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Dysfunctional Vγ9Vδ2 T cells are negative prognosticators and markers of dysregulated mevalonate pathway activity in chronic lymphocytic leukemia cells

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The role of Vγ9Vδ2 T cells in chronic lymphocytic leukemia (CLL) is unexplored, although these cells have a natural inclination to react against B-cell malignancies. Proliferation induced by zoledronic acid was used as a surrogate of γδ TCR-dependent stimulation to functionally interrogate Vγ9Vδ2 T cells in 106 untreated CLL patients. This assay permitted the identification of responder and low-responder (LR) patients. The LR status was associated with greater baseline counts of Vγ9Vδ2 T cells and to the expansion of the effector memory and terminally differentiated effector memory subsets. The tumor immunoglobulin heavy chain variable region was more frequently unmutated in CLL cells of LR patients, and the mevalonate pathway, which generates Vγ9Vδ2 TCR ligands, was more active in unmutated CLL cells. In addition, greater numbers of circulating regulatory T cells were detected in LR patients. In multivariate analysis, the LR condition was an independent predictor of shorter time-to-first treatment. Accordingly, the time-to-first treatment was significantly shorter in patients with greater baseline numbers of total Vγ9Vδ2 T cells and effector memory and terminally differentiated effector memory subpopulations. These results unveil a clinically relevant in vivo relationship between the mevalonate pathway activity of CLL cells and dysfunctional Vγ9Vδ2 T cells. (*Blood*. 2012; 120(16):3271-3279)

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Introduction

The clinical course of chronic lymphocytic leukemia (CLL) is highly heterogeneous and strongly influenced by intrinsic tumor cell features such as the mutational status of the immunoglobulin heavy chain variable (IGHV) genes.[1,2] The IGHV status also imprints the reactivity to the local microenvironment: purified unmutated (UM) CLL cells have greater rates of spontaneous apoptosis in vitro than mutated (M) CLL cells, but exogenous stimuli such as soluble factors, stromal cells, and autologous T cells can prevent apoptosis and maintain UM CLL cell viability.[3]

Conventional αβ T cells play an enigmatic double-cross with CLL cells. On the one hand, they harbor tumor-reactive CD4+ and CD8+ cells potentially able to hold CLL cells in check, especially in patients with nonprogressive disease[4]; on the other hand, oligoclonal CD4+ and CD8+ cells can stimulate the growth and survival of CLL cells.[5,6] Regulatory T (Treg) cells also have been reported to influence CLL progression, especially in patients with advanced disease who have increased Treg cell numbers in the peripheral blood.[7-9]

Much less is known about the role of unconventional T cells, such as γδ T cells, on disease progression and clinical outcome.[6,10] γδ T cells play a key role in innate immune responses against microbial pathogens, stressed cells, and tumor cells. In humans, γδ T cells consist of 2 main subsets: Vδ1 T cells, which are represented mainly in spleen, mucosae, and epithelial tissues, and Vγ9Vδ2 T cells, which represent the majority of circulating γδ T cells. Both γδ T-cell subsets are equipped with natural killer (NK)-like receptors (NKR) to recognize stress-induced self-ligands like MHC class I–related molecules (MICA/B), and UL-16–binding proteins (ULBP), which are induced or up-regulated on the surface of several tumor cell types. Vδ1 T cells are increased in patients whose CLL cells express ULBP2 and ULBP3, and NKR-dependent Vδ1 T-cell activation has been reported to delay the progression of disease.[11] Unlike Vδ1 T cells, whose TCR ligands are unknown, Vγ9Vδ2 T cells sense the presence of infectious agents via the TCR-dependent recognition of intermediate metabolites (phosphoantigens; pAgs) generated by the mevalonate (Mev) and non-Mev pathway of microbial pathogens. The Mev pathway of mammalian cells also generates pAgs such as isopentenyl pyrophosphate (IPP), which activates Vγ9Vδ2 T cells as efficiently as the microbial ligands. Cell stress and transformation increase Mev pathway activity and accelerate IPP generation, whose accumulation in excess of physiologic levels is detected by Vγ9Vδ2 T cells.[12] Such accumulation of pAgs and subsequent Vγ9Vδ2 T-cell proliferation can be pharmacologically induced by zoledronic acid (ZA), a specific inhibitor of the farnesyl pyrophosphate synthase (FPPS) in the Mev pathway.[13]

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Table 1. Clinical and biologic features of CLL patients at diagnosis

No. patients	N = 106[a]
Median age, y (range)	64 (32-84)
Female, no. (%)	41 (39)
Binet stage, no. (%)	
A	89 (84)
B	12 (11)
C	5 (5)
Lymphocytes, no./μL[b,c]	14 900 ± 1800
Lymphocytes, %[b,d]	65.4 ± 1.5
Hb, g/dL[b,c]	13.9 ± 0.2
Plts, no./μL[b,e]	201000 ± 6000
LDH, U/mL[b,f]	358 ± 11
β2M, g/dL[b,g]	2.2 ± 0.1
% B CLL clone[b,h]	72 ± 2
CD38+, no. (%)[b,i]	37 (35)
ZAP70+, no. (%)[e,j]	27 (26)
K-light chain, no. (%)[h]	57 (57)
Cytogenetics[k]	
Normal karyotype, no. (%)	38 (38)
del (11)	10 (10)
del (13)	44 (44)
del (17)	13 (13)
IGHV unmutated, no. (%)[l]	34 (35)

CLL indicates chronic lymphocytic leukemia; IGHV, immunoglobulin heavy chain variable; LDH, lactate dehydrogenase; and Plts, platelets.

[a]All cases were evaluable for each of the variables, if not otherwise specified.
[b]Data are expressed as mean ± SEM.
[c]Data were available in 105 patients.
[d]Data were available in 103 patients.
[e]Data were available in 104 patients.
[f]Data were available in 93 patients.
[g]Data were available in 53 patients.
[h]Data were available in 100 patients.
[i]CD38 expression was considered positive when > 30%.
[j]ZAP70 expression was considered positive when > 20%.
[k]Data were available in 101 patients.
[l]Data were available in 97 patients.

Vγ9Vδ2 T cells are naturally inclined to recognize tumor cells of B-cell origin,[14,15] but only anecdotal data have been reported in CLL. In this work we have performed a comprehensive analysis of Vγ9Vδ2 T cells in 106 patients with untreated CLL and unveiled for the first time a relationship between Vγ9Vδ2 T cells and the Mev pathway of CLL cells, predicting a shorter time-to-first treatment (TTFT), especially in patients with UM CLL.

Methods

Patients

Between January 2004 and February 2008, peripheral blood samples were collected from 106 patients with untreated CLL after their informed consent, in accordance with the Declaration of Helsinki and approval by the local institutional review board. The median follow-up of survivors was 50 months (range, 3-277 months). Biologic and clinical features of CLL patients are summarized in Table 1. The IGHV M or UM status was determined as previously reported.[16] The control group consisted of 24 healthy donors (HD) older than 50 years of age, kindly provided by the local blood bank.

Cells and culture conditions

PBMCs were purified from HDs and patients with CLL as previously reported.[17] To investigate the Mev pathway, a B Cell Isolation Kit II (Miltenyi Biotec) was used to further purify CLL clonal cells if their percentage was less than 91% in PBMCs.

To evaluate pAgs-induced Vγ9Vδ2 T-cell proliferation, IPP accumulation was induced in monocytes with ZA as previously reported.[13] ZA induces Vγ9Vδ2 T-cell proliferation in the presence of low doses of exogenous IL-2.

PBMCs from patients with CLL and HDs were cultured in 96-well, round-bottomed plates in standard culture medium as previously reported.[17] Culture conditions included 10 IU/mL IL-2 (Eurocetus) and 1μM ZA (kindly provided by Novartis Pharma) + 10 IU/mL IL-2. On day 7, cells were harvested and analyzed for cell number and viability by trypan blue staining. Viable Vγ9Vδ2 T cells were counted, and the fold increase was calculated in each individual patient by dividing their counts after stimulation with ZA + IL-2 versus IL-2 alone. We defined a median fold increase of 3.62 as cut-off value to categorize patients as responder (R; > 3.62) or low-responder (LR; < 3.62).

mAbs and cytofluorometric analysis

Two- and three-color flow cytometry was performed with the appropriate combinations of FITC, R-phycoerythrin (PE), Tri-color, peridinin-chlorophyll-protein complex (PerCP), or allophycocyanin (APC)–conjugated mAbs, and subsequent analysis was performed with the FACScan cell sorter and CELLQuest software (Becton Dickinson).

CLL cells were identified and characterized with the use of anti-CD5–APC (DAKO SpA), anti-CD19–PerCP (Beckman Coulter), anti-CD38–PE (Becton Dickinson), and anti-ZAP70–Alexa Fluor 488 (Caltag Laboratories).

γδ T cells and Vγ9Vδ2 T cells were identified and characterized by the use of anti-CD3–APC (Ancell Corporation); anti-CD3–PerCP, anti-TCR γδ-PE, anti-TCR γδ-FITC, anti-TCR Vγ9-PE (all from Becton Dickinson); anti-TCR Vδ1–FITC (Endogen-Pierce); and anti-CD6–PE (Ancell). Vγ9Vδ2 T-cell subset distribution was evaluated by the use of anti-TCR Vγ9-PE (Becton Dickinson), anti-CD27–FITC (Dako Cytomation), and anti-CD45RA–APC (Caltag Laboratories).[17,18]

NKR expression patterns were determined by 7-color flow cytometry and analyzed with the CyAn ADP flow cytometer (Beckman Coulter) and FlowJo software (TreeStar Inc). The following NKR were evaluated (number of patients tested and mAb used in parenthesis): NKG2A (n = 32; anti-CD159a–PE); NKp44 (n = 23; anti-CD336–PE); NKp46 (n = 23; anti-CD335–PE); NKp30 (n = 23; anti-CD337–PE); p50.3 (n = 32; anti-CD158i–PE); p58.1 (n = 32; anti-CD158a,h–PE); p58.2 (n = 32; anti-CD158b,j–PE); p70 (n = 32; anti-CD158e–PE); ILT2 (n = 32; anti-CD85j–PE); FcγRIII (n = 32; anti-CD16–PE-Cy7; all from Beckman Coulter); NKG2C (n = 32; anti-CD159c–PE); and NKG2D (n = 32; anti-CD314–PE; both from R&D Systems). Presentation of data on a logarithmic scale was used when data covered a large range of values.

CD6 expression on Vγ9Vδ2 T cells (n = 19) was analyzed using an anti-CD6–PE mAb (Ancell). In 29 samples from patients with CLL, NKR ligands surface expression was evaluated with anti–HLA-G–FITC (Serotec; Kidlington) and anti-MICA/B (R&D) mAb. CD166 expression also was evaluated in the CLL cells of 19 patients via the use of anti-CD166–PE mAb (Ancell).

Treg cells were quantified by 4-color flow cytometry in 24 patients with CLL and 15 with HD. Treg were identified by surface staining with anti-CD3–PerCP (Becton Dickinson), anti-CD4–APC (Beckman Coulter), anti-CD25–PE (Caltag Laboratories), and intracellular staining with anti-FOXP3–FITC mAb as previously reported.[13]

Gene profiling data analysis

Metabolic activity of CLL cells was retrospectively evaluated with the use of gene expression profiling data available from 13 patients with M CLL and 4 patients with UM CLL. RNA extraction procedure and oligonucleotide microarray assay were performed as previously reported.[19] The detailed protocol for sample preparation and microarray processing is reported elsewhere (http://www.affymetrix.com). Each gene expression data were log2 transformed and then Z-scored (gene value-whole chip's mean value/whole chip SD value). Using this conversion, we characterized more than-expressed genes by positive Z-scoring values in the 0 to +3 range, whereas negative values in the 0 to −3 range correspond to less

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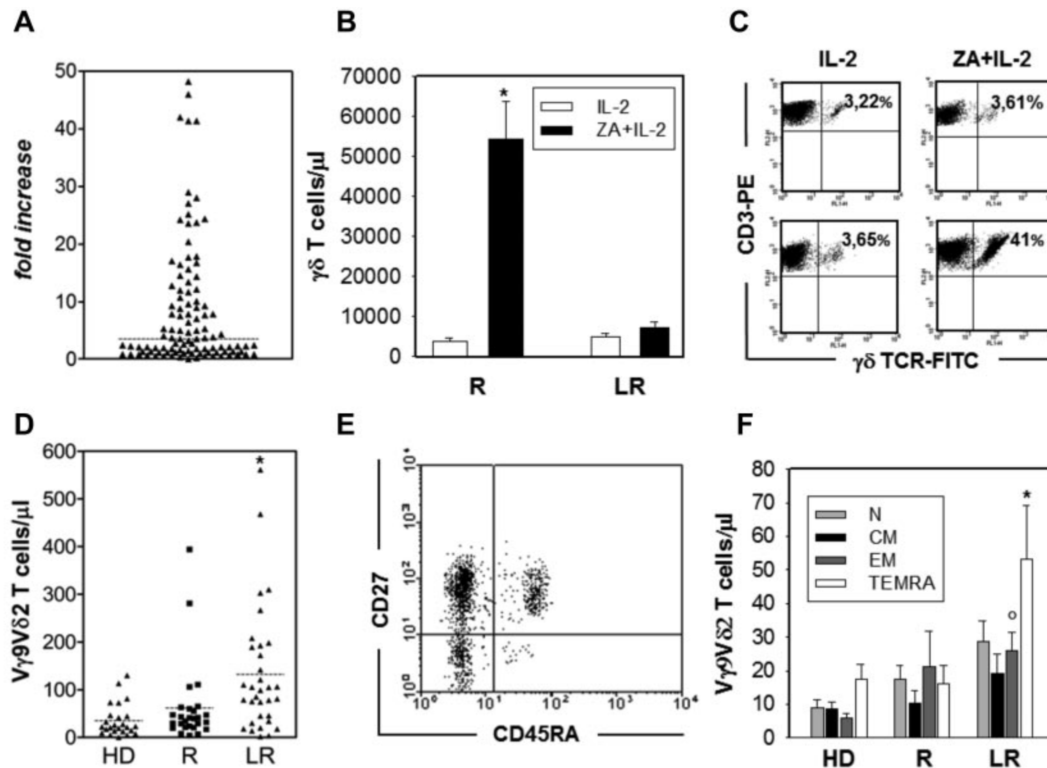

Figure 1. Proliferative response of γδ T cells to ZA + IL-2 identifies 2 subsets of CLL patients. (A) Increase in the absolute number of γδ T cells after 7 days stimulation with ZA + IL-2. Fold increase was calculated for each patient as a ratio between the absolute number of viable γδ T cells in PBMCs stimulated with ZA + IL-2 and that in PBMCs stimulated with IL-2 alone [(ZA + IL-2)/IL-2] on day 7. Median value of fold increase (3.62) was selected as a cut-off value to define R and LR patients. (B) Absolute numbers of γδ T cells in R (n = 53) and LR (n = 53) CLL patients after stimulation with ZA + IL-2 or IL-2 alone. After 7 days of culture in ZA + IL-2 conditioned medium, the total count of γδ T cells was significantly greater in R than in LR patients (*$P < .001$). (C) γδ T-cell proliferation was evaluated after 7 days' stimulation with ZA + IL-2. Representative dot plots from a LR (top) and from an R (bottom) patient are shown. Percent γδ T cells of total $CD3^+$ T cells is indicated. (D) Baseline total counts of Vγ9Vδ2 T cells in HD (n = 24), R (n = 27), and LR (n = 33) patients. LR patients showed significantly greater numbers of circulating Vγ9Vδ2 T cells compared with HD and R patients (*$P < .001$ and $P = .005$, respectively). (E) Representative cytofluorimetric analysis of Vγ9Vδ2 T cell subsets according to the expression of CD27 and CD45RA markers. Four Vγ9Vδ2 T-cell subsets are identified: $CD45RA^+CD27^+$ N cells (top right); $CD45RA^-CD27^+$ CM cells (top left); $CD45RA^-CD27^-$ EM cells (bottom left); and $CD45RA^+CD27^-$ TEMRA (bottom right). (F) Vγ9Vδ2 T cells subsets distribution in HD (n = 20), R (n = 23), and LR (n = 28) patients. The absolute number of EM and TEMRA Vγ9Vδ2 T cells was significantly greater in LR CLL patients compared with HD (°$P = .009$ and *$P = .03$, respectively) and to R CLL patients (°$P = .018$ and *$P = .004$, respectively). In panels A and D, horizontal lines represent median value. In panels B and F, bars represent mean values ± SEM.

than-expressed genes (http://194.57.223.128/crct/). The Z-scores of gene expression level for enzymes involved in each of the following pathways were summed: Mev pathway, glycolysis, Krebs cycle, and pyruvate pathway. The metabolic Z-score of each pathway was computed for each cell sample, and the Z-scores of UM and M CLL cell samples were compared.

Biochemical Mev pathway quantification

CLL cells from 33 M and 20 UM patients were incubated for 24 hours with 1 µCi of [^{3}H]acetate (3600 mCi/mmol; Amersham International) to measure the synthesis of cholesterol and farnesyl pyrophosphate (FPP) and with 1 µCi of [^{14}C]mevalonic acid (60 mCi/mmol; PerkinElmer) to detect the levels of intracellular and extracellular IPP (IPP_{in} and IPP_{ex}, respectively), as reported.[13] According to the titration curves, the results are expressed as fmol/L $\times 10^6$ cells for cholesterol, FPP, and IPP_{in}, and as pmol/L $\times 10^6$ cells for IPP_{ex}.

In a set of experiments, cells were incubated 24 hours with known pharmacologic inhibitors of Mev pathway (10µM simvastatin [Sim] or 1µM ZA), then grown for an additional 24 hours with [^{3}H]acetate or [^{14}C]mevalonic acid.

Statistical analysis

Statistical analysis was performed with the SigmaStat 3.11 and SigmaPlot 9.01 software (Systat Software Inc) and MedCalc 7.3 (MedCalc Software). Results are expressed as mean ± SEM or median value, as indicated. Continuous variables were compared by *t* test and Mann-Whitney rank sum test, whereas categorical variables were compared by χ^2 test. TTFT was defined as time from diagnosis to first-line treatment, death, or last follow-up and was estimated by Kaplan-Meier plots; differences between groups were investigated by log-rank test. Multivariate analysis was performed by use of the Cox proportional hazard regression test. A $P < .05$ was considered significant.

Results

Identification of R and LR patients

ZA-induced Vγ9Vδ2 T-cell proliferation was evaluated in 106 patients with untreated CLL. The Vγ9Vδ2 T-cell fold increase in individual patients varied from 0 to 48. The median fold increase (3.62) was selected as the cut-off value, and 53 patients were classified as R and 53 as LR (Figure 1A). As expected, total counts of Vγ9Vδ2 T cells were significantly greater in R versus LR patients after ZA + IL-2 stimulation but not after stimulation with IL-2 alone (Figure 1B). Representative Vγ9Vδ2 T-cell cytofluorometric analysis from 1 LR and R patient are shown in Figure 1C.

The LR status was not because of lower baseline Vγ9Vδ2 T-cell counts because LR patients had significantly greater values than HD ($P < .001$) and R CLL ($P = .005$; Figure 1D), whereas the difference between HD and R CLL was not significant.

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Table 2. Features of NK receptors tested

NKR	CD	Gene name	Function	Ligand
p58.1	CD158a,h	KIR2DL1	Inhibitory	HLA-C
		KIR2DS1	Activating	
p58.2	CD158b,j	KIR2DL2	Inhibitory	HLA-C/B
		KIR2DS2	Activating	
p50.3	CD158i	KIR2DS4	Activating	HLA-C/A11
p70	CD158e	KIR3DS1	Inhibitory	HLA-A/B
		KIR3DL1	Activating	
NKG2A	CD159a	KLRC1	Inhibitory	HLA-E
NKG2C	CD159c	KLRC2	Activating	HLA-E
NKG2D	CD314	KLRK1	Activating	MICA/B
				ULBP1 (bacteria)
ILT2	CD85j	LILRB1	Inhibitory	HLA-B/C/G
				UL18 (CMV)
FcγRIIIa	CD16	FCGR3A	Activating	Fc portion of IgG Ab
NKp46	CD335	NCR1	Activating	?
NKp44	CD336	NCR2	Activating	?
NKp30	CD337	NCR3	Activating	?

NK indicates natural killer.

Vγ9Vδ2 T-cell subset distribution was evaluated according to CD27 and CD45RA expression in 23 R and 28 LR patients and compared with HD (Figure 1E). Differences were not statistically different between HD and R patients, whereas LR patients showed significantly greater total counts of naive (N), effector memory (EM), and terminally differentiated effector memory (TEMRA) cells than did HDs (always $P \leq .03$), and those of EM and TEMRA cells also were significantly greater than R patients ($P = .018$ and $P = .004$, respectively; Figure 1F).

The activation threshold of Vγ9Vδ2 T cells is fine-tuned by the interactions of activating and inhibitory NKR with the corresponding ligands on tumor cells (see Table 2 for NKR nomenclature, functions, and ligands).[20] NKR profiles were available in 32 patients. NKG2D, an activating NKR, was significantly up-regulated on Vγ9Vδ2 T cells of R patients ($P = .04$; supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article), whereas none of the other NKRs showed a statistically different distribution between R and LR Vγ9Vδ2 T cells (data not shown).

The expression of MICA/B and HLA-G as prototypic counter-receptors of activating and inhibitory NKR also was investigated in CLL cells without us detecting any difference between R and LR patients (data not shown).

CD166 expression on tumor cells provides a potent costimulatory signal for TCR-dependent Vγ9Vδ2 T-cell activation by recruiting CD6 at the center of the immune synapse.[21] R patients showed a significantly greater percentage of CD166$^+$ CLL cells than LR patients (supplemental Figure 1B), whereas CD6 expression on Vγ9Vδ2 T cells was not statistically different (data not shown).

Association between R/LR and IGHV mutational status

We asked whether the R/LR status was associated with clinically relevant CLL features. CLL cell counts, disease stage, β2-microglobulin, LDH serum levels, CD38 and ZAP70 expression, and cytogenetics abnormalities by FISH were not statistically different between R and LR patients (Table 3). By contrast, the IGHV mutational status was significantly different between R and LR CLL: 63 of 97 evaluable CLL (65%) were M, whereas 34 of 97 were UM (35%). Forty of 50 R CLL were M (80%), whereas 24 of 34 of UM CLL were LR (71%; $P = .003$). These patients were termed concordant because they showed a clear-cut association with the IGHV mutational status (R-M, LR-UM). Thirty-three patients did not fall into the concordant categories: CLL cells were M in 23 LR patients (LR-M) and UM in 10 R patients (R-UM), and these patient were termed discordant.

NKR profiles were reassessed according to the IGHV mutational status. NKp44, an activating NKR, was significantly more expressed on Vγ9Vδ2 T cells from M than UM patients ($P = .04$; supplemental Figure 1C). We also investigated NKR profiles after the patients' categorization according to both R/LR and M/UM status. The results showed that R-M tended to have a more favorable NKR activating profile than LR-UM patients (supplemental Figure 1D-H).

UM CLL cells show greater levels of Mev pathway activity than M CLL cells

Next, we asked whether Vγ9Vδ2 T-cell alterations in LR patients were driven by a chronic pAgs stimulation. A preliminary comparison of gene expression (measured by the use of Affimetrix HG U133 plus 2 microarrays) showed that the Z-scores for glycolysis, Krebs cycle, and pyruvate pathway were not statistically different between the cell samples from 11 M and 4 UM patients. In contrast, the Z-score for the Mev pathway was significantly greater in UM than M CLL cells ($P < .01$), suggesting a greater metabolic activity in the former (Figure 2).

To validate this finding, we conducted a prospective analysis and biochemically quantified the amounts of FPP, IPP_{in}, IPP_{ex}, and cholesterol produced by CLL cells isolated from 20 UM and 33 M patients. In line with the transcriptome-based conclusions, UM CLL cells generated significantly greater amounts of cholesterol, FPP, IPP_{in}, and IPP_{ex} than M CLL cells (always $P \leq .03$;

Table 3. Comparison of clinical and biologic features in R and LR CLL patients

	R (n = 53)[a]	LR (n = 53)[a]	P
Median age, y (range)	63 (35-82)	64 (32-84)	NS
Female, no. (%)	21 (40)	20 (38)	NS
Binet stage, no. (%)			
A	44 (83)	45 (85)	NS
B	7 (13)	5 (9)	NS
C	2 (4)	3 (6)	NS
Lymphocytes, no./μL[b]	14 200 ± 2900	15 700 ± 2100[c]	NS
Lymphocytes, %[b]	64.5 ± 2[c]	66.4 ± 2.3[d]	NS
Hb, g/dL[b]	14.2 ± 0.2[d]	13.6 ± 0.3	NS
Plts, no./μL[b]	202 000 ± 8000[d]	200 000 ± 9000	NS
LDH, U/mL[b]	342 ± 10[e]	373 ± 20[f]	NS
β2M, g/dL[b]	2.1 ± 0.1[g]	2.2 ± 0.3[h]	NS
% B CLL clone[g]	73 ± 3[d]	72 ± 3[i]	NS
CD38$^+$, no. (%)[j]	18 (34)	19 (37)[c]	NS
ZAP70$^+$, no. (%)[k]	14 (26)	13 (25)[e]	NS
K-light chain, no. (%)	26 (51)[e]	31 (63)[i]	NS
del(11) and/or del(17), no. (%)	8 (15)	12 (25)[l]	NS
IGHV unmutated, no. (%)	10 (20)[m]	24 (51)[n]	.003

CLL indicates chronic lymphocytic leukemia; IGHV, immunoglobulin heavy chain variable; LDH, lactate dehydrogenase; LR, low responder; NS, nonsignificant; and R, responder.

[a]All cases were evaluable for each of the variables, if not otherwise specified.
[b]Data expressed as mean ± SEM.
[c]Data were available in 52 patients.
[d]Data were available in 51 patients.
[e]Data were available in 47 patients.
[f]Data were available in 46 patients.
[g]Data were available in 27 patients.
[h]Data were available in 26 patients.
[i]Data were available in 49 patients.
[j]CD38 expression was considered positive when > 30%.
[k]ZAP70 expression was considered positive when > 20%.
[l]Data were available in 48 patients.
[m]Data were available in 50 patients.
[n]Data were available in 47 patients.

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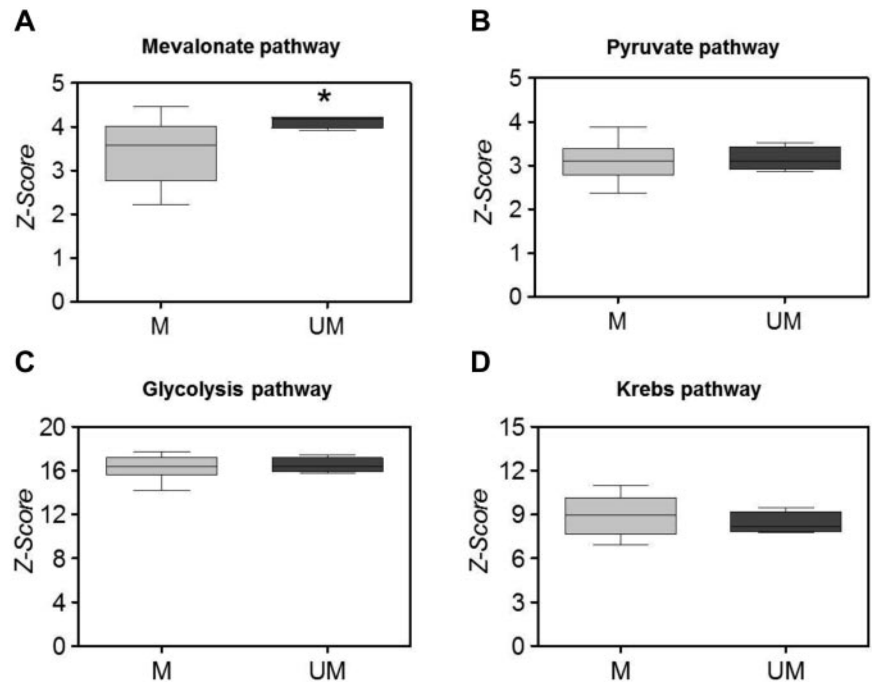

Figure 2. Gene expression profiling of CLL cells' metabolic activity. The expression level of enzymes involved in the main cellular metabolic pathways was retrospectively analyzed in M (n = 13) and UM (n = 4) CLL cells. Between-groups comparison of Z-scores indicates that the Mev pathway was significantly greater in UM CLL cells than in M cells (median Z-score 4.2 and 3.6, respectively, $^*P < .01$; A). No significant differences were found in the analysis of the pyruvate pathway (B), glycolysis (C), and Krebs cycle (D). Box and whiskers plot represent median values, first and third quartiles, and minimum and maximum values for each dataset.

Figure 3). In particular, IPP_{ex} levels, which are even more critical than IPP_{in} to activate Vγ9Vδ2 T cells,[13] were uniformly greater in UM patients irrespective of their R/LR status: both concordant LR-UM and discordant R-UM CLL cells showed significantly greater IPP_{ex} levels than R-M and LR-M CLL cells, whereas discordant LR-M CLL cells showed a Mev pathway activity similar to that of R-M CLL (supplemental Figure 2).

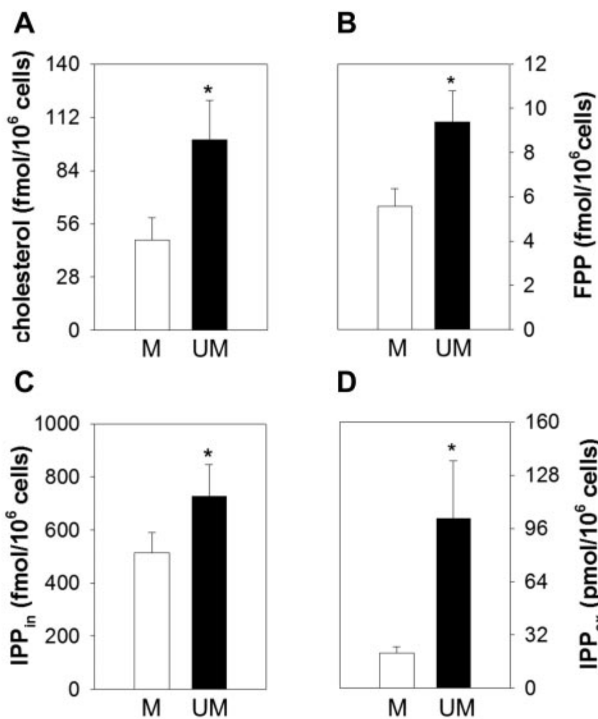

Figure 3. UM CLL cells have a significantly greater activity of the Mev pathway compared with M CLL cells. Mev pathway metabolites were measured in CLL cells cultures from M and UM patients. (A) Cholesterol production. UM CLL cells (n = 20) produced significantly greater levels of cholesterol compared with M CLL cells (n = 33; $^*P = .034$). (B) FPP production. UM CLL cells (n = 20) produced significantly greater levels of FPP compared with M CLL cells (n = 33; $^*P = .026$). (C) IPP_{in} production. IPP_{in} was significantly greater in UM (n = 17) than in M (n = 28) CLL cells ($^*P = .019$). (D) IPP_{ex} production. IPP_{ex} was significantly greater in UM (n = 19) than in M (n = 27) CLL cells ($^*P < .001$). Bars represent mean values ± SEM.

The Mev pathway can be pharmacologically inhibited using statins like Sim or aminobisphosphonates like ZA. The former switches off the whole pathway, whereas ZA switches off the pathway downstream to FPPS and thereby induces IPP accumulation. The Mev pathway was equally targetable by Sim, which significantly decreased cholesterol, FPP, IPP_{in}, and IPP_{ex} levels in both M and UM CLL cells. ZA also inhibited cholesterol and FPP synthesis in a comparable way in UM and M CLL cells, but not IPP_{in} and IPP_{ex} levels, which were significantly greater in the former (supplemental Figure 3). These data indicate that the Mev pathway is amenable to pharmacologic manipulation in both M and UM CLL cells, but they also demonstrate that the accelerated activity of the Mev pathway in UM CLL cells generates significantly greater amounts of IPP_{in} and IPP_{ex} on ZA-induced FPPS inhibition.

Increased Treg-cell numbers in patients with CLL

Vγ9Vδ2 T-cell proliferation can be antagonized by Treg and an excess of Treg over Vγ9Vδ2 T cells can be responsible for the LR status reported in some cancers.[22] Thus, we evaluated the involvement of Treg cells in the LR status of CLL patients (Figure 4). LR patients showed the greatest counts (104 ± 21 Treg cells/μL), significantly greater than R patients (75 ± 25 Treg cells/μL, $P = .018$), and HDs (8 ± 2 Treg cells/μL, $P < .001$). The difference between R patients and HD also was statistically significant ($P < .001$; Figure 4B), whereas no difference was detected between M (94 ± 27 Treg cells/μL) and UM patients (89 ± 10 Treg cells/μL; data not shown).

Prognostic value of R/LR status

The median TTFT of all CLL patients was 82 months. Binet stage, FISH abnormalities, and M/UM status were statistically significant

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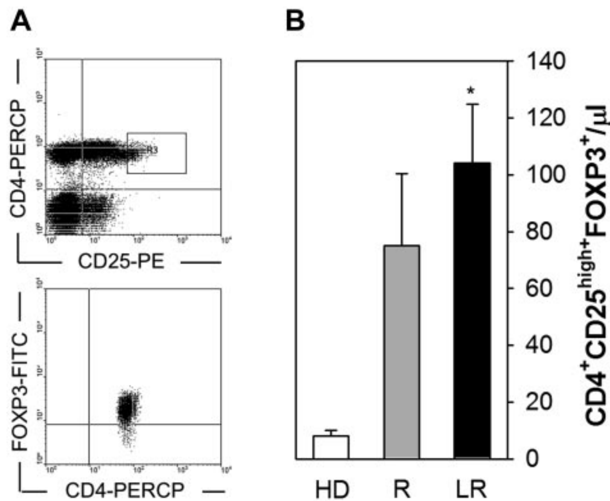


Figure 4. Treg cells are significantly expanded in LR patients. (A) Gating strategy used to quantify Treg cells. $CD4^+CD25high^+$ T cells (R3) were identified in total lymphocytes (top) and evaluated for their expression of FOXP3 (bottom). (B) Absolute numbers of circulating $CD4^+CD25high^+FOXP3^+$ cells. Treg total counts were significantly greater in the peripheral blood of LR patients (n = 14) compared both with healthy donors (HD, n = 15) and R patients (n = 10; $^*P < .001$ and $P = .018$, respectively). Bars represent mean values ± SEM.

TTFT predictors by univariate analysis (Figure 5A-C). Interestingly, the R/LR status was also a significant TTFT predictor in univariate analysis: the median TTFT of LR CLL was 59 months, whereas that of R CLL was not reached ($P = .026$; Figure 5D). The multivariate analysis showed that the negative prognostic value of LR status was maintained after adjustment for the other variables ($P = .03$; Figure 5E).

Because an advanced Binet stage at diagnosis is an indication per se to initiate treatment, the multivariate analysis also was performed after we removed the Binet stage B and C covariates. Even in this model, the LR status confirmed its value as a negative TTFT predictor (data not shown).The median TTFT was statistically different between the concordant LR-UM and R-M patients (29 months vs 82 months; $P < .001$; Figure 6). The R/LR status also was useful to refine the prognosis within UM patients because R-UM had a significantly longer TTFT than LR-UM patients (not reached vs 29 months, $P = .02$).

We also evaluated the impact of baseline Vγ9Vδ2 T-cell and EM + TEMRA cell counts on TTFT. The median values (Vγ9Vδ2 T cells: 57.5/μL; EM+TEMRA cells: 29.3/μL) were used as the cut-off values to divide patients into high and low categories. TTFT was significantly longer in $V\gamma9V\delta2_{low}$ and $EM + TEMRA_{low}$ patients compared with $V\gamma9V\delta2_{high}$ (median TTFT not reached for $V\gamma9V\delta2_{low}$ vs 42 months for $V\gamma9V\delta2_{high}$, $P = .014$) and $EM + TEMRA_{high}$ patients (median TTFT not reached for $EM + TEMRA_{low}$ vs 42 months for $EM + TEMRA_{high}$, $P = .02$; Figure 7). A receiver operating characteristic analysis using treatment status as state variable further corroborated the reliability of median values (data not shown).

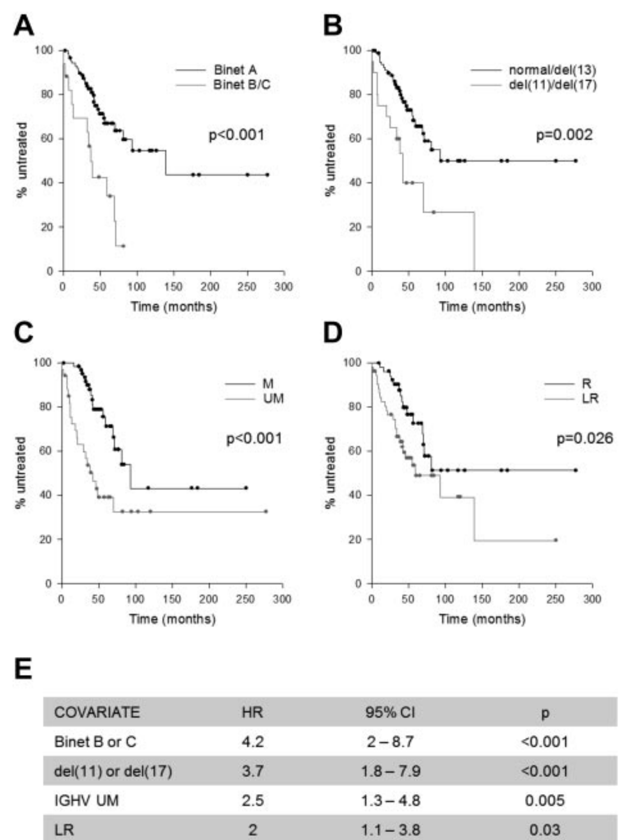
For both Vγ9Vδ2 and EM+TEMRA Vγ9Vδ2 T-cell counts, the association with the IGHV status was maintained, even if it did not reach a statistical significance ($P = .051$ and $P = .055$, respectively).

Discussion

Vγ9Vδ2 T cells are intrinsically inclined to recognize and kill tumor cells of B-cell origin, but their contribution to the immune regulation and clinical outcome of CLL patients has been unexplored. To close this gap, we performed a comprehensive analysis of Vγ9Vδ2 T cells in 106 patients with untreated CLL.

Proliferation of Vγ9Vδ2 T cells to ZA-induced stimulation was used as a surrogate of pAgs-induced, TCR-dependent activation. This assay permitted the separation of patients with CLL into LR and R. Provocatively, the former showed significantly greater baseline peripheral Vγ9Vδ2 T-cell counts than the latter, ruling out a quantitative defect. Monocytes, which are essential to generate endogenous IPP to induce Vγ9Vδ2 T-cell proliferation after ZA stimulation,[17] also displayed similar counts in HD, R, and LR patients (data not shown).

Vγ9Vδ2 T cells are divided into subsets according to their phenotype, proliferative capacity, and effector functions. N and central memory (CM) cells display high proliferative capacity but low effector functions, whereas EM and TEMRA cells display the opposite pattern.[18] LR patients showed a significant accumulation of EM and TEMRA cells, whereas Vγ9Vδ2 T-cell subset distribution was well-shaped in R patients as in HD. Of note, this subset imbalance is peculiar to CLL. Approximately 50% of untreated multiple myeloma are also LR, but the most relevant alteration in these patients is the decrease of N and CM cells rather than accumulation of EM/TEMRA cells.[17]



COVARIATE	HR	95% CI	p
Binet B or C	4.2	2 – 8.7	<0.001
del(11) or del(17)	3.7	1.8 – 7.9	<0.001
IGHV UM	2.5	1.3 – 4.8	0.005
LR	2	1.1 – 3.8	0.03

Figure 5. R/LR status is predictive of TTFT in CLL. Kaplan-Meier survival curves for TTFT in our cohort of patients stratified on the basis of Binet staging system, cytogenetic abnormalities, IGHV mutational status, and γδ T-cell proliferative response are shown. *P* values were calculated by log-rank test. By univariate analysis, advanced Binet stage (B or C; stage B/C, n = 17; stage A, n = 89; A), unfavorable cytogenetic abnormalities (del(11) or del(17) [del(11)/del(17)], n = 20; normal/del(13), n = 81; B), UM IGHV mutational status (UM, n = 34; M, n = 63; C), and LR status (LR, n = 53; R, n = 53; D) correlated with shorter TTFT. By Cox multivariate analysis all the aforementioned parameters were independently associated with shorter TTFT (E).

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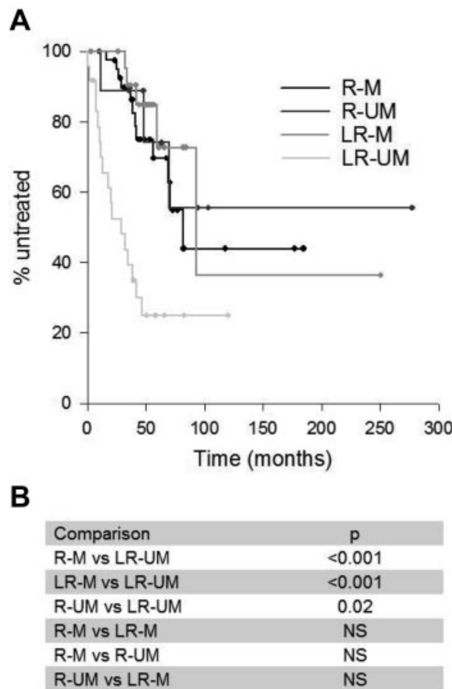

Comparison	p
R-M vs LR-UM	<0.001
LR-M vs LR-UM	<0.001
R-UM vs LR-UM	0.02
R-M vs LR-M	NS
R-M vs R-UM	NS
R-UM vs LR-M	NS

Figure 6. The combination of IGHV mutational profile and R/LR status identifies groups of CLL patients with different TTFT. Patients were divided into 4 groups on the basis of their IGHV mutational profile and their R/LR status. For each group median TTFT was calculated and compared by Kaplan-Meier analysis. Median TTFT was 29 months for LR-UM (n = 24), 82 months for R-M (n = 40), 93 months for LR-M (n = 23), and was not reached for R-UM patients (n = 10). (A) R-M and LR-UM patients retained a significant difference ($P < .001$). Among UM CLL patients, the R/LR status was found to further discriminate those who were likely to progress and those who would remain stable, as R patients had a significantly longer TTFT than LR ($P = .021$). (B) Between-groups comparisons are shown. P values were calculated by log-rank test.

Vγ9Vδ2 T cells have the prerogative to be activated not only via TCR-dependent stimuli but also via TCR-independent interactions of stress pathway receptors, such as NKR, with the corresponding ligands on tumor cells. NKG2D is an activatory NKR recognizing ligand such as MICA/B and ULBP, which are expressed on the cell surface of several hematologic malignancies including CLL.[11,23] Interestingly, significantly greater proportions of Vγ9Vδ2 T cells expressed NKG2D in R compared with LR patients, whereas no differences were observed in the expression of the corresponding ligands MICA/B on CLL cells from R and LR patients.

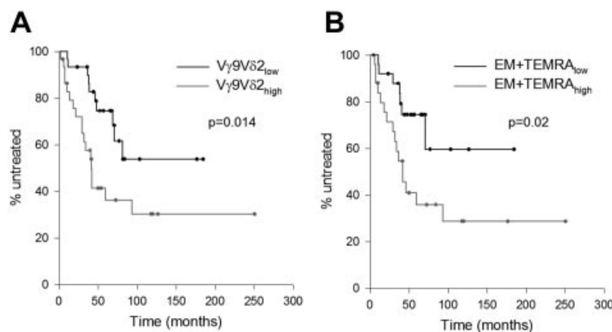

Figure 7. Baseline levels of total Vγ9Vδ2 T cells and EM + TEMRA subsets affect TTFT. Patients were allocated to $V\gamma9V\delta2_{low}$/$V\gamma9V\delta2_{high}$ and EM + $TEMRA_{low}$/EM + $TEMRA_{high}$ categories on the basis of their baseline count of total Vγ9Vδ2 T cells and EM + TEMRA subpopulations. Median values of baseline count of total Vγ9Vδ2 T cells and EM + TEMRA subsets calculated for the entire group of patients were used as cut-off level. (A) TTFT was significantly longer in $V\gamma9V\delta2_{low}$ compared with $V\gamma9V\delta2_{high}$ patients (median TTFT not reached and 42 months, respectively, $P = .014$). (B) TTFT was significantly longer in EM + $TEMRA_{low}$, compared with EM + $TEMRA_{high}$ patients (median TTFT not reached and 42 months, respectively, $P = .02$).

Besides TCR and NKR signaling, Vγ9Vδ2 T-cell activation is also regulated by the interactions of cell surface receptors with adhesion molecules such as ICAM-1 and CD166.[21,24] The latter was significantly more expressed on CLL cells from R than LR patients, and the interaction between CD6 on Vγ9Vδ2 T cells and CD166 on tumor cells facilitates TCR-mediated Vγ9Vδ2 T-cell activation and the generation of effective antitumor immune responses.[21]

This lower NKG2D and CD166 expression could partially explain the defective ZA-induced Vγ9Vδ2 T-cell responses of LR patients but not the increased numbers of EM/TEMRA Vγ9Vδ2 T cells, which rather suggested antigen-driven differentiation. To gain some insights into this apparent discrepancy, we tried to correlate the R/LR status with some of the most important clinical and biologic features of CLL patients. Interestingly, the IGHV mutational status was the only feature significantly different in R and LR patients: 80% of R patients were M, whereas 70% of UM patients were LR.

Given the unique pAgs-reactivity of Vγ9Vδ2 T cells, we investigated whether M and UM cells had different Mev pathway activity. The Z-score in silico approach showed that Mev pathway was the only one among the glycolysis, Krebs cycle, and pyruvate pathway to be significantly different in M and UM CLL cells. A biochemical analysis confirmed that UM CLL cells have a significantly accelerated Mev pathway activity compared with M CLL cells. The Mev pathway activity is increased in tumor cells with greater metabolic demands, which is in line with the greater proliferation rate and reactivity to prosurvival signals of UM compared with M CLL cells.[25] A serum metabolome analysis recently performed in CLL in which the authors used nuclear magnetic resonance has shown that serum cholesterol levels are significantly greater in UM patients and that serum cholesterol is the metabolite scoring the highest difference between UM and M patients.[26]

The accelerated Mev pathway activity in UM CLL cells results in increased pAgs production such as IPP_{in} and IPP_{ex}, which are very potent TCR-dependent Vγ9Vδ2 T-cell activators. IPP generation was boosted by ZA treatment, especially in UM CLL cells, and the amounts of IPP_{ex} released from these cells was almost 5-fold that released by M CLL cells but still insufficient to induce Vγ9Vδ2 T-cell proliferation in LR patients.

One possible explanation is that Vγ9Vδ2 T cells have become functionally exhausted in LR patients as a consequence of the long-lasting exposure to supraphysiologic IPP levels produced by UM CLL cells, especially in the absence of adequate IL-2 costimulation. Indeed, several in vitro and in vivo data indicate that chronic Vγ9Vδ2 T-cell activation may induce functional exhaustion or anergy. Anergy has been reported in HIV-infected individuals,[27] whereas functional exhaustion has been described in a preclinical nonhuman primate model after repeated stimulations with the synthetic pAgs BrHPP and low-dose IL-2.[28] Functional exhaustion has been associated with a differentiation shift, which is well in line with the accumulation of EM/TEMRA Vγ9Vδ2 T cells in LR patients.

To identify other possible mechanisms underlying Vγ9Vδ2 T-cell dysfunction, we have focused on Treg cells. These cells have been reported to be increased in the peripheral blood of untreated CLL patients, especially in those with extended or progressive

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disease,[7-9] and greater frequencies of Treg cells have been correlated with decreased CD8$^+$ T-cell immune responses against viral and tumor antigens.[8] Treg cells also have been reported to inhibit pAgs-induced Vγ9Vδ2 T-cell proliferation, and the LR status of selected cancer patients also has been attributed to an excess of Treg cells over Vγ9Vδ2 T cells.[22] We have confirmed that patients with untreated CLL have increased counts of circulating Treg cells, and, interestingly, LR patients showed greater values than R patients.

Therefore, this set of data shows that the defective ZA-induced Vγ9Vδ2 T-cell proliferation in LR patients can be interpreted as a multifactorial dysfunction to which contribute the NKR pattern, the accumulation of EM/TEMRA cells, driven by the supra-physiologic IPP amounts generated by UM CLL cells, and the Treg-cell excess.

The next question was the clinical relevance of such Vγ9Vδ2 T-cell dysfunction. Intuitively, the accumulation of antigen-experienced TEMRA Vγ9Vδ2 T cells with a powerful cytotoxic potential could be considered beneficial in terms of disease control, but the strong association between LR and UM status argued against this interpretation. Indeed, LR patients showed a significantly shorter TTFT than R patients. As expected, Binet stage C, unfavorable cytogenetics, and IGHV UM status also were negative prognosticators and included in a multivariate analysis. The LR status remained statistically significant in the multivariate analysis, highlighting its value as an independent prognosticator sizing the "seed" and "soil" contribution to disease progression in CLL.

When the IGHV and R/LR status were combined together, the concordant R-M and LR-UM patients showed very different TTFT, as expected. Interestingly, the discordant R-UM patients had a significantly better TTFT than LR-UM. Thus, the combination of R/LR and M/UM status is helpful to identify a subgroup of UM patients with a more favorable outcome.

Because LR patients had significantly greater counts of Vγ9Vδ2 T cells than R patients, mainly because of the accumulation of EM/TEMRA cells, we investigated whether these counts could be used as an alternative to predict TTFT instead of the more cumbersome and time-consuming functional R/LR status evaluation. Indeed, TTFT was significantly shorter in patients with greater numbers of circulating Vγ9Vδ2 T cells, a negative prognosticator that maintained its predictive value also when counts were limited to EM + TEMRA cells only.

The negative prognostic value of EM + TEMRA Vγ9Vδ2 T-cell counts was reminiscent of data previously reported in conventional CD4$^+$ cells and CD8$^+$ cells. An increase of CM/EM CD4$^+$ T cells was reported in UM patients and associated with advanced stage, progressive disease, and shorter TTFT. In multivariate analysis, the relative numbers of CM/EM CD4$^+$ cells remained a negative TTFT prognosticator after correction for CD38 expression, IGHV status, genomic alterations, and disease stage.[29] The emergence of EM and TEMRA cells also has been reported in CD8$^+$ cells, particularly in CLL patients with inverted CD4:CD8 ratio who showed progressive disease and shorter TTFT than patients without TEMRA accumulation.[30] TEMRA CD8$^+$ cells in these patients displayed the phenotypic hallmarks of functional exhaustion, further supporting the concept that long-lasting tumor-induced chronic activation leads to the undesired accumulation of cells unable to exert effective antitumor activity.

Several drugs are available to selectively target the Mev pathway, such as statins and aminobisphosphonates, and we have shown that the Mev pathway activity of UM patients, although accelerated, is amenable to drug manipulation as effectively as in M patients. Statins induce apoptosis and inhibit proliferation of CLL and lymphoma cells in vitro and increase the susceptibility of both drug-sensitive and drug-resistant CLL and lymphoma cells to dexamethasone and cytotoxic drugs.[31] Thus, statins are worth investigating in association with other drugs and eventually revert drug-resistance especially in UM patients.

Shanafelt et al have reported that TTFT in CLL patients taking statins was not different compared with patients who were not, but the study included only 10 UM patients under statin treatment.[32] Thus, it remains an open question whether Mev pathway inhibition can be considered a valuable chemoprevention strategy especially in UM patients, given their accelerated Mev pathway activity and shorter TTFT.

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Authorship

Contribution: M.C. and C.V. designed the research, performed the experiments, analyzed the data, provided patient care, and wrote the paper; M.F. performed Treg experiments and reviewed the manuscript; S.P., M.R., V.G., B.C., and A.G. assisted with the experiments; D.A. and L.B. performed phenotypic characterization of NKR expression; S.C. performed gene profiling analyses; C.R. performed Mev pathway activity analysis and reviewed the manuscript; D.D. and M.L. performed IGHV mutational status analysis; A.B., R.F., M.B., and J.-J.F. reviewed the manuscript; M.M. contributed to the research design, supervised the study, and wrote and revised the paper; and all authors approved final manuscript.

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