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FCRL6 distinguishes mature cytotoxic lymphocytes and is upregulated in patients with B cell chronic lymphocytic leukemia

Daniel M. Schreeder[3], **Jicun Pan**[1,2], **Fu Jun Li**[1,2], **Eric Vivier**[5,6,7,8], and **Randall S. Davis**[1,2,3,4]

[1] Division of Hematology/Oncology, University of Alabama at Birmingham, Birmingham, AL, USA

[2] Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA

[3] Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

[4] Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL, USA

[5] Centre d'Immunologie de Marseille-Luminy, Université, de la Méditerranée, case 906, Campus de Luminy, 13288 Marseille, France

[6] INSERM, U631, case 906, Campus de Luminy, 13288 Marseille, France

[7] CNRS, UMR6102, case 906, Campus de Luminy, 13288 Marseille, France

[8] Hôpital de la Conception, Assistance Publique – Hôpitaux de Marseille, 147 bd Baille 13005, Marseille France

Abstract

Fc receptor-like 6 (FCRL6), the most recently characterized member of the FCRL family, is a cell surface glycoprotein with tyrosine-based regulatory potential. An extensive survey of human hematopoietic tissues disclosed that FCRL6 expression by NK and T cell subpopulations increases as a function of differentiation and is remarkably restricted to mature lymphocytes with cytotoxic capability. In particular, FCRL6 distinguishes perforin-expressing $CD56^{dim}$ NK cells, $V\delta1^+$ and $V\delta2^+$ γδ T cells, effector and effector memory $CD8^+$ T cells, and rare cytotoxic $CD4^+$ T cells in adult tissues. Analysis of this receptor in B cell chronic lymphocytic leukemia (CLL) was also performed. FCRL6 was found to mark significantly expanded populations of cytotoxic $CD8^+$ T, $CD4^+$ T, and NK cells in patients with CLL. Despite sequence homology with the known Fc receptors for IgG and IgE, FCRL6 did not bind immunoglobulin. Although FCRL6 can be tyrosine-phosphorylated, its antibody-mediated ligation was unable to influence cellular activation. Collectively these results demonstrate that FCRL6 is a distinct indicator of cytotoxic effector lymphocytes that is upregulated in diseases characterized by chronic immune stimulation.

Keywords

Human NK cells; Human cytotoxic T cells; Fc Receptor-like; Chronic lymphocytic leukemia

Corresponding author: Randall S. Davis, University of Alabama at Birmingham, 1825 University Blvd SHEL 412, Birmingham, AL 35294-2182, USA; Tel: (+1) 205-934-1522; Fax: (+1) 205-934-1875; rsdavis@uab.edu.

CONFLICT OF INTEREST DISCLOSURE

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INTRODUCTION

Cytotoxic lymphocytes are important effector components of cell-mediated immunity that are principally responsible for defense against virally infected and malignantly transformed cells. Natural killer (NK) cells and $CD8^+$ αβ T cells represent the main lymphocytes involved in cell-mediated effector responses, but other populations including γδ T cells and $CD4^+$ αβ T cells have been shown to exert direct cytotoxic effects on stressed or transformed cells. For all of these cell types, cytolytic function is restricted to the later stages of development and is a hallmark of their differentiation [1–3]. Sequential stages of cellular maturation are also accompanied by the expression of distinct surface receptors important for regulating and localizing immune responses via their ability to target stressed cells and spare normal bystander cells [4–6].

A recently described gene family related to the Fc receptors (FcR) for IgG and IgE by chromosomal location, genetic organization, and sequence identity have been termed Fc receptor-like (*FCRL*) [7–9]. Human *FCRL1-5* encode type I transmembrane receptors with variable numbers of extracellular immunoglobulin (Ig)-like domains and cytoplasmic tails with immunoreceptor tyrosine-based activation motifs (ITAM), inhibition motifs (ITIM), or both. Studies of the five initially identified family members reveal that these molecules are differentially expressed in the later stages of B cell development and possess potent co-activating or inhibitory capability [9–11].

We and others have identified a sixth member of this extended family [12–14]. Human *FCRL6* (previously termed *FcRH6* or *IFGP6*) encodes a type I transmembrane receptor with three extracellular Ig-like domains, an uncharged transmembrane region, and a cytoplasmic tail with a consensus ITIM or a noncanonical 'ITAM-like' sequence [9]. Using an FCRL6-specific monoclonal antibody, we found that in contrast to the B lineage-expressed FCRL1-5 molecules, FCRL6 is found on mature, cytotoxic NK and T lineage cells and marks expanded populations of cytotoxic lymphocytes in patients with chronic lymphocytic leukemia.

RESULTS AND DISCUSSION

FCRL6 is expressed by cytotoxic cells at a mature developmental stage

Studies of *FCRL6* transcription demonstrated preferential expression by NK and T cell populations in the blood and spleen (data not shown). To examine its protein expression, a panel of mouse anti-human FCRL6 monoclonal antibodies (mAb) was generated. After screening a series of human FCRL1-6 and FcγRI-III transfected cell lines, the 7B7 subclone (mouse IgG1κ) was found to be FCRL6-specific (Fig S1).

To explore its ontogeny and cellular distribution, an extensive analysis of fetal and adult hematopoietic tissues was performed. FCRL6 could not be detected on NK cells from developmental sites such as the fetal liver, fetal bone marrow, or tonsils [1;15;16]; however, considerable expression was identified on NK cells from the adult spleen and blood (Fig. 1A). A flow cytometric analysis of adult blood NK cells with distinguishing markers indicated the mature phenotype of $FCRL6^+$ cells (Fig. S2). FCRL6 was distinctly confined to $CD16^+CD56^{dim}$ NK cells, which represent mature cytotoxic NK cells (Fig. 1B) [17], and correlated with perforin expression (Fig. 1C). $CD56^{dim}FCRL6^+$ NK cells also expressed higher levels of PEN5 compared to $CD56^{dim}FCRL6^-$ NK cells (Fig. 1D). It has been hypothesized that the late stages of NK cell differentiation involve the increased expression of PEN5 [18]. Thus, these findings suggest that terminal differentiation may also correlate with FCRL6 upregulation.

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Previous studies indicate that CD16 is a marker of maturity for the NK lineage [19]. To compare CD16 and FCRL6 expression as a function of ontogeny, NK cells from umbilical cord blood and adult blood were analyzed. Although no significant difference was found in the percentage of NK cells that express CD16 between these two cohorts, the percentage of $CD16^+$ NK cells expressing FCRL6 was significantly higher in adult blood compared to cord blood (Fig. 1E). Furthermore, the mean fluorescence intensity of FCRL6 staining was higher on adult $CD16^+$ NK cells (data not shown). This analysis indicates that FCRL6 is expressed later in ontogeny than CD16.

Similar results were obtained upon characterizing γδ T cell subsets. FCRL6 could not be detected on γδ T cells from the thymus or tonsils; however, it was identified on these innate-like T cells in spleen and blood isolates (Fig. 1F, S3A). Two major subsets of human γδ T cells have been described. $V\delta1^+$ γδ T cells and Vγ9Vδ2 T cells ($V\delta2^+$ γδ T cells) differ in their TCR composition, tissue distribution, and antigen recognition strategy, but both populations are capable of cytolytic function [2;20;21]. In a survey of blood samples from five healthy donors, FCRL6 was variably expressed by both Vδ1 and Vδ2-expressing γδ T cells (Fig. 1F). As with NK cells, FCRL6 levels on blood γδ T cells correlated with cytoplasmic perforin expression (Fig 1G). Angelini and colleagues have demonstrated that $CD16^+$ Vγ9Vδ2 T cells are more cytotoxic than their $CD16^-$ counterparts [2], and in line with this observation, FCRL6 was found at higher levels on $CD16^{high}$ rather than $CD16^{low}$ $V\delta2^+$ T cells (Fig. 1H).

FCRL6 expression on conventional αβ T cells was additionally assessed. Consistent with the hypothesis that it is confined to only mature lymphocyte populations, we were unable to detect FCRL6 on thymocytes or tonsillar T cell subpopulations (Fig S3B). However, FCRL6 could be found on a subset of $CD8^+$ T cells in the spleen and blood (Fig. S3B). Using CD45RA and CCR7 to better discriminate blood $CD8^+$ T cell subsets [22], we observed that FCRL6 was primarily confined to $CD45RA^+CCR7^-$ effector and $CD45RA^-CCR7^-$ effector memory $CD8^+$ T cells (Fig. 2A). In contrast, little if any expression was identified on $CD45RA^+CCR7^+$ naive or $CD45RA^-CCR7^+$ central memory $CD8^+$ T cells. Among 13 healthy adult blood donors, the percentage of $FCRL6^+$ naive, central memory, effector memory, and effector $CD8^+$ T cells averaged 2.6%, 5.1%, 13.3%, and 41.5%, respectively. In addition to the CD45RA/CCR7 format, it has been shown that cytotoxic T cells are preferentially $CD27^-$ [23]. Indeed, the majority of $CD27^-CCR7^-$ cells were $FCRL6^+$ (Fig. 2A). Similar to its pattern on NK cells and γδ T cells, FCRL6 also positively correlated with perforin expression in $CD8^+$ T cells (Fig 2B).

In an examination of a series of healthy adult blood donors, several individuals were found to have a small but appreciable population of circulating $FCRL6^+CD4^+$ T cells. In 3 of 18 samples, greater than 2% of $CD4^+$ T cells stained positive for FCRL6. This unusual T cell subset, which is characterized by intermediate levels of CD4 expression, also expresses perforin, CD57, and NKG2D, but lacks surface CCR7 (Fig. 2C). CD45RA expression on $FCRL6^+CD4^+$ T cells was found to vary among individuals (data not shown). These cells were negative for CD25, FoxP3, Vα24 and γδ TCR, implying that they are not regulatory T, NKT, or γδ T cells (data not shown). The first evidence for cytotoxic $CD4^+$ T cells came from Krensky et al who generated $CD4^+$ CTL clones by stimulating PBMCs with B cell lines and demonstrated that they were capable of killing in an MHC class II-restricted fashion [24]. Appay and colleagues have confirmed the in vivo existence of cytotoxic $CD4^+$ T cells, which express perforin but lack CCR7 expression and express intermediate levels of CD4 [3]. Two recent studies have also characterized CMV-specific $CD4^+$ T cells that express perforin, CD57, and NKG2D, and possess cytotoxic function [25;26]. Collectively, these results indicate that FCRL6 marks a rare population of $CD4^+$ T cells at a distinctly late developmental stage with cytotoxic potential.

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In addition to NK and T cells, B cells, monocytes, and granulocytes were also examined for FCRL6 expression, but found to be negative at both the transcript and protein levels. These data suggest that FCRL6 is distinctly restricted to mature lymphocytes with a cytotoxic phenotype.

FCRL6-expressing CD8$^+$ T cells, CD4$^+$ T cells, and NK cells are expanded in CLL

The function of T cells in individuals with B cell chronic lymphocytic leukemia (CLL) has been a subject of long-standing debate. The T cell compartment is expanded in the blood of CLL patients [27;28] suggesting that the cellular arm of the immune system is mounting a response against the malignant clone. Moreover, oligoclonal T cell populations that respond to autologous tumor cells have also been characterized [29;30]. However, several groups have indicated that T cells derived from CLL patients are dysfunctional compared to those isolated from normal individuals, as they proliferate poorly in response to mitogens and exhibit diminished ex vivo cytotoxic capability [28;31]. In light of this work, and because other FCRL members are upregulated on CLL cells [32], FCRL6 expression in this lymphoproliferative disorder was investigated. In pilot studies, FCRL6 was not detected on normal polyclonal or leukemic blood B cells, but was prominently expressed on T and NK cells from CLL donors. An expanded analysis of blood mononuclear cells from 11 CLL patients and 13 age-matched volunteers was therefore performed.

A significantly higher percentage of FCRL6-expressing CD8$^+$ T cells were found among the CLL cohort compared to normal donors (Fig. 3A). Several related factors could contribute to this observed increase and collectively imply that FCRL6$^+$CD8$^+$ T cells are expanded in CLL patients. First, the absolute number of T cells were increased (mean 2,100 cells/μL; normal range 1000 – 1500 cells/μL), a finding that corroborates earlier studies [27;28]. Second, the constitution of the blood CD8$^+$ T cell pool in CLL patients is remarkably different than that of normal individuals. The percentage of CD45RA$^+$CCR7$^+$ naive CD8$^+$ T cells was significantly lower and the percentage of CD45RA$^+$CCR7$^-$ effector and CD45RA$^-$CCR7$^-$ effector memory CD8$^+$ T cells was significantly higher among individuals with CLL compared to normal cohort donors (Fig. 3B). Finally, the percentage of effector and effector memory cells that are FCRL6$^+$ was significantly higher in patients with CLL compared to age-matched healthy individuals (Fig. 3C).

In addition to an increase of FCRL6$^+$CD8$^+$ T cells, FCRL6 was also expressed by higher percentages of CD4$^+$ T cells and CD16$^+$ NK cells (Figs. 3D and E). CLL patients' FCRL6$^+$CD4$^+$ T cells had the same cytotoxic phenotype as those identified from unaffected donors, and were characterized by intermediate levels of CD4 expression, and high levels of CD57 and perforin (data not shown). These data suggest that FCRL6-expressing cytotoxic lymphocyte subsets are expanded in patients with CLL; however, it is unclear whether this increase in FCRL6 expression is the product of a healthy anti-tumor immune response or rather a manifestation of the reported dysfunction of cytotoxic lymphocytes in this disease. Similar to the expansion of FCRL6$^+$ cells in CLL patients reported here, Wilson et al's study demonstrated FCRL6 expression by a higher percentage of CD8$^+$ and CD4$^+$ T cells in patients with HIV [14]. This finding indicates that FCRL6 expansion is not unique to CLL and may be a feature common to conditions characterized by chronic immune stimulation.

FCRL6 is not an Fc receptor

Because of its similarity to Fc receptors, the Ig-binding capability of FCRL6 was tested. Vector-only control or FCRL6 transduced BW5147 cells, as well as human FcγRIIB transfected A20IIA1.6 cells, were incubated with purified monomeric human Igs including IgG1-4, IgD, IgE, IgM, and IgA isotypes as well as polymeric IgA and heat aggregated IgG, and analyzed for binding by flow cytometry. Despite IgG isotype staining by FcγRIIB

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transfectants, FCRL6 transduced cells did not demonstrate evidence of Ig association (data not shown). These data indicate that FCRL6 is not an immunoglobulin receptor.

FCRL6 can be phosphorylated but does not possess typical ITIM or ITAM functionality

FCRL6 has two tandemly positioned tyrosine residues (Y356, Y371) in its cytoplasmic tail. The sequence surrounding the membrane distal tyrosine (Y371) fits an ITIM consensus [33], but together, the sequence encompassing these two tyrosines could also comprise a non-canonical ITAM [9]. To explore its tyrosine-based functional capacity, we first determined whether FCRL6 can be phosphorylated following treatment with the phosphatase inhibitor sodium pervanadate. Phosphotyrosine could be clearly detected by Western blotting FCRL6 immunoprecipitates from pervanadate-treated but not untreated BW5147 transductants (Fig. S4A). A series of BW5147 T cell transductants was then used to directly test the potential activating function of FCRL6. Using plate bound mAbs, FCRL6 was ligated and transductants were assayed for IL-2 production. Whereas CD16 stimulation resulted in robust IL-2 production by the BW5147 cells, FCRL6 ligation did not (Fig. S4B). The ability of FCRL6 to influence cytotoxic granule release or IFN-γ production by primary NK cells was also investigated in re-directed killing assays using antibody-coated P815 cells as targets. FCRL6 stimulation alone resulted in sporadic, minimal degranulation by NK cells; and furthermore, had no or only a minimally enhancing effect on CD16-mediated IFN-γ production and degranulation. In clear contrast and consistent with prior reports [34], co-ligation with LAIR-1, an Ig domain-containing receptor with two functional ITIMs, resulted in complete abrogation of CD16-mediated activation (Fig. S4C). Similar results were obtained when plate bound mAbs were used for cell stimulation rather than P815 cells (data not shown).

Although the cytoplasmic portion of FCRL6 contains a consensus ITIM or a non-canonical ITAM, and is capable of being tyrosine-phosphorylated, studies using FCRL6 mAbs to interrogate its function have failed to demonstrate convincing evidence of inhibitory or activating tyrosine-based function for this receptor. Our data are consistent with those of Wilson et al who found that the putative Y371 ITIM motif was capable of tyrosine phosphorylation and SHP-2 recruitment following pervanadate treatment; however, antibody crosslinking of FCRL6 had a minimal effect on cytotoxicity, cytokine production, apoptosis, or proliferation by primary or transduced cells [14]. While its structural features and expression profile suggest that FCRL6 may influence the effector function of cytotoxic lymphocytes, its distinct biological role requires further study. We hope that future identification of the ligand(s) for this receptor will yield exciting insight into its function as well as the role it may play in cell-mediated immunity.

CONCLUDING REMARKS

A detailed investigation using a novel FCRL6-specific monoclonal antibody found that this FCRL family member is restricted to subsets of mature cytotoxic NK and T cells, distinctly correlates with perforin expression, and emerges at a later stage of NK cell maturation than CD16. Importantly, FCRL6 marks expanded populations of cytotoxic $CD8^+$ and $CD4^+$ T lymphocytes and $CD16^+$ NK cells in patients with chronic lymphocytic leukemia. Although FCRL6 can be tyrosine phosphorylated, its ligation was unable to influence IL-2 production, cytotoxic granule release, or IFN-γ production in primary or transduced cells and does not possess Ig binding capability. Collectively, these data indicate FCRL6 is an orphan cell surface receptor with a discriminating expression pattern reflective of the cytotoxic maturation program of NK and T cells.

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MATERIALS AND METHODS

Tissues

Human tissues were acquired from the University of Washington Birth Defects Research Laboratory and the UAB Tissue Procurement program with University of Alabama at Birmingham Institutional Review Board (IRB) approval. Venous blood samples were obtained after informed consent with IRB approval according to the Declaration of Helsinki from healthy adult volunteers or patients diagnosed with CLL according to standard NCI criteria [35] from the UAB Health Clinics. Mononuclear cell suspensions were generated from mechanically disassociated tissues by passage through a 40μm nylon filter and density gradient separation using Lymphocyte Separation medium (Mediatech).

Flow Cytometry

FCRL6 expression on primary human lymphocytes was characterized using the anti-FCRL6 7B7 (mouse IgG1κ) mAb or an isotype-matched control mAb labeled with Alexa Fluor 647 (Invitrogen) in addition to discriminating human cell surface markers including: CD3 FITC, CD4 PE, CD8 PE, CD8 FITC, CD57 FITC, CD25 PE-Cy7, CD27 PE, anti-CCR7 PE-Cy7, anti-γδ TCR PE, anti-Vγ9 TCR PE, CD56 PE, CD16 PE, CD69 PE, streptavidin PE-Cy7, mouse IgG1κ PE, mouse IgG1κ FITC, mouse IgG1κ APC, mouse IgG1κ PE-Cy7, and rat IgG2aκ PE-Cy7 (all from BD Biosciences); CD3 PE-Cy7 (SBA); CD158a/h PE, CD158e PE, CD158i PE, NKp30 PE, NKp44 PE, NKp46 PE, NKG2A PE, CD94 PE, CD62L PE, CD161 PE, CD16 PE, CD25 PE, DNAM-1 PE, CD244 PE, CD56 PECy7, CD16 FITC and CD45RA FITC (Beckman Coulter); NKG2D PE, and NKG2C PE (R&D Systems); pan-γδ TCR FITC, anti-Vδ1 TCR FITC, and anti-Vδ2 TCR FITC (Pierce); biotin-labeled NKG2D (BioLegend); and CD4 FITC (eBioscience). To evaluate PEN5 expression, cells were stained with unlabeled anti-PEN5 (clone 5H10, mouse IgM) followed by counterstaining with goat anti-mouse IgM FITC (SBA). Stained cells were washed and resuspended in cold PBS 0.5% BSA before analysis on a Cyan Cytometer (DAKO Cytomation) or FACSCanto (BD Bioscience). Propidium iodide was used to exclude dead cells. To examine intracellular perforin expression, PBMCs were stained for surface markers then fixed and permeabilized using the Fix and Perm Kit (Invitrogen) followed by staining with anti-perforin FITC or an isotype-matched control (BD Biosciences). For Ig binding analysis, cells were incubated on ice with 5μg of IgG1, IgG2, IgG3, IgG4, IgM, monomeric IgA (all from Sigma), IgD (Calbiochem), IgE (a gift from Dr. Max Cooper, Emory University), or heat aggregated IgG (a gift from Dr. Hiromi Kubagawa, University of Alabama at Birmingham) for 15 minutes, then washed and counterstained with goat anti-human Ig PE (SBA). Flow cytometry figures were plotted using Flowjo (Treestar) or Summit software (Dako Cytomation).

Statistics

Statistical analyses were performed using Microsoft Excel and Statdisk software and *p* values less than. 05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FCRL	Fc Receptor-like
CLL	Chronic lymphocytic leukemia

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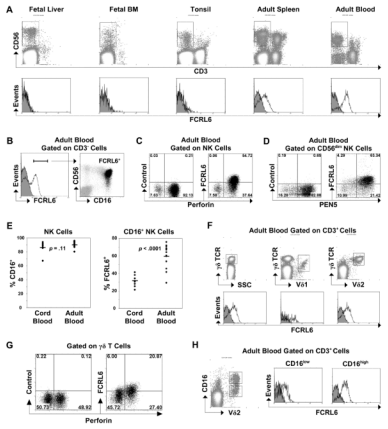

Figure 1. FCRL6 is expressed by mature CD56^{dim} NK cells and cytotoxic Vδ1^{+} and Vδ2^{+} γδ T cells

(A) Gated CD3^{-}CD56^{+} NK cells located within the boxed regions detailed in dot plots from the indicated tissues were analyzed for FCRL6 expression. Histograms below represent staining with an isotype-matched control mAb (grey shade) or the anti-FCRL6 7B7 mAb (black line). (B) The adult blood CD3^{-}FCRL6^{+} cells gated within the histogram and indicated by the horizontal line, are highlighted in black in the dot plot and plotted against CD56 and CD16 expression. Total CD3^{-} lymphocytes are plotted in grey. (C) Gated adult blood CD3^{-}CD56^{+} NK cells were analyzed with an isotype control mAb (left panel) or the anti-FCRL6 7B7 mAb (right panel). After staining for the indicated surface markers, cells were fixed, permeabilized, and analyzed for perforin expression. Percentages of cells staining positive are shown in the quadrants of each dot plot. (D) Adult blood CD3^{-}CD56^{dim} NK cells were gated and analyzed for PEN5 and FCRL6 or isotype-matched control staining. (E) CD3^{-}CD56^{+} NK cells from umbilical cord blood (n=7) or adult blood (n=13) were compared for CD16 expression (left panel) and CD3^{-}CD56^{+}CD16^{+} NK cells were examined for FCRL6 expression (right panel) measured as the percentage of cells that stain positive relative to an isotype-matched control mAb. The mean CD16^{+} or FCRL6^{+} percentage for each cohort is depicted by a horizontal bar and the *p* value was calculated using the Student's t test. (F) Analyses of γδ T cell subsets from healthy adult blood samples for FCRL6 using mAb specific for the indicated molecules are presented in a comparable manner to the plots in (A). (G) Gated adult blood CD3^{+}γδ TCR^{+} cells were assessed for FCRL6 and perforin expression as in (C). (H) CD3^{+}Vδ2^{+} T cell subsets were stratified by CD16 expression levels (indicated by the boxed regions) and examined for FCRL6 expression. For all panels at least three independent samples were analyzed and one representative plot is shown.

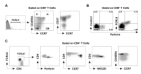

Figure 2. FCRL6 is expressed by effector and effector memory CD8$^+$ T cells and cytotoxic CD4$^+$ T cells

(A) CD8^{bright} cells from adult blood were gated and the FCRL6$^+$ cells, highlighted in black, plotted against CD45RA vs CCR7 and CD27 vs CCR7 expression. Total CD8$^+$ T cells are labeled grey in the dotplots. In the CD45RA/CCR7 plot, naive (N), central memory (CM), effector memory (EM), and effector (E) CD8$^+$ T cells are distinguished as previously described [22]. (B) Gated adult blood CD3$^+$CD8^{bright} cells were analyzed for FCRL6 and perforin expression as in Figure 1. (C) FCRL6$^+$CD4$^+$ T cells from one of three representative donors are gated and highlighted in black. This subset was further analyzed for perforin, CD57, NKG2D, CD45RA, and CCR7 expression. Total CD4$^+$ T cells are labeled grey in the dotplots.

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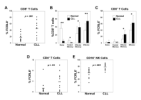

Figure 3. FCRL6-expressing CD8$^+$ T cells, CD4$^+$ T cells, and NK cells are expanded in CLL

(A) Blood T cells from 13 age-matched healthy adults were compared with those from 11 CLL patients to assess the percentage of CD8^{bright} T cells staining positive for FCRL6 compared to staining with an isotype-matched control mAb. Dots represent the percentage of FCRL6$^+$ cells from an individual donor and mean percentages from each cohort are depicted as horizontal bars. (B, C) Age-matched control and CLL cohort samples were analyzed to determine the composition of the CD8$^+$ T cell pool (B) and the percentage of CD8$^+$FCRL6$^+$ cells (C) among different subsets. Naive, central memory, effector memory, and effector cells were assessed by determining CD45RA and CCR7 expression levels as depicted in Figure 2. FCRL6 expression on central memory cells was not determined (ND) because their numbers in the CLL cohort were too low to allow for a meaningful evaluation. Bars represent the mean percentages and error bars represent SD. (D) Blood CD4$^+$ T cells (Normal, n=13 and CLL, n=11) or (E) CD16$^+$ NK cells (Normal, n=13 and CLL, n=9) were similarly evaluated for the percentage of FCRL6$^+$ cells compared to staining with an isotype-matched control mAb. The *p* values from all panels were calculated using Student's t test (* $p<0.02$; ** $p<0.002$) to compare normal versus CLL samples in each subset.

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