable additions to the therapeutic arsenal of chronic lymphocytic leukemia (CLL), these agents are not curative and continuous treatment is required, which invokes toxicities and resistance[1].

Allogeneic hematopoietic stem cell therapy (allo-SCT) has demonstrated that T cell-based therapy has curative potential in CLL[2,3]. However, current autologous T cell-based approaches, such as checkpoint inhibition and chimeric antigen receptor T cells, have yielded limited response rates in CLL[4-8], specifically when compared to their activity in more aggressive lymphoproliferative diseases (e.g. acute lymphocytic leukemia)[9-11]. Activation of autologous T cells is constrained both by low immunogenicity of CLL cells and by acquired T cell dysfunction that progresses throughout the disease[12]. T cell abnormalities include altered cytokine secretion profiles, an exhausted phenotype, and compromised cytotoxicity of $CD8^+$-T cells[13,14], in addition to a subset distribution skewed towards an effector memory phenotype, particularly in CMV-positive patients[15].

One to ten % of $CD3^+$-T cells in the peripheral blood (PB) carries a highly conserved γδ-T cell receptor. Vγ9Vδ2-T cells form the predominant γδ-T cell subset present in the PB. In contrast to αβ-T cell antigen recognition, Vγ9Vδ2-T cells respond to stress molecules in malignant cells, in a T cell receptor (TCR)-dependent yet major histocompatibility complex (MHC)-independent process[16,17]. The Vγ9Vδ2-TCR is activated via non-peptidic phosphorylated antigens, or phosphoantigens, produced as intermediate metabolites in the mevalonate pathway during cellular stress, malignant transformation, or upon treatment with aminobisphosphonates[18,19]. Upon activation, Vγ9Vδ2-T cells have potent cytolytic activity against a wide variety of malignant cells including lymphoma cells[20-24]. Moreover, strategies based on Vγ9Vδ2-T cells rather than total CD3 activation have low toxicity[24-30]. Vγ9Vδ2-T cells could therefore represent an attractive alternative source of autologous effector T cells for CLL immunotherapy[20-24].

In support of the relevance of Vγ9Vδ2-T cells in CLL, Coscia et al. have previously described a correlation between a low proliferative response of Vγ9Vδ2-T cells to the aminobisphosphonate zolendronate and short time to first treatment (TTFT) status in CLL[31]. Diminished proliferation was mostly seen in CLL patients with unmutated immunoglobulin genes (U-CLL), who had higher phosphoantigen levels and a hyperactive mevalonate pathway in comparison to patients with mutated immunoglobulin genes (M-CLL). The mechanism responsible for the shorter TTFT, and the ability of Vγ9Vδ2-T cells from CLL patients to eradicate leukemic cells remains unexplored. Moreover, little is known about functional characteristics of this subset beyond proliferation and whether perturbations demonstrated in αβ-T cells extend to Vγ9Vδ2-T cells.

We therefore extensively characterized CLL-derived Vγ9Vδ2-T cells and studied whether they can be exploited as effector lymphocytes in CLL.

Materials and methods

Patient material

PB mononuclear cells (PBMCs) were isolated from PB samples from untreated CLL patients or age-matched healthy controls (HCs) or HC buffy coats from Sanquin Blood Supply (Amsterdam, the Netherlands) and cryopreserved as described previously (Table 1)[32]. The presence of monoclonal B-cell lymphocytosis was excluded in HCs by CD5, CD19, κ and λ immunophenotyping. The study was approved by the medical ethics committee at the Academic Medical Center. Written informed consent from all subjects was obtained in accordance with the Declaration of Helsinki.

Flow cytometry

Thawed PBMCs were stained with monoclonal antibodies (details in Supplementary text, Supplementary Table 1) and measured on an LSRFortessa cytometer (BD Biosciences). Samples were analyzed with Flowjo MacV10.

Cytotoxicity

$CD3^+$TCR-$Vγ9^+$TCR-$Vδ2^+$ cells were sorted from PBMCs, depleted of $CD19^+$ cells with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany; CD19 fraction <5%), on a FACSAria IIu 3-laser (BD Biosciences)**.** Thawed allogeneic CLL PBMCs (>95% $CD5^+CD19^+$) or Daudi (ATCC) were used as target cells, labeled with carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific, Waltham, MA, USA) and subsequently pretreated with 25 μM aminobisphosphonates (ABP) for 2 hours (pamidronate, TEVA Pharmachemie, Haarlem, the Netherlands) as indicated before coculture with sorted Vγ9Vδ2-T cells (purity >95%). Where indicated, Vγ9Vδ2-T cells were pretreated with 100 nM concanamycin A (Sigma-Aldrich, St. Louis, MO, USA) or DMSO control for 2 hours and washed before coculture.

Viability was measured using Mitotracker Orange (Invitrogen) and To-pro-3 (Invitrogen) on a FACSCanto (BD Biosciences).

Cytokine and degranulation assays

Target cells, thawed allogeneic CLL PBMCs ($CD5^+CD19^+$ >95%) or Daudi cells, were pretreated with 25 μM ABP or medium for 2 hours. CD19-depleted PBMCs were stimulated with target cells in a 1:5 Vγ9Vδ2-T cell effector:target cell ratio with anti-CD28 (2 μg/mL; 15E8, Sanquin) for 16-18 hours, or with phorbol-12-myristate-13-acetate (PMA; 10 ng/mL; Sigma-Aldrich) and ionomycin (1 μg/mL; Sigma-Aldrich) for 4 hours at 37°C. Brefeldin A (10 μg/mL; Invitrogen), GolgiStop and anti-CD107a APC (BD Biosciences) were present during the final 4-6 hours[33,34].

Where indicated, CD19-depleted PBMCs were pretreated with ibrutinib (Pharmacyclics, Sunnyvale, CA, USA) or CC-292 (Selleckchem, Houston, TX, USA) for 30 minutes before coculture.

Coculture assay

CLL or healthy B cells were isolated with anti-CD19 magnetic microbeads and cocultured with allogeneic CD19-depleted HC PBMCs for 36 hours in a 1:10 ratio. CD25 expression was measured and cells were cocultured with ABP-pretreated Daudi cells or stimulated with PMA/ionomycin for cytokine and degranulation assays as above.

moDC-based *ex vivo* Vγ9Vδ2-T cell activation and expansion

$CD3^+TCR\text{-}V\gamma9^+TCR\text{-}V\delta2^+$ cells were FACS-sorted as above and cultured with moDCs, as previously described[35]. In short, $CD14^+$ cells were isolated from HC or CLL PBMCs (anti-CD14 magnetic microbeads, Miltenyi) and cultured for 7 days in the presence of IL-4 (20 ng/mL, R&D Systems, Minneapolis, MN, USA) and granulocyte-macrophage colony-stimulating factor (100U/mL, Genzyme, Cambridge, MA, USA) ($0.45*10^6$ cells/mL) to generate immature moDCs. These immature moDCs were matured with LPS (100 ng/mL, Sigma-Aldrich) for 48 hours and ABP (100 µM) during the final 2 hours. Mature moDCs were then irradiated (5000 Rad) and cocultured with sorted Vγ9Vδ2-T cells in the presence of IL-7 (10 U/mL) and IL-15 (10 ng/mL, R&D Systems) for 2 weeks ($1*10^6$ Vγ9Vδ2-T cells and $0.2*10^6$ moDCs/mL). Vγ9Vδ2-T cells were restimulated with irradiated moDCs weekly. When using autologous moDCs, cultures were performed in medium supplemented with 10% autologous filter-sterilized serum.

Vγ9Vδ2-T cell generation from ABP/IL-2 treated PBMCs

CD19-depleted PBMCs were treated with ABP (25 µM) and IL-2 (1000 IU/mL, PeproTech, Rocky Hill, NJ, USA) for 2 weeks. The medium was refreshed with ABP and IL-2 containing medium 3 times per week.

RNA sequencing and data analysis

RNA was isolated (NucleoSpin RNA kit, Macherey Nagel, Düren, Germany) from FACS-sorted $CD3^+TCR\text{-}V\gamma9^+TCR\text{-}V\delta2^+$ cells. cDNA synthesis and amplification was performed with the Ovation RNA-seq System V2 (Nugen, San Carlos, CA, USA) followed by library preparation with the Ovation Ultralow System V2 (Nugen). Single-end 75 basepair sequencing was performed on an Illumina NextSeq 500 sequencer by GenomeScan (Leiden, the Netherlands).

RNA sequencing analyses were performed using R (v.3.4.3) and Bioconductor (v3.6) (see Supplementary text). Geneset enrichment analysis was performed using CAMERA, with a combination of all hallmark (collection H) and Biocarta (collection C2) genesets retrieved from the Molecular Signatures Database (MSigDB v6.1; Entrez Gene ID version) and 10 manually generated (Supplementary Table 2) genesets. Sequence data has been deposited at the European Genome-phenome Archive (EGA) under accession number EGAS00001003193.

Ibrutinib pull-down

1 µM biotinylated ibrutinib-derivative[36] was coupled to avidin agarose (Thermo Fisher Scientific; 30 minutes, room temperature). Remaining binding sites were blocked with 10 mM biotin (Sigma-Aldrich, 15 minutes, room temperature). HC-Vγ9Vδ2-T cells or Mec-1 (DSMZ) cells were lysed with NP-40 lysis buffer. Lysates were treated with 1 µM ibrutinib or 1 µM CC-292 (30 minutes, room temperature) and incubated overnight with ibrutinib-coupled or uncoupled control agarose at 4°C. Proteins were eluted by heating to 95°C in Laemmli buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 100 mM DTT) for 10 minutes and analyzed by Western blotting as described previously[37]. Blots were probed with anti-human BTK (611116, BD Biosciences) or IL-2-inducible T-cell kinase (ITK; 2380S, Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

Data were checked for normality with D'Agostino-Pearson normality tests and analyzed using two-sided paired or unpaired *t* tests, Mann-Whitney U test or one-way ANOVA (followed by Bonferroni or Dunnett's post hoc test) as indicated, with significance set at $P<0.05$ using GraphPad Prism software version 5.0. Statistical analyses of RNA sequencing data were performed using the edgeR and limma R/Bioconductor packages (see Supplementary text). Data are presented as mean and standard error of mean.

Results

Vγ9Vδ2-T cells are cytotoxic against CLL cells, yet cytotoxic function is impaired in CLL-derived Vγ9Vδ2-T cells

The capacity of CLL cells to activate Vγ9Vδ2-T cells was determined by measuring the expression of CD25 after coculture. HC-derived Vγ9Vδ2-T cells were activated by CLL cells, as demonstrated by robust induction of CD25 expression. In contrast, culture with allogeneic healthy B cells did not alter CD25 expression (Figure 1A and B).

The cytotoxic potency of Vγ9Vδ2-T cells against CLL was studied by measuring target cell death after coculture of sorted HC Vγ9Vδ2-T cells with CLL cells. The Burkitt lymphoma cell line Daudi was used as a positive control target (Supplementary Figure 1A). Vγ9Vδ2-T cells from HCs induced CLL cell death (Figure 1C). Cell death was granzyme-dependent since pretreatment of Vγ9Vδ2-T cells with concanamycin A, which prevents granule exocytosis, abrogated target cell death (Figure 1D).

Similarly, the cytotoxicity of CLL-derived Vγ9Vδ2-T cells towards allogeneic CLL cells was measured. Vγ9Vδ2-T cells from CLL patients were significantly less effective at inducing cell death in both CLL and Daudi target cells (Figure 1E).

To assess whether the impaired cytotoxicity of CLL-derived Vγ9Vδ2-T cells reflects diminished activation, expression of the activation marker CD69 was measured after coculture with Daudi cells. V9Vδ2-T cells from CLL patients were activated by the Daudi cells, but not as strongly as the HC Vγ9Vδ2-T cells (Figure 1F).

Taken together, these results indicate activation of Vγ9Vδ2-T cells by CLL, resulting in a granzyme-dependent cytotoxic response. However, cytotoxic function is impaired in Vγ9Vδ2-T cells from CLL patients, suggesting phenotypic and functional alterations of Vγ9Vδ2-T cells in the context of CLL.

CLL-derived Vγ9Vδ2-T cells are more differentiated and express less granzyme B

A phenotypical analysis of CLL-derived versus HC-Vγ9Vδ2-T cells was performed directly *ex vivo*. The absolute number as well as the proportion of Vγ9Vδ2-T cells was comparable in CLL patients and age-matched HCs (Figure 2A and B). Vγ9Vδ2-T cells can be divided into naïve, central memory (CM), effector memory (EM) and RA re-expressing effector memory (EMRA) subsets based on surface expression of CD27 and CD45RA[38-40]. Functionally, CM and naïve cells have a larger proliferative response, whereas EM and EMRA have a higher cytokine production and cytotoxic capacity. CLL-derived Vγ9Vδ2-T cell subsets were skewed, with a higher prevalence of EM cells, at the expense of naïve type cells (Figure 2C, Supplementary Figure 1B and C). In $CD8^+$ T cells from CLL patients, subset distribution is skewed, particularly in CMV-positive patients[15]. The subset distribution of Vγ9Vδ2-T cells was similar in CMV-positive and –negative CLL patients (Supplementary Figure 1D and E).

Similar to αβ-T cells, the expression of exhaustion markers correlates with dysfunction in Vγ9Vδ2-T cells[41,42]. Expression of PD-1, BTLA, CD160, CD244 and LAG-3 was therefore determined (Figure 2D). Of these markers, only CD160 was expressed higher on CLL-Vγ9Vδ2-T cells as compared to HC-Vγ9Vδ2-T cells. There were no significant differences between CMV-positive and –negative patients in the expression of exhaustion markers (Supplementary Figure 1F).

To obtain mechanistic insight into the impaired cytotoxic function of CLL-derived Vγ9Vδ2-T cells, the cytotoxic effector molecules granzyme B and K were quantified in unstimulated Vγ9Vδ2-T cells. CLL-derived Vγ9Vδ2-T cells contained less granzyme B than HC-derived Vγ9Vδ2 T cells. In contrast, the percentage of Vγ9Vδ2-T cells expressing granzyme K did not differ between HCs and CLL patients (Figure 2E).

The function of Vγ9Vδ2-T cells can be attenuated through natural killer cell receptors (NKRs) and NKG2D in particular promotes effector functions[43]. The functional impairments observed in Vγ9Vδ2-Tcells from CLL patients do not merely reflect lower NKG2D expression levels, which were in fact somewhat higher on CLL Vγ9Vδ2-T cells (Supplementary Figure 1G).

Diminished effector type cytokine production and cytotoxic potential of CLL-derived Vγ9Vδ2-T cells

Next, functional differences between HC- and CLL- Vγ9Vδ2-T cells in cytokine production were determined. Upon TCR-independent stimulation with PMA/ionomycin, Vγ9Vδ2-T cells from both HCs and CLL patients produced mainly effector type cytokines IFN-γ and TNF-α (Figure 3A). Significantly less CLL-Vγ9Vδ2-T cells produced IFN-γ (30.0% versus 51.4%) and TNF-α (30.0% versus 55.2%) than their healthy counterparts. As IFN-γ and TNF-α production was diminished, the possibility of T helper 2 (T_H2) skewing was analyzed. Vγ9Vδ2-T cells from CLL patients produced as much IL-4 as HC (Figure 3A).

Recently, a tumor-promoting role has been attributed to IL-17 producing γδ-T cells in solid malignancies[44]. The percentage of IL-17 producing Vγ9Vδ2-T cells was negligible in both HCs and CLL following PMA/ionomycin stimulation (Figure 3A).

Cytokine production upon contact with malignant B cells was then measured in Vγ9Vδ2-T cells. Coculture with Daudi cells triggered production of IFN-γ and TNF-α (Figure 3B). IFN-γ and TNF-α production was significantly impaired in CLL-Vγ9Vδ2-T cells as compared to HC-Vγ9Vδ2-T cells.

Moreover, expression of CD107a, a marker for degranulation, was significantly lower in Vγ9Vδ2-T cells from CLL patients in response to PMA/ionomycin (Figure 3C) as well as CLL cells (Figure 3D).

In summary, the production of IFN-γ and TNF-α as well as degranulation in response to both TCR-independent stimulation and malignant B cells is diminished in CLL Vγ9Vδ2-T cells, resulting in a diminished T_H1 response and cytotoxic capability.

CLL cells can induce dysfunction in healthy Vγ9Vδ2-T cells

We hypothesized that functional impairments in Vγ9Vδ2-T cells result from CLL-related immune suppression rather than an intrinsic Vγ9Vδ2-T cell defect. This was supported by the observation that cytotoxicity (Supplementary Figure 2A), effector type cytokine production (Supplementary Figure 2B), and degranulation (Supplementary Figure 2C) correlated inversely with absolute leukocyte count. The cytotoxic capacity, degranulation and effector type cytokine production of Vγ9Vδ2-T cells from U-CLL and M-CLL patients was comparable (Supplementary Figure 2D-F).

To test whether CLL cells could induce functional impairments in healthy Vγ9Vδ2-T cells, HC-Vγ9Vδ2-T cells were cultured for 48 hours in the presence of CLL cells or allogeneic healthy B cells. After subsequent coculture with Daudi cells as targets, Vγ9Vδ2-T cells that were cocultured with CLL cells produced significantly less IFN-γ and TNF-α than Vγ9Vδ2-T cells that were cocultured with healthy B cells or medium alone (Figure 4A). Similarly, degranulation was impaired in CLL-cocultured Vγ9Vδ2-T cells (Figure 4B).

Production of effector type cytokines (Figure 4C) and degranulation (Figure 4D) was also impaired upon TCR-independent stimulation with PMA/ionomycin in healthy Vγ9Vδ2-T cells that were cocultured with CLL cells. IL-4 production was not affected by CLL coculture.

In conclusion, CLL cells can induce dysfunction in healthy Vγ9Vδ2-T cells.

ABP pretreatment of CLL cells modestly augments cytotoxicity

Since ABP treatment can increase target cell recognition by Vγ9Vδ2-T cells through upregulation of phosphoantigen levels[18] and as ABP was previously shown to increase phoshoantigen production by CLL cells[31], we examined whether ABP treatment of target cells could overcome the observed dysfunction in CLL-derived Vγ9Vδ2-T cells.

First, we confirmed the expression of CD277/BTN3A1, the transmembrane protein required for phosphoantigen recognition[18], on CLL cells (Supplementary Figure 3A and B). Pretreatment of Daudi cells with ABP led to more IFN-γ producing Vγ9Vδ2-T cells and lysis of Daudi cells (Supplementary Figure 3C-F). In contrast, ABP pretreatment of CLL cells generated only a trend towards increased IFN-γ and TNF-α production by CLL-derived Vγ9Vδ2-T cells (Supplementary Figure 3C and D), yet did enhance degranulation (Supplementary Figure 3E). Correspondingly, CLL-derived Vγ9Vδ2-T cells lysed allogeneic CLL cells more efficiently after ABP treatment (Supplementary Figure 3F), although not to the level of HC- Vγ9Vδ2-T cells (Figure 1E).

Thus, ABP pretreatment of CLL cells has a modest sensitizing effect on lysis by Vγ9Vδ2-T cells in the context of dysfunctional Vγ9Vδ2-T cells from CLL patients.

Vγ9Vδ2-T cell dysfunction is reversible upon *ex vivo* activation

To examine the plasticity of Vγ9Vδ2-T cell dysfunction and the feasibility of Vγ9Vδ2-T cell expansion in CLL patients, sorted Vγ9Vδ2-T cells from HCs or CLL patients were activated *ex vivo* by culturing them for 2 weeks with phosphoantigen-expressing HC-derived moDCs in the presence of IL-7 and IL-15[35]. Samples from patients with a previously confirmed dysfunctional phenotype (see Figure 1E and Figure 3) were used.

Following *ex vivo* culture, cytokine production and degranulation upon coculture with Daudi cells was measured. The percentage of Vγ9Vδ2-T cells that produced IFN-γ, TNF-α or IL-4 did not differ between CLL- and HC-derived cultured Vγ9Vδ2-T cells (Supplementary Figure 4A), which was also true regarding degranulation (Supplementary Figure 4B). Furthermore, cultured Vγ9Vδ2-T cells from HCs and CLL patients killed an equal amount of Daudi cells (Supplementary Figure 4C). Thus, Vγ9Vδ2-T cell dysfunction in CLL patients is fully reversible upon *ex vivo* activation.

To adapt this activation method to a clinically achievable setting, the reversibility of the dysfunction was subsequently tested using autologous moDCs and serum. The proliferative capacity of Vγ9Vδ2-T cells was similar with allogeneic and autologous moDCs (mean expansion factor 87.00 ± 16.46 versus 76.42 ± 8.55) and was not impaired in CLL-derived Vγ9Vδ2-T cells in comparison to HC-derived Vγ9Vδ2-T cells (Figure 5A). Vγ9Vδ2-T cells from CLL patients and HCs were equally activated after culture as measured by CD25 expression (Supplementary Figure 4D).

After autologous moDC-activation, the number of cells that produced IFN-γ, TNF-α or IL-4 in response to Daudi cells was equivalent in CLL- and HC-derived Vγ9Vδ2-T cells (Figure 5B). Moreover, the reduction in degranulating CLL-derived Vγ9Vδ2-T cells (Figure 5C) and lysis of Daudi cells (Supplementary Figure 4C) was no longer observed after autologous moDC-activation. In line with this, granzyme B levels in Vγ9Vδ2-T cells increased during *ex vivo* activation and did not differ between HC- and CLL-derived Vγ9Vδ2-T cells (Figure 5D). To assess the long-term potential of expanded Vγ9Vδ2-T cells, the differentiation status after *ex vivo* activation was assessed. The Vγ9Vδ2-T cells were mainly differentiated towards a memory phenotype after moDC-activation (Figure 5E). Moreover, there was a trend towards diminished CD160 expression on both HC- and CLL-derived Vγ9Vδ2-T cells after *ex vivo* activation (Figure 5F).

Since the generation of moDCs complicates this *ex vivo* activation method, an expansion protocol based on previous clinical trials was also tested[28,30,45,46]. In short, PBMCs were depleted from B cell and CLL cells (magnetic bead depletion) and subsequently cultured in

the presence of ABP and IL-2 for 2 weeks. Using this protocol we could generate functional Vγ9Vδ2-T cells from both HCs and CLL patients (Supplementary Figure 4A-D). Yet, the expansion of the Vγ9Vδ2-T cells was inferior in comparison to the moDC-based protocol (Figure 5A).

In conclusion, *ex vivo* expansion of CLL-derived Vγ9Vδ2-T cells using a clinically feasible activation method resulted in fully functional Vγ9Vδ2-T cells with a memory phenotype.

The transcriptional profile of Vγ9Vδ2-T cells from CLL patients is globally altered

To gain more mechanistic insight into the observed differences between Vγ9Vδ2-T cells from HCs and CLL patients before activation, we performed RNA sequencing analyses on Vγ9Vδ2-T cells directly after thawing and after autologous moDC-based activation.

Although there was considerable inter-donor variation, as could be expected using primary T cells, principal component analysis (PCA) showed that Vγ9Vδ2-T cells from CLL patients cluster apart from Vγ9Vδ2-T cells from HCs (Figure 6A). *Ex vivo* activation had a strong effect on the transcriptional profile of the Vγ9Vδ2-T cells and Vγ9Vδ2-T cells from both CLL patients and HCs clustered together after *ex vivo* activation. Thus, transcriptomics fitted with the previously observed altered function of Vγ9Vδ2-T cells from CLL patients in comparison to HC Vγ9Vδ2-T cells, which was no longer the case after *ex vivo* activation.

Increased expression of 83 genes was observed in CLL Vγ9Vδ2-T cells in comparison to HC Vγ9Vδ2-T cells prior to expansion (Figure 6B). The expression of considerably more genes (n=430) was decreased in these CLL Vγ9Vδ2-T cells. In line with PCA, no genes were differentially expressed between CLL and HC Vγ9Vδ2-T cells after *ex vivo* activation.

We hypothesized that the most relevant candidate genesets underlying our functional observations would be differentially expressed between CLL and HC Vγ9Vδ2-T cells before *ex vivo* activation, but comparably expressed after *ex vivo* activation (Figure 6C). Geneset enrichment analysis indicated that the concerned genesets were primarily immune-related and included TNF-α signaling via NFκB and adhesion and diapedesis of lymphocytes.

Next, we compared the expression levels of selected genes hypothesized to be most relevant to Vγ9Vδ2-T cell function (as shown in Supplementary Table 2) between Vγ9Vδ2-T cells from CLL patients and HCs. Although the statistical power was limited, these data point to differential expression of genes that relate to T cell memory, inhibition and exhaustion, costimulation and synapse formation and adhesion (Figure 6D). Although genes involved in costimulation and memory were differentially expressed by CLL Vγ9Vδ2-T cells before activation, the same differences persisted after *ex vivo* activation (Supplementary Figure 5A-C), making these unlikely candidates to explain our functional observations. We then zoomed in on genes related to synapse formation and adhesion, most of which were downregulated in CLL Vγ9Vδ2-T cells as compared to HC Vγ9Vδ2-T cells before *ex vivo* activation (Figure

6E), but not after *ex vivo* activation (Supplementary Figure 5F). Although there were some exceptions, genes attributed to T cell inhibition and exhaustion were generally upregulated in CLL Vγ9Vδ2-T cells (Figure 6F), most clearly before *ex vivo* activation (Figure 6F).

Thus, RNA sequencing confirmed that Vγ9Vδ2-T cells from CLL patients have a transcriptional profile that is distinct from HC Vγ9Vδ2-T cells and implicated impaired synapse formation and increased inhibitory molecules as conceivable contributors to compromised Vγ9Vδ2-T cell function in CLL patients.

Ibrutinib promotes T_H1 skewing of Vγ9Vδ2-T cells

The BTK inhibitor ibrutinib not only targets malignant B cells, but also skews αβ-T cells towards a tumor suppressive T_H1 phenotype through inhibition of ITK[47]. Whether ITK is expressed and exerts a similar role in Vγ9Vδ2-T cells has not been studied.

CLL-derived Vγ9Vδ2-T cells that were pretreated with ibrutinib produced significantly more TNF-α upon coculture with ABP-treated Daudi cells (Figure 7A). In contrast, IL-4 producing Vγ9Vδ2-T cell numbers declined after ibrutinib treatment. Ibrutinib treatment did not impair the degranulation of Vγ9Vδ2-T cells (Figure 7B). To study whether effects of ibrutinib indeed depended on inhibition of targets other than BTK, Vγ9Vδ2-T cells from CLL patients were pretreated with the highly BTK-specific inhibitor CC-292[48]. In contrast to ibrutinib, CC-292 did not alter cytokine production by Vγ9Vδ2-T cells (Figure 7C).

To test whether ITK was bound by ibrutinib in Vγ9Vδ2-T cells, we performed competitive pull-down experiments utilizing biotinylated ibrutinib coupled to avidin-agarose. Specific binding of BTK to biotinylated ibrutinib was confirmed in the CLL-derived Mec-1 cell line, which could be reverted by ibrutinib or CC-292 pretreatment (Figure 7D). ITK was bound by biotinylated ibrutinib in Vγ9Vδ2-T cells. Pretreatment with ibrutinib, but not with CC-292, blocked ITK binding.

Taken together, these experiments demonstrate that ibrutinib, but not CC-292, promotes a T_H1 phenotype in Vγ9Vδ2-T cells from CLL patients, likely mediated by ITK inhibition.

Discussion

Vγ9Vδ2-T cells are attractive effector cells for immunotherapy, capable of MHC-independent recognition of malignant cells. In line with the cytotoxic capacity of Vγ9Vδ2-T cells towards other malignant B cells[18,21-24], we demonstrate Vγ9Vδ2-T cell cytotoxicity towards CLL cells.

Clinical application of autologous Vγ9Vδ2-T cells however seems hampered by the observed dysfunction in Vγ9Vδ2-T cells. Indeed, CLL cells have previously been found to have an active immunosuppressive function[49,50]. In agreement with these findings, we show here that CLL cells also induce dysfunction in healthy donor-derived Vγ9Vδ2-T cells. Although the expression of exhaustion markers, especially PD-1, correlates with αβ-T cell dysfunction in CLL[13], Vγ9Vδ2-T cells do not express increased transcriptional or protein levels of PD-1. On the other hand, CD160 expression was upregulated on Vγ9Vδ2-T cells from CLL patients and genes related to T cell exhaustion were generally expressed at higher transcriptional levels in CLL Vγ9Vδ2-T cells. Although the functional role of CD160 in γδ-T cells has only been described in the context of CD3-stimulation[51], it is plausible that CD160 has an inhibitory role in Vγ9Vδ2-T cells, as was described in αβ-T cells[52]. Gene expression profiling also implicated alterations in synapse formation in the observed cytotoxic impairment, as has previously also been demonstrated for αβ-T cells in CLL[14]. Vγ9Vδ2-T cell anergy can arise as a result of chronic phosphoantigen overstimulation[53,54]. A similar mechanism in CLL is supported by our observation that naïve Vγ9Vδ2-T are lost and dysfunction increases with disease stage, as well as increased Vγ9Vδ2-T cell numbers in advanced stage CLL in comparison to MBL and Rai 0[55]. The possibility of chronic overstimulation of Vγ9Vδ2-T cells due to a hyperactive state of the mevalonate pathway in U-CLL cells was previously proposed[31].

We explored several approaches to improve the function of CLL-derived Vγ9Vδ2-T cells. Pharmacological enhancement of phosphoantigen levels is clinically feasible and *in vivo* induction of Vγ9Vδ2-T cell proliferation using administration of ABP or synthetic phosphoantigens and IL-2 has been achieved in solid[25-27] and lymphoid[24] malignancies. However, ABP treatment only led to a modest lysis-sensitizing effect of primary CLL cells *in vitro*, which might be explained by overactivity of the mevalonate pathway in CLL.

Next, we examined *ex vivo* activation of Vγ9Vδ2-T cells in light of adoptive transfer strategies. Clinical trials have demonstrated safety[28,29,56] and efficacy[45,56-58]of *ex vivo* expanded Vγ9Vδ2-T cell administration. In line with our data, others have previously shown that sufficient Vγ9Vδ2-T cell numbers can be achieved by expansion using coculture with moDCs[59], as well as through adding synthetic phosphoantigens or ABP to PBMCs[57]. Moreover, we demonstrate that it is possible to generate Vγ9Vδ2-T cells with regained functionality in an autologous *ex vivo* culture system that is clinically feasible. The function of Vγ9Vδ2-T cells improved for all patients tested, although the number of patients tested was

limited. Activation with moDCs was performed in the presence of IL-15, which was previously shown to be superior to IL-2 for induction of sustained proliferation, T_H1 skewing and degranulation[46,60]. Most Vγ9Vδ2-T cells had a memory phenotype after *ex vivo* activation, which is favorable in light of long-term functionality[61-63].

Finally, we investigated combination strategies with novel BTK inhibitors, which allow for selective elimination of CLL cells while largely sparing T cells. In fact, ibrutinib has been shown to increase T cell numbers and reduce the immunosuppressive features of CLL[64,65], conceivably favoring subsequent autologous T cell therapy. Specifically, ibrutinib promotes a favorable anti-tumor T_H1 profile in Vγ9Vδ2-T cells derived from CLL patients, similar to what was previously shown for $CD4^+$-T cells[47,64]. As T_H1 skewing did not occur with CC-292, the effect is likely mediated by inhibition of ITK, as was supported by our pull-down experiments[48,66]. Hence, ibrutinib may be a suitable candidate to combine with autologous Vγ9Vδ2-T cell-based therapy.

In conclusion, Vγ9Vδ2-T cells are cytotoxic towards CLL cells and dysfunction of autologous Vγ9Vδ2-T cells is fully reversible upon *ex vivo* expansion. As autologous Vγ9Vδ2-T cells can be expanded to high numbers and ibrutinib stimulates an anti-tumor T_H1 pattern, further exploration of combining Vγ9Vδ2-T cell adoptive transfer with ibrutinib treatment is justified.

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Authorship

Contribution: IdW designed and performed experiments, analyzed data and wrote the manuscript. TH, RL, SE and RCGdB performed experiments and reviewed the manuscript. AJ and PDM analyzed data and reviewed the manuscript. NL and MvdS provided biotinylated ibrutinib. LMF and MDL provided patient samples and reviewed the manuscript. EBMR, IJMtB, EE, SHT and TDdG contributed to the design of experiments and reviewed the manuscript. HJvdV and APK designed the study and wrote the manuscript.

Conflict of interest disclosure: The authors declare no competing financial interests.

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Table 1. Patient characteristics

	Immuno-phenotype	Cytokine assay	Cytotoxicity assay	*Ex vivo* activation
Healthy controls				
Number	20	12	5	4
Sex, % male	45	58	60	50
Age, years (range)	72 (53-84)	67 (53-79)	68 (62-73)	67 (62-73)
Vγ9Vδ2, % of CD3 (range)	1.4 (0.1-4.9)	1.5 (0.1-4.9)	1.2 (0.5-1.5)	1.2 (0.5-1.5)
CMV positive, %	55	50	40	50
CLL patients				
Number	39	14	5	8
Sex, % male	67	50	60	50
Age, years (range)	64 (41-87)	67 (55-83)	64 (57-69)	64.9 (57-69)
Vγ9Vδ2, % of CD3 (range)	1.0 (0.1-6.2)	0.8 (0.1-1.8)	1.3 (0.6-2.6)	1.2 (0.6-2.6)
CMV positive, %	64.3	50	60	62.5
ALC, $*10^9$ cells/L (range)	80.4 (15.3-358.7)	77.4 (19.8-226.1)	51.3 (28.0-95.6)	44.3 (10.6-95.6)
U-CLL, %	43.6	42.9	40	50
Rai stage 0+1, %	72	64	60	62.5

CLL indicates chronic lymphocytic leukemia; CMV, cytomegalovirus; ALC, absolute leukocyte count; U-CLL, unmutated immunoglobulin genes.

Figure legends

Figure 1. Vγ9Vδ2-T cell recognition and lysis of CLL cells. (A) Representative plot of geometric MFI of CD25 expression measured after 36 hours coculture of HC Vγ9Vδ2-T cells with CLL cells (red solid line), allogeneic healthy B cells (blue dashed line) or Vγ9Vδ2-T cells alone (grey histogram). (B) As in A, scatter plot summarizing results for 6 donors. (C-E) Cell death of CFSE-labeled target cells after overnight coculture with Vγ9Vδ2-T cells, measured by Mitotracker Orange and To-pro-3. (C) Specific lysis of CLL cells after coculture with Vγ9Vδ2-T cells from HCs at the indicated effector:target ratios (n=9). Specific lysis was calculated as: (% cell death in stimulated cells)−(% cell death in unstimulated cells)/(% viable cells in unstimulated cells) * 100. (D) Healthy Vγ9Vδ2-T cells were pretreated for 2 hours with 100nM concanamycin A or DMSO and washed before coculture with CLL cells at a 1:1 ratio (n=9). (E) Specific lysis of allogeneic CLL and Daudi cells after coculture with Vγ9Vδ2-T cells from CLL patients (n=6) or HCs (n=9) at a 1:1 ratio. (F) CD69 expression on Vγ9Vδ2-T cells from CLL patients (n=8) or HCs (n=4) after overnight coculture with Daudi cells in a 1:5 ratio. Data are presented as mean and standard error of mean. **P* <0.05; ***P* <0.01, ****P* <0.001, *****P*<0.0001 (B, E, F: one-way ANOVA followed by (B) Dunnett's or (E, F) Bonferroni correction; D: paired t-test).

Figure 2. Frequency and immune phenotype of Vγ9Vδ2-T cells in CLL patients and HCs. Immunophenotyping of Vγ9Vδ2-T cells from untreated CLL patients and age-matched HCs, directly *ex vivo*. (A) Frequency of $V\gamma9^{+}V\delta2^{+}$ cells within $CD3^{+}$-T lymphocytes (CLL n=39, HC n=20). (B) Absolute number of Vγ9Vδ2-T cells (CLL n=39, HC n=6). (C) Distribution of differentiation subsets within Vγ9Vδ2-T cells based on CD27 and CD45RA expression (CLL n=39, HC n=20). Naïve T_N: $CD27^{+}CD45RA^{+}$; central memory T_{CM}: $CD27^{+}CD45RA^{-}$; effector memory T_{EM}: $CD27^{-}CD45RA^{-}$; CD45RA positive effector memory T_{EMRA}: $CD27^{-}$ $CD45RA^{+}$. (D) Expression of exhaustion markers on Vγ9Vδ2-T cells. Geometric MFI of PD-1 and BTLA expression (CLL n=39, HC n=20) and CD244, CD160 and LAG-3 expression (CLL n=16, HC, n=11). (E) Frequency of granzyme B^{+} and granzyme K^{+} cells within Vγ9Vδ2-T cells (CLL n=7, HC n=7). Data are presented as mean and standard error of mean. **P* <0.05 (A, B, D, E: Student's t-test).

Figure 3. Impaired production of effector cytokines and degranulation in CLL-derived Vγ9Vδ2-T cells. (A) Production of IFN-γ, TNF-α, IL-17 (n=14 CLL, n=12 HCs) and IL-4 (n=4 CLL and HCs) by Vγ9Vδ2-T cells after stimulation with PMA/Ionomycin for 4 hours. (B) CD19-depleted PBMCs from CLL patients (n=8) or HCs (n=8) were co-cultured with allogeneic CLL cells or Daudi cells for 16-18 hours. During the last 6 hours of coculture, Brefeldin A and GolgiStop were added to measure cytokine production in Vγ9Vδ2-T cells. (C)

CD107a expression in Vγ9Vδ2-T cells after PMA/ionomycin stimulation for 4 hours (n=14 CLL patients, n=12 HCs). (D) CD107a expression after coculture with malignant B cells as in B. Data are presented as mean and standard error of mean. *P <0.05; (A: one-way ANOVA followed by Bonferroni post hoc test; B-D: Student's t-test).

Figure 4. CLL cells induce Vγ9Vδ2-T cell dysfunction. (A) Cytokine production and (B) CD107a expression by HC Vγ9Vδ2-T cells after coculture with allogeneic healthy B cells or CLL cells for 36 hours in a 1:10 ratio and subsequent coculture with ABP-pretreated Daudi cells (n=6). (C) Cytokine production and (D) CD107a expression by HC Vγ9Vδ2-T cells after coculture and subsequent stimulation with PMA/ionomycin (n=6). Data are presented as mean and standard error of mean. *P <0.05; **P <0.01; ***P <0.001, ****P<0.0001 (one-way ANOVA followed by Bonferroni post hoc test).

Figure 5. Vγ9Vδ2 T cell dysfunction is reversible upon *ex vivo* activation and expansion. Vγ9Vδ2-T cells from CLL patients (n=8), with previously confirmed impaired function, and Vγ9Vδ2-T cells from HCs (n=4) were sorted by FACS and subsequently cultured for 2 weeks with phosphoantigen-expressing moDCs in the presence of IL-7 and IL-15. Alternatively, Vγ9Vδ2-T cells (CLL n=4, HC n=4) were generated from CD19-depleted PBMCs by culture in the presence of ABP and IL-2 for 2 weeks. (A) Expansion factor of Vγ9Vδ2-T cells calculated by dividing the amount of Vγ9Vδ2-T cells after 2 weeks culture with allogeneic HC-derived moDCs (allo-moDC) or autologous moDCs (auto-moDCs) by the Vγ9Vδ2-T cell number at the start of culture. (B) Cytokine production and (C) CD107a expression by CLL-derived and HC-derived Vγ9Vδ2-T cells after culture with autologous moDCs. Vγ9Vδ2-T cells were cocultured with ABP-pretreated Daudi cells 16-18 hours and Brefeldin A and GolgiStop were added during the last 6 hours of coculture. (D) Granzyme B and (F) CD160 expression on Vγ9Vδ2-T cells before and after culture with autologous moDCs. (E) Distribution of differentiation subsets within Vγ9Vδ2-T cells after culture with autologous moDCs based on CD27 and CD45RA expression. Naïve T_N: $CD27^+CD45RA^+$; central memory T_{CM}: $CD27^+CD45RA^-$; effector memory T_{EM}: $CD27^-CD45RA^-$; CD45RA positive effector memory T_{EMRA}: $CD27^-$ $CD45RA^+$. Data are presented as mean and standard error of mean. **P <0.01. A: Mann-Whitney test, C: Student's t-test; B: one-way ANOVA followed by Bonferroni post hoc test; D, F: paired t-test.

Figure 6. Altered Vγ9Vδ2-T cell transcriptional profile in CLL patients. RNA sequencing was performed on paired Vγ9Vδ2-T cells from 4 CLL patients and 4 HCs, both directly after thawing and after autologous moDC-based expansion. (A) Multidimensional scaling plot of gene expression data. Each symbol represents an individual donor. (B) Number of genes

differentially expressed (false discovery rate <0.05) in the indicated comparisons. (C) Geneset enrichment analysis of CLL versus HC Vγ9Vδ2-T cells, before (x-axis) and after *ex vivo* expansion (y-axis). Each symbol represents a geneset (derived from the Hallmark (H) or Biocarta (B) genesets from the Molecular Signatures Database, or self-generated genesets). Genesets differentially expressed (*P*<0.01) before, but not after expansion (*P*>0.1), are highlighted in orange and purple. (D) Geneset enrichment analysis of CLL versus HC Vγ9Vδ2-T cells directly prior to *ex vivo* expansion. The red dashed line indicates a *p*-value of 0.05. The figure indicates per geneset whether it is up- or downregulated in CLL in comparison to HC Vγ9Vδ2-T cells. (E, F) The gene expression of CLL Vγ9Vδ2-T cells was compared to HC Vγ9Vδ2-T cells. Each symbol represents an individual gene. Genes related to synapse and adhesion (E) or inhibitory and exhaustion molecules (F) are highlighted in red. Genes with a *p*-value <0.05 and/or a fold change ≤-1.75 or ≥1.75 are annotated. FC: fold change; TCR: T cell receptor; NK: natural killer cell.

Figure 7. **Ibrutinib promotes T_H1 phenotype in Vγ9Vδ2-T cells.** (A, B) CD19-depleted PBMCs were pretreated with 0, 10 or 100 nM ibrutinib for 30 minutes and subsequently cocultured with ABP-pretreated (25 μM pamidronate, 2 hours) Daudi cells. During the last 6 hours of coculture, Brefeldin A and GolgiStop were added to measure (A) cytokine production and (B) CD107a expression in Vγ9Vδ2-T cells (n=10). (C) Cytokine production as in (A) after pretreatment with 0, 10 or 100 nM CC-292 (n=6). Data are presented as mean and standard error of mean. (D) Pull-down with biotinylated ibrutinib coupled to avidin agarose; uncoupled avidin agarose was used as a control. Lysates from healthy Vγ9Vδ2-T cells or control Mec-1 B cells were pretreated with 1 μM ibrutinib or 1 μM CC-292 before pull-down as indicated. Representative result of 3 different donors in 2 independent experiments is shown. **P* <0.05 (A-C: one-way ANOVA followed by Dunnett's post hoc test).

Figure 1

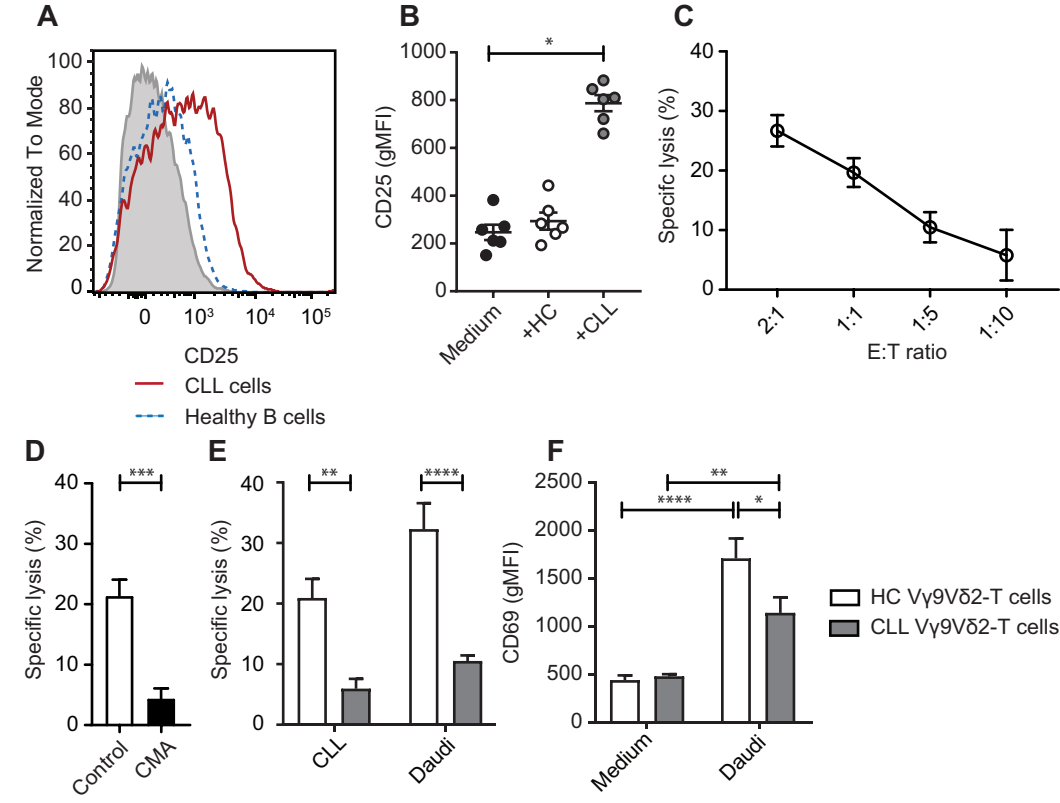

A
Normalized To Mode
100
80
60
40
20
0
0
10³
10⁴
10⁵
CD25
CLL cells
Healthy B cells
B
CD25 (gMFI)
1000
800
600
400
200
0
*
Medium
+HC
+CLL
C
Specifc lysis (%)
40
30
20
10
0
2:1
1:1
1:5
1:10
E:T ratio
D
Specific lysis (%)
40
30
20
10
0
***
Control
CMA
E
Specific lysis (%)
40
30
20
10
0
**
****
CLL
Daudi
F
CD69 (gMFI)
2500
2000
1500
1000
500
0
**
****
*
Medium
Daudi
HC Vγ9Vδ2-T cells
CLL Vγ9Vδ2-T cells

Figure 2

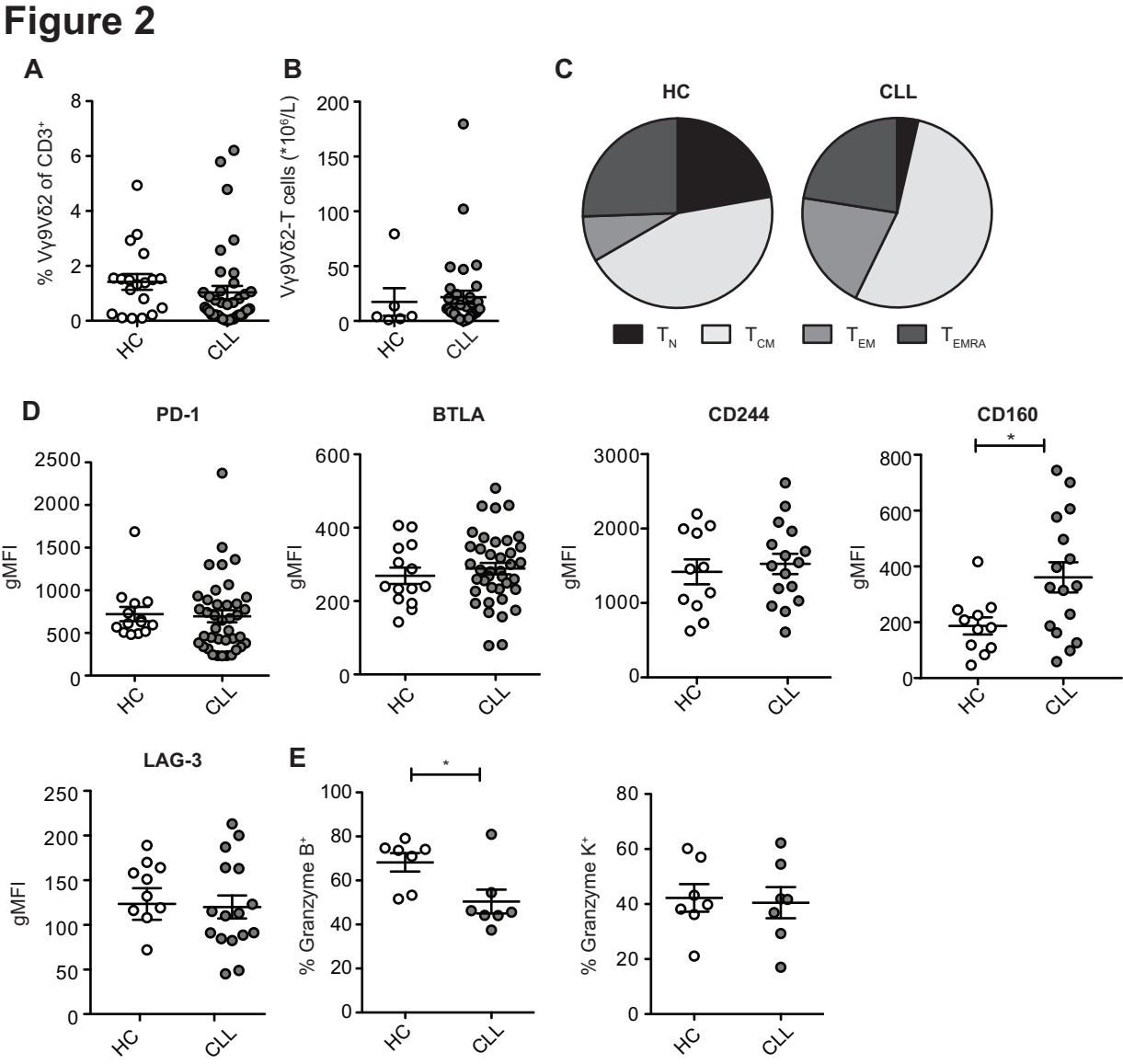

A
% Vγ9Vδ2 of CD3+
HC
CLL
B
Vγ9Vδ2-T cells (*10^6/L)
HC
CLL
C
HC
CLL
T_N
T_CM
T_EM
T_EMRA
D
PD-1
BTLA
CD244
CD160
LAG-3
gMFI
HC
CLL
*
E
% Granzyme B+
% Granzyme K+
HC
CLL
*

Figure 3

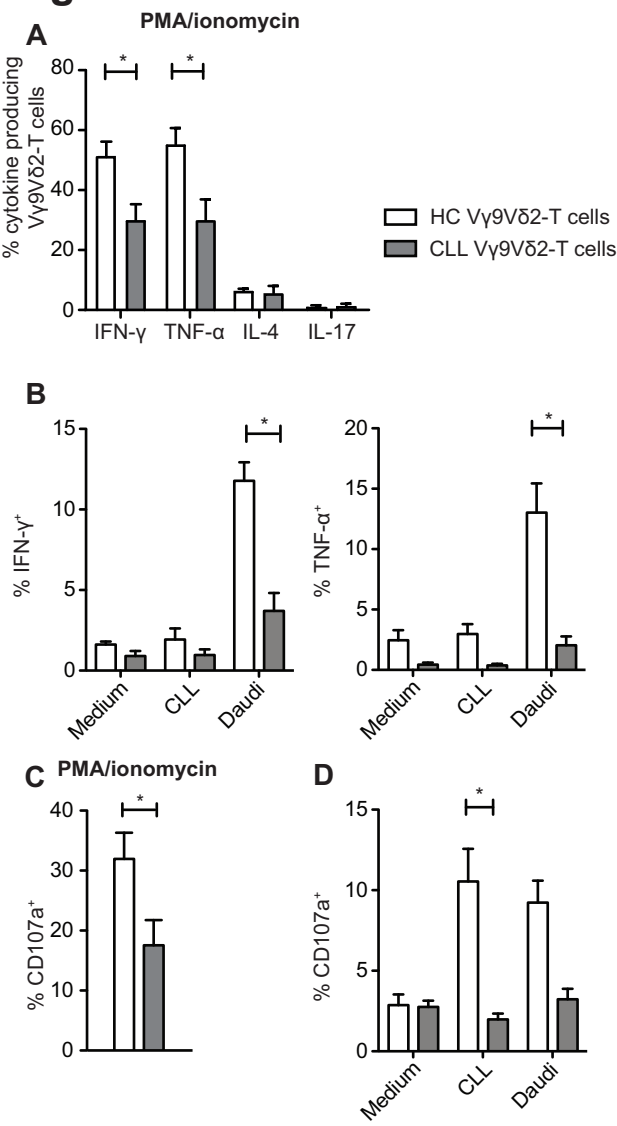

PMA/ionomycin
A
% cytokine producing Vγ9Vδ2-T cells
80
60
40
20
0
*
*
IFN-γ
TNF-α
IL-4
IL-17
HC Vγ9Vδ2-T cells
CLL Vγ9Vδ2-T cells
B
% IFN-γ+
15
10
5
0
*
Medium
CLL
Daudi
% TNF-α+
20
15
10
5
0
*
Medium
CLL
Daudi
C
PMA/ionomycin
% CD107a+
40
30
20
10
0
*
D
% CD107a+
15
10
5
0
*
Medium
CLL
Daudi

Figure 4

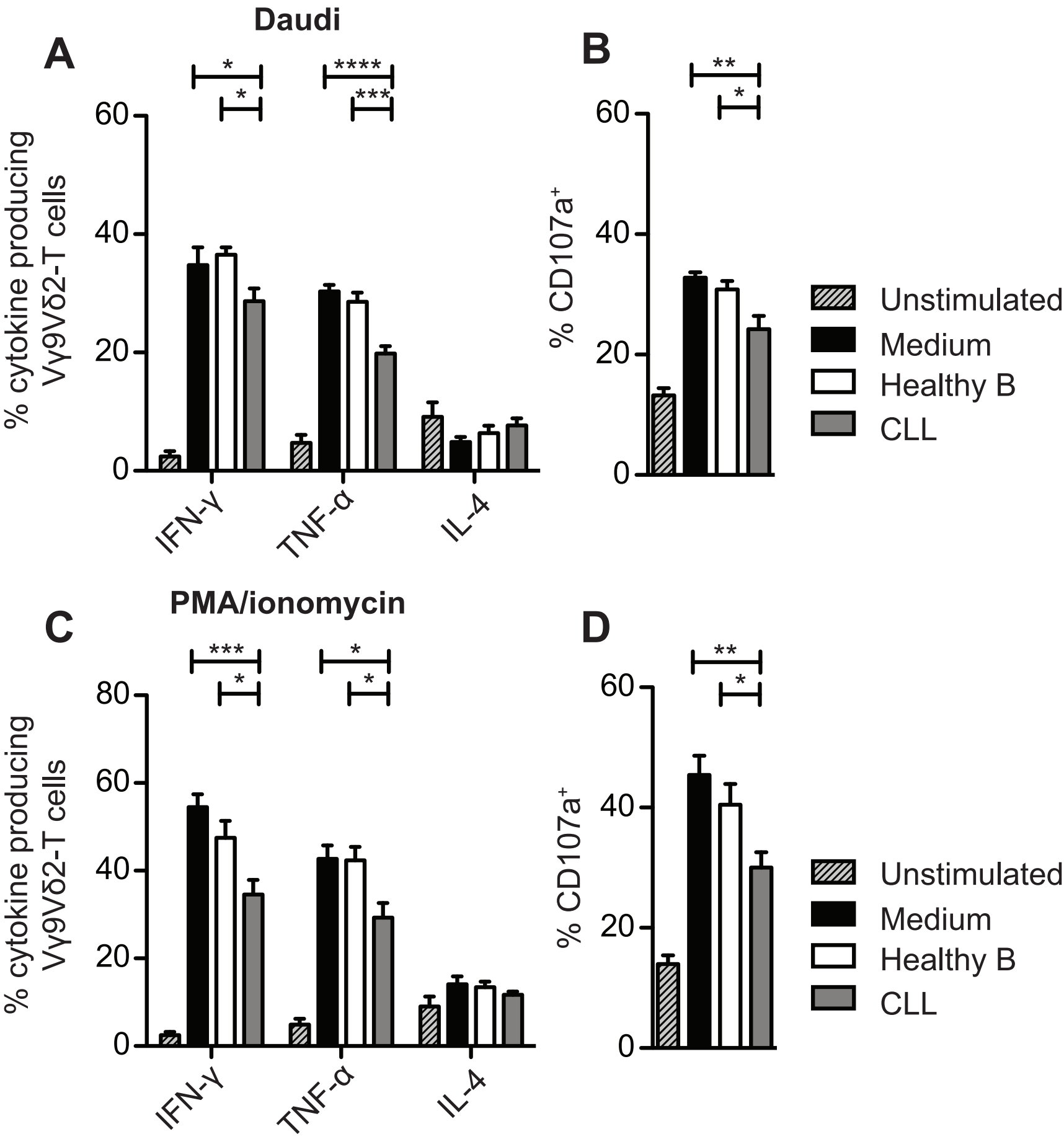
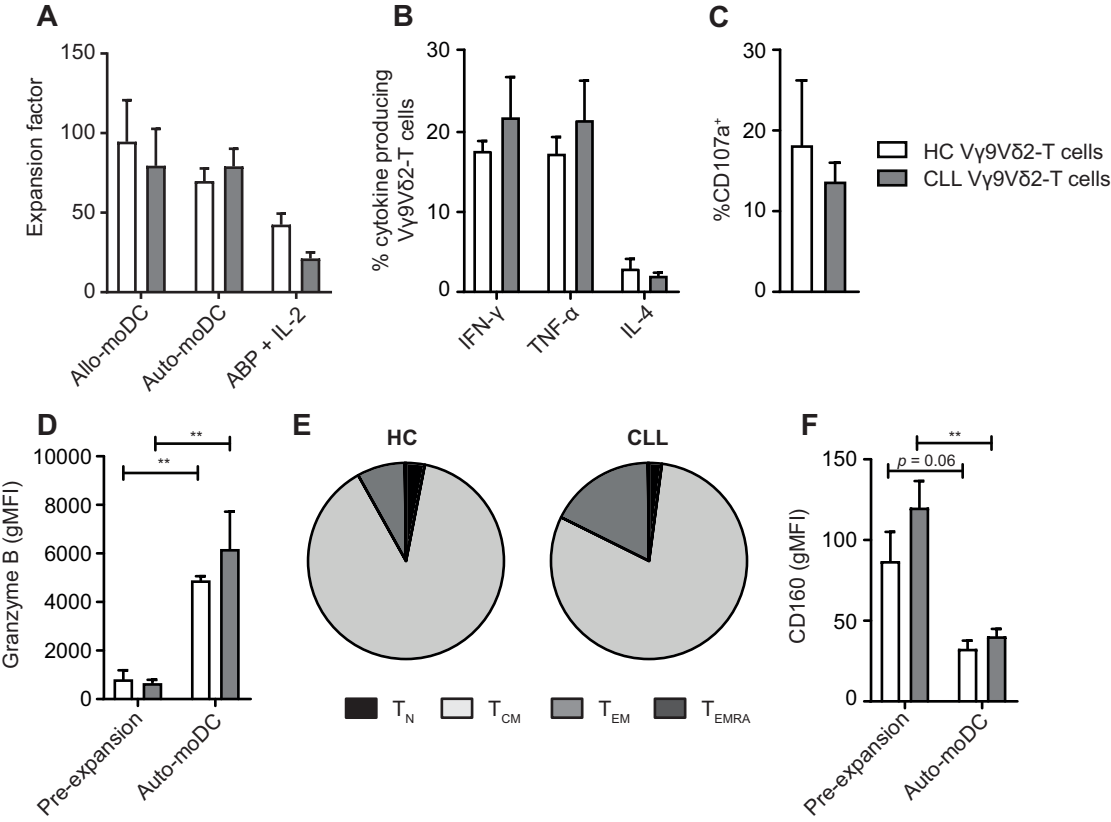

Daudi
A
B
C
D
PMA/ionomycin
% cytokine producing Vγ9Vδ2-T cells
% CD107a+
IFN-γ
TNF-α
IL-4
Unstimulated
Medium
Healthy B
CLL
*
**
***
****

Figure 5


A
Expansion factor
150
100
50
0
Allo-moDC
Auto-moDC
ABP + IL-2
B
% cytokine producing Vγ9Vδ2-T cells
30
20
10
0
IFN-γ
TNF-α
IL-4
C
%CD107a+
30
20
10
0
HC Vγ9Vδ2-T cells
CLL Vγ9Vδ2-T cells
D
Granzyme B (gMFI)
10000
8000
6000
4000
2000
0
**
**
Pre-expansion
Auto-moDC
E
HC
CLL
TN
TCM
TEM
TEMRA
F
CD160 (gMFI)
150
100
50
0
**
p = 0.06
Pre-expansion
Auto-moDC

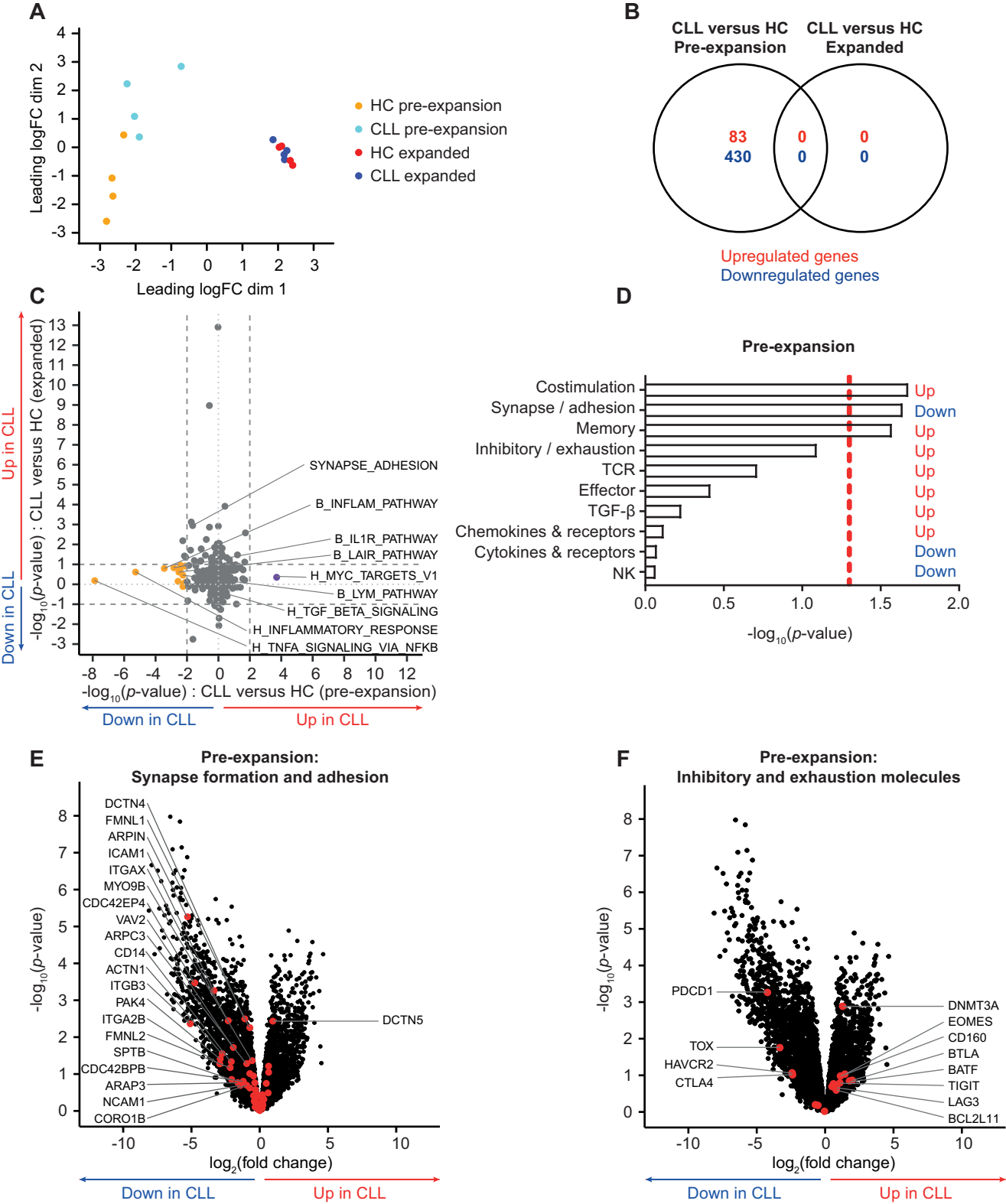
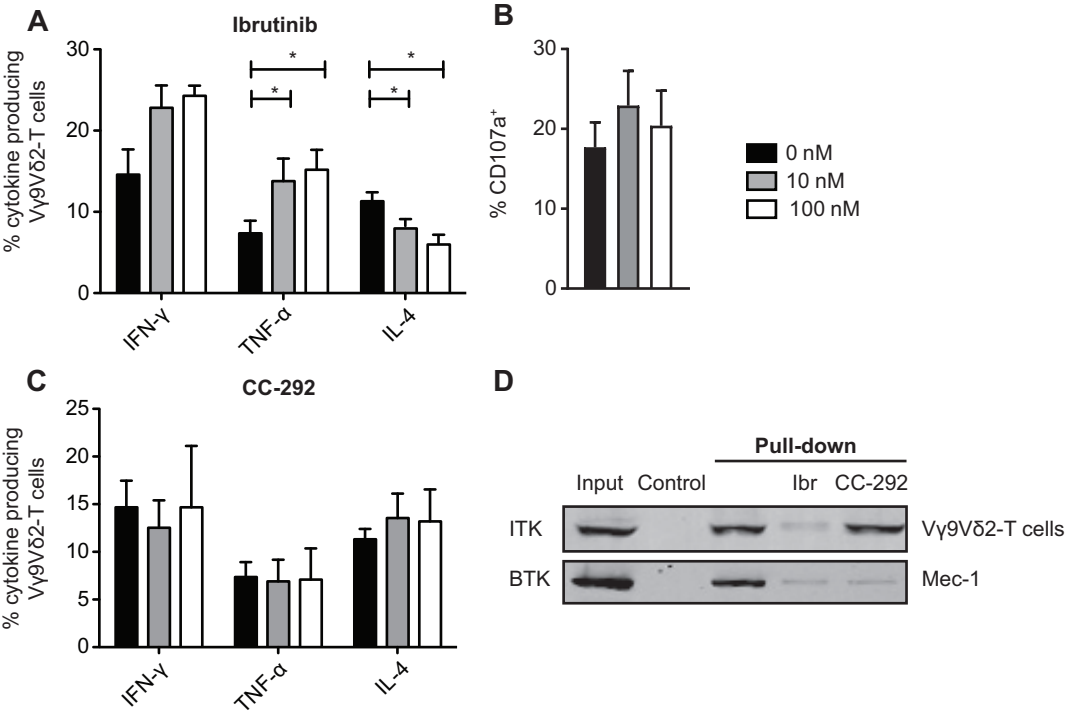

A
Leading logFC dim 2
Leading logFC dim 1
HC pre-expansion
CLL pre-expansion
HC expanded
CLL expanded
B
CLL versus HC
Pre-expansion
CLL versus HC
Expanded
83
430
0
0
0
0
Upregulated genes
Downregulated genes
C
Up in CLL
Down in CLL
-log10(p-value) : CLL versus HC (expanded)
SYNAPSE_ADHESION
B_INFLAM_PATHWAY
B_IL1R_PATHWAY
B_LAIR_PATHWAY
H_MYC_TARGETS_V1
B_LYM_PATHWAY
H_TGF_BETA_SIGNALING
H_INFLAMMATORY_RESPONSE
H_TNFA_SIGNALING_VIA_NFKB
-log10(p-value) : CLL versus HC (pre-expansion)
Down in CLL
Up in CLL
D
Pre-expansion
Costimulation
Synapse / adhesion
Memory
Inhibitory / exhaustion
TCR
Effector
TGF-β
Chemokines & receptors
Cytokines & receptors
NK
Up
Down
Up
Up
Up
Up
Up
Up
Down
Down
-log10(p-value)
E
Pre-expansion:
Synapse formation and adhesion
DCTN4
FMNL1
ARPIN
ICAM1
ITGAX
MYO9B
CDC42EP4
VAV2
ARPC3
CD14
ACTN1
ITGB3
PAK4
ITGA2B
FMNL2
SPTB
CDC42BPB
ARAP3
NCAM1
CORO1B
DCTN5
-log10(p-value)
log2(fold change)
Down in CLL
Up in CLL
F
Pre-expansion:
Inhibitory and exhaustion molecules
PDCD1
TOX
HAVCR2
CTLA4
DNMT3A
EOMES
CD160
BTLA
BATF
TIGIT
LAG3
BCL2L11
-log10(p-value)
log2(fold change)
Down in CLL
Up in CLL

Figure 7


A
Ibrutinib
% cytokine producing Vγ9Vδ2-T cells
30
20
10
0
*
*
*
*
IFN-γ
TNF-α
IL-4
B
% CD107a+
30
20
10
0
0 nM
10 nM
100 nM
C
CC-292
% cytokine producing Vγ9Vδ2-T cells
25
20
15
10
5
0
IFN-γ
TNF-α
IL-4
D
Pull-down
Input
Control
Ibr
CC-292
ITK
BTK
Vγ9Vδ2-T cells
Mec-1

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Improving CLL Vγ9Vδ2-T cell fitness for cellular therapy by ex vivo activation and ibrutinib

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