Th17 and non-Th17 interleukin-17-expressing cells in chronic lymphocytic leukemia: delineation, distribution, and clinical relevance

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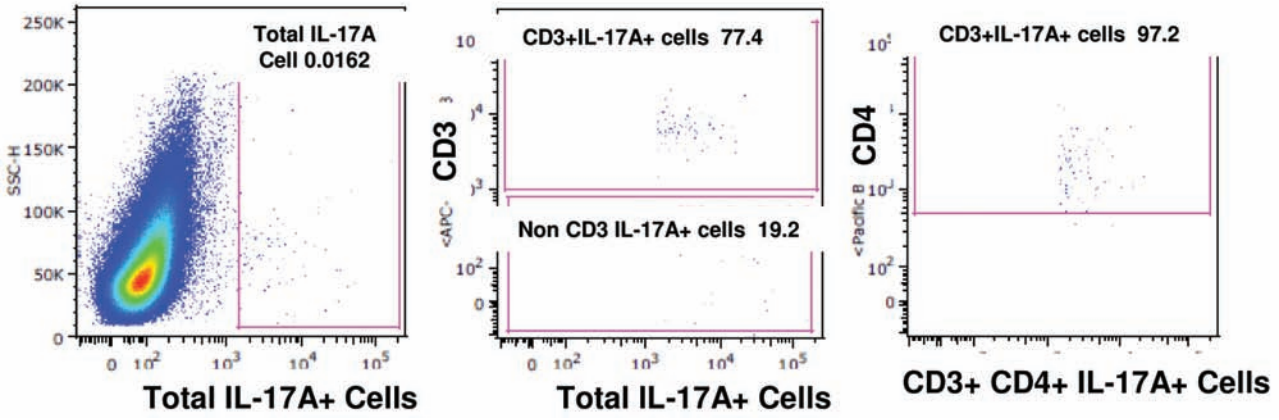
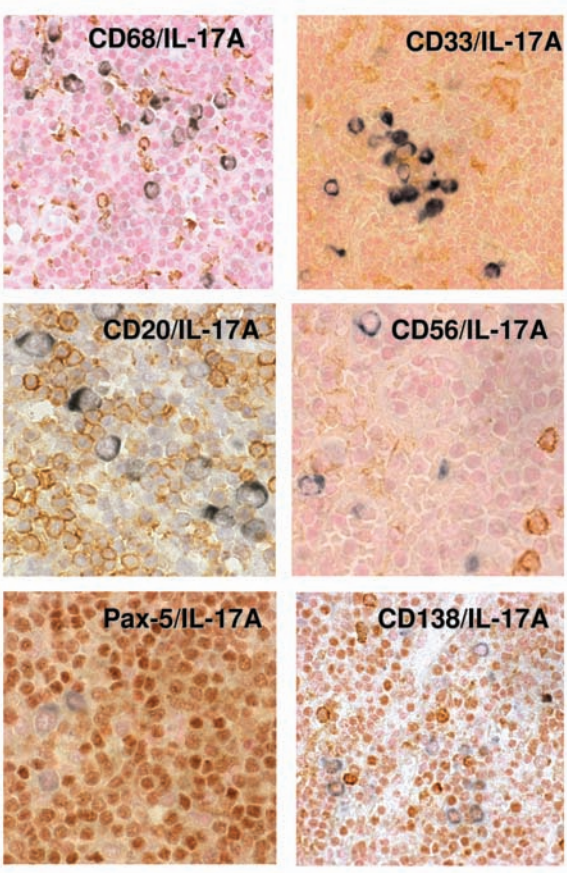
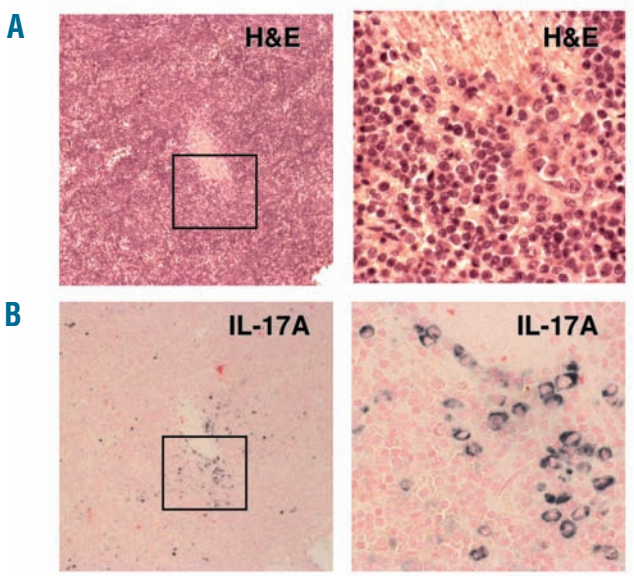


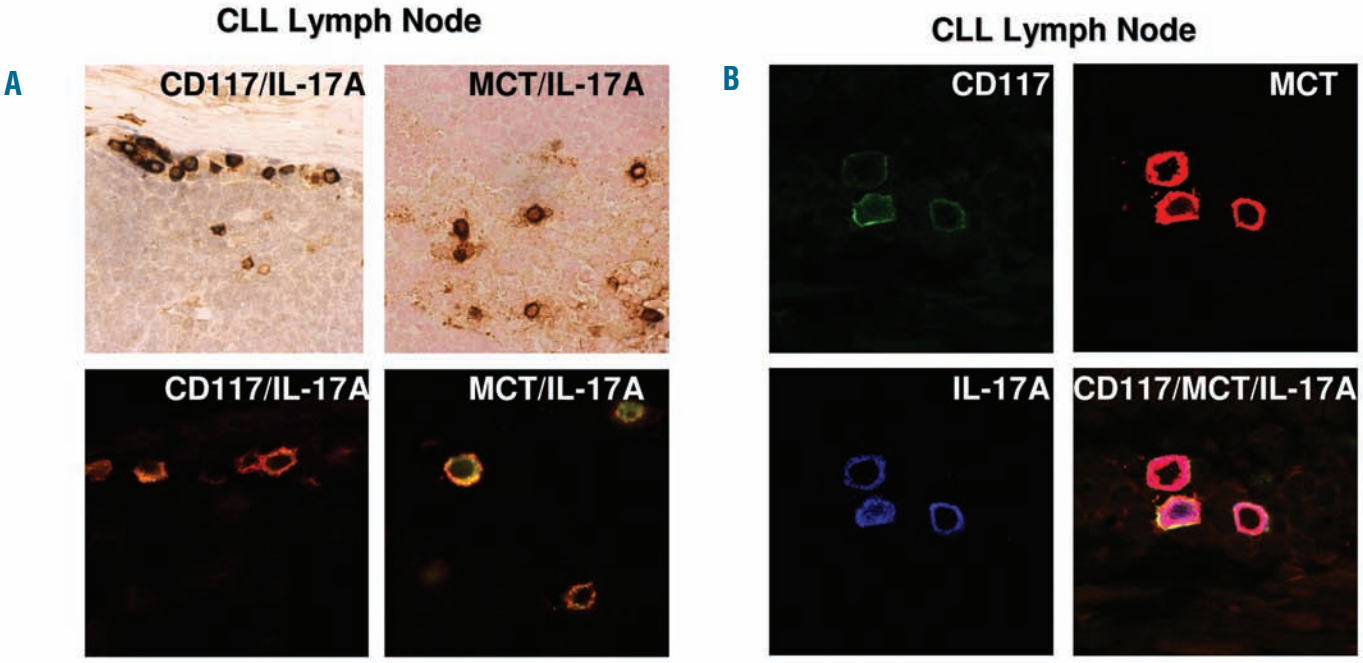
Online Supplementary Figure S1. Total IL-17A-containing cells in spleen suspensions from CLL patients. The figure shows a representative dot plot of the distribution of Th17 and non-Th17 cells in spleen suspensions from CLL patients.



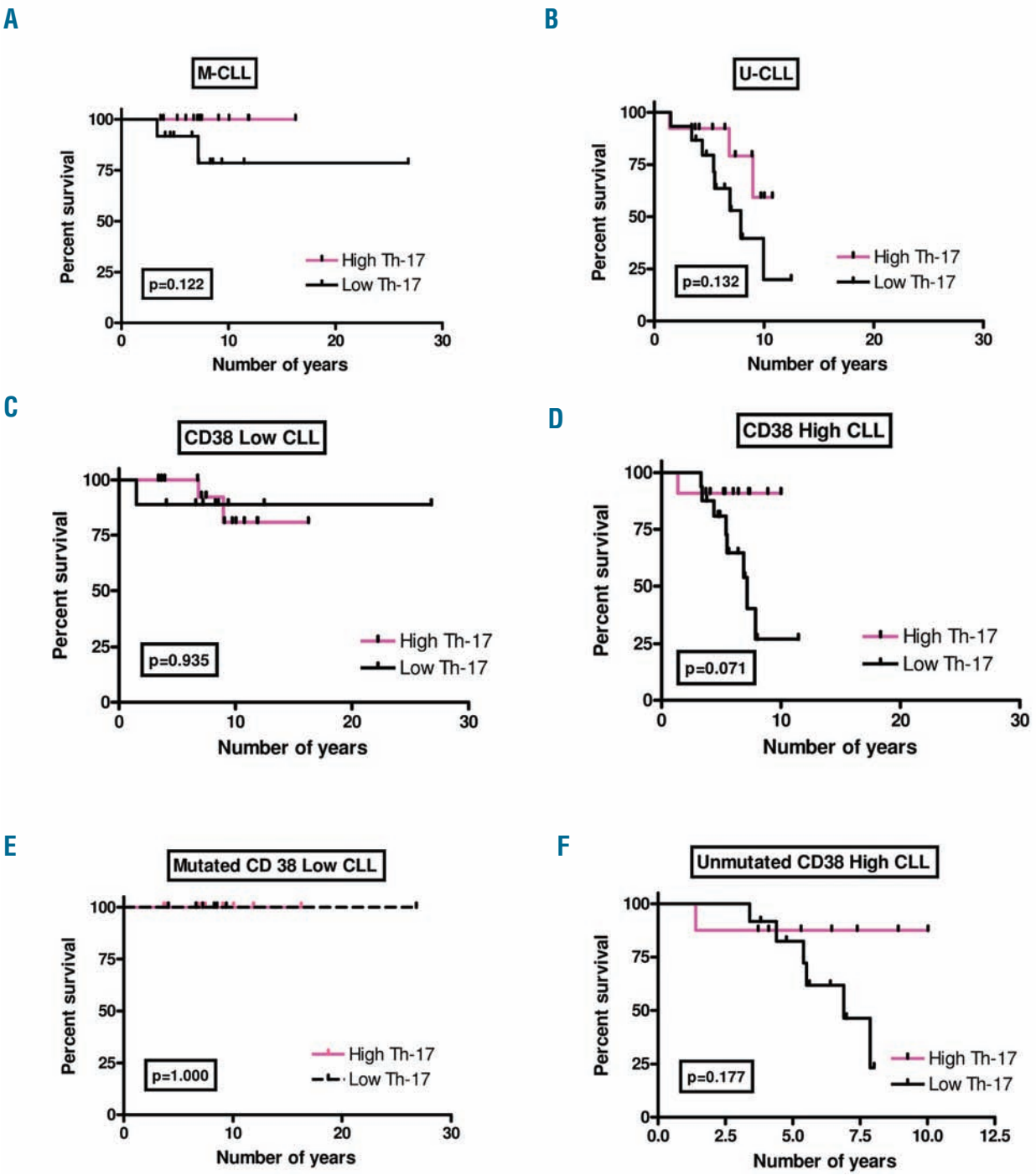
Online Supplementary Figure S2. Phenotype of IL-17A-containing cells in spleens from CLL patients. IL-17A$^+$ cells do not express CD68, CD33, CD20, CD56, CD138 and Pax-5. All images were obtained from double-stained slides and were obtained at 600X magnification using a 60X oil immersion objective lens; images were taken by light microscopy (Zeiss Axiovert 200M, Axiovision 4 software) at room temperature.



Online Supplementary Figure S3. Localization of IL-17A-expressing cells in CLL spleen. (A) H&E staining of CLL spleen showing the distribution of cells (see box) at low magnification (100X) and high magnification (600X) using 60X oil immersion objective lens. (B) The same area with (single staining) high (X600) magnification view of CLL spleen pointing out the IL-17A cells. Note that these cells are mostly in clusters in and around sinusoids and vessel walls. All images were obtained at 600X magnification using a 60X oil immersion objective lens at room temperature.



Online Supplementary Figure S4. IL-17A$^+$ mast cells in CLL lymph nodes. (A) Immunohistochemistry showing all IL-17A-containing cells in CLL lymph nodes coexpress CD117 and MCT. (B) Co-expression of CD117 and MCT by IL-17A$^+$ cells in CLL lymph nodes by immunofluorescence suggests that they are mast cells and not the immature proliferating myeloid cells as seen in CLL spleens. All images were obtained at 600X magnification using a 60X oil immersion objective lens, with a light microscope (Zeiss Axiovert 200M, Axiovision 4 software) and confocal laser scanning biological microscope (Olympus Fluoview 300, Fluoview software) at room temperature.



Online Supplementary Figure S5. Relationship between absolute numbers of circulating Th17 cells and prognostic markers in patients who were untreated at the time of blood collection. (A) Correlation of absolute numbers of Th17 cells with overall survival (OS) in M-CLL patients. M-CLL patients were divided into $Th17^{Low}$ (n=12) and $Th17^{High}$ (n=15) subgroups. Median survival for both M-CLL groups was not reached. (B) Correlation of absolute numbers of Th17 cells with survival in U-CLL patients. U-CLL patients were divided into $Th17^{Low}$ and $Th17^{High}$ subgroups. U-CLL patients with higher levels of Th17 cells (n=13; median OS not reached) lived longer than $Th17^{Low}$ U-CLL patients (n=15; median survival = 7.87 years; *P*=0.132). (C) Correlation of absolute numbers of Th17 cells with survival in $CD38^{Low}$ CLL patients. $CD38^{Low}$ CLL patients were divided into $Th17^{Low}$ (n=9) and $Th17^{High}$ (n=17) subgroups. Median survival for both $CD38^{Low}$ CLL groups was not reached (D) Correlation of absolute numbers of Th17 cells with survival in $CD38^{High}$ CLL patients. $CD38^{High}$ CLL patients were divided into $Th17^{Low}$ and $Th17^{High}$ subgroups. $CD38^{High}$ CLL patients with higher levels of Th17 cells (n=11; median OS not reached) had a longer outcome than $Th17^{Low}$ U-CLL patients (n=16; median survival = 7.18 years; P=0.071). (E) Correlation of absolute numbers of Th17 cells with survival in $M/CD38^{Low}$ CLL patients. $M/CD38^{Low}$ CLL patients were divided into $Th17^{Low}$ and $Th17^{High}$ subgroups. The survival curve for $M/CD38^{Low}$ CLL patients with higher levels of Th17 cells (n=12; median OS not reached) was identical to the survival curve for $Th17^{Low}$ $M/CD38^{Low}$ patients (n=5; median survival = 7.76 years). The dotted black line indicates the $Th17^{Low}$ subgroup. (F) Correlation of absolute numbers of Th17 cells with survival in $U/CD38^{High}$ CLL patients. $U/CD38^{High}$ CLL patients were divided into $Th17^{Low}$ and $Th17^{High}$ groups. $U/CD38^{High}$ CLL patients with higher levels of Th17 cells (n=8; median OS not reached) had a longer survival than $Th17^{Low}$ $U/CD38^{High}$ patients (n=12; median survival = 6.89 years; *P*=0.177).

Online Supplementary Table S1. Clinical and laboratory characteristics of CLL patients listed in order of increasing absolute number of circulating Th17 ($CD3^+CD4^+IL\text{-}17A^+$) cells.

CLL Number	*IGHV* Mutation Status	CD38 level	Fluorescence *in situ* hybridization (FISH)*	Rai stage	Duration of disease (years)	Treated (T) or untreated (NT) at time of sample collection	Time-to-first treatment (years) **	% Th17 cells*	Absolute number of Th17 cells**
0514	NA	Low	NA	2	19.14#	T	0.25	0	0
0569	U	High	Normal	2	5.51	NT	5.38	0	0
0692	M	High	Trisomy 12	0	11.47#	NT	8.02	0	0
0797	M	High	Trisomy 12	0	7.18	NT	5.92	0	0
0967	M	High	Normal	2	4.53#	NT	NT	0	0
0973	U	High	del17p,del11q	4	4.39	NT	2.35	0	0
1090	M	Low	Normal	0	4.10#	NT	NT	0.020	0
1278	U	High	Trisomy 12	2	6.89	NT	5.55	0	0
0717	U	Low	NA	1	1.50	NT	0.14	0.480	0.1
1041	U	High	Normal	3	14.1	T	4.93	0.1	0.2
0394	U	High	del 11q	3	8.70	T	3.76	0.015	0.2
0710	M	High	Normal	3	3.32	NT	0.55	0.371	0.3
0404	M	Low	NA	0	26.82#	NT	NT	0.2	0.3
0156	U	NA	del13q,del11q	3	9.96	NT	5.67	0.09	0.5
1013	U	High	Trisomy 12	0	4.76#	NT	NT	0.120	0.5
0776	M	Low	Trisomy 12	4	9.40	NT	5.05	0.2	0.5
0321	U	Low	Trisomy 12	0	12.48#	NT	NT	0.084	0.6
1140	U	High	del13q	2	3.80#	NT	1.09	0.137	1.0
0815	U	High	del11q	1	5.40	NT	3.08	0.1	1.1
1191	U	High	Trisomy 12,del11q,del6q	0	6.40#	NT	3.01	0.160	1.3
1156	U	High	del13q,del17p	1	3.40	NT	2.09	0.059	1.3
0847	U	High	NA	1	5.62#	NT	1.11	0.4	1.5
1381	M	Low	NA	1	8.29#	NT	NT	0.402	1.7
1071	U	NA	del13q	2	4.55#	NT	2.94	0.4	2.4
0631	U	High	del11q	0	6.99#	NT	NT	0.222	2.5
0865	U	High	NA	1	7.87	NT	4.56	0.2	2.9
0515	U	High	Normal	2	8.02	NT	4.89	0.5	3.6
0700	M	Low	del 13q	0	6.61#	NT	NT	0.839	4.0
1167	M	Low	del13q	0	7.22#	NT	5.34	0.620	4.6
0445	M	Low	Normal	0	8.54#	NT	NT	2.4	4.6
1153	U	High	Normal	0	4.46	T	2.58	0.589	5.0
1016	U	High	NA	3	3.85	T	0.20	0.479	5.2
0705	U	High	del17p.del13q	4	1.71	T	0.08	1.030	5.4
1028	M	Low	NA	1	9.07#	NT	4.50	0.650	5.8
1178	M	Low	del13q	0	3.94#	NT	NT	0.590	6.7
1380	U	Low	del13q	1	3.40#	NT	NT	1.290	7.1
0373	U	Low	del13q	2	9.73#	NT	NT	1.5	7.3
1163	U	High	del11q	1	3.73#	NT	NT	1.047	7.5
0721	U	Low	Trisomy 12	3	12.57#	T	3.55	1.720	7.6
0712	M	Low	NA	1	25.84#	T	9.90	0.550	7.8
0678	M	Low	NA	1	6.76#	NT	2.15	0.4	7.9
1104	U	High	Fusion 14q and Trisomy 12	1	4.10#	NT	NT	0.690	8.7
0896	U	High	NA	0	5.31#	NT	0.88	0.730	9.6
0745	U	High	del 13q,del 11q	1	6.44#	NT	5.76	2.3	10.6
1099	M	Low	del13q	0	10.06#	NT	NT	0.800	11.4
1025	U	High	del13 q	0	11.09#	T	2.80	0.775	12.2
1008	U	High	Trisomy 12,del11q	4	13.10	T	1.97	0.768	14.4
0699	M	Low	del13q	1	7.47#	NT	NT	2.010	14.9
1042	M	Low	NA	0	7.10#	NT	NT	0.591	15.1
1168	M	Low	del13q	1	11.87#	NT	8.92	1.346	16.3

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CLL Number	*IGHV* Mutation Status	CD38 level	Fluorescence *in situ* hybridization (FISH)*	Rai stage	Duration of disease (years)	Treated (T) or untreated (NT) at time of sample collection	Time-to-first treatment (years) **	% Th17 cells+	Absolute number of Th17 cells++
0974	U	High	del 13q,del 11q	0	7.39#	NT	NT	2.800	17.9
1180	M	Low	Normal	1	3.65#	NT	NT	0.620	18.6
0834	U	Low	del13q	1	10.78#	NT	8.82	0.6	20.4
0726	M	High	del13 q	0	7.29#	NT	3.46	2.710	21.7
1283	U	High	del17p.del13q,del11q	3	1.40	NT	NT	2.3	24.1
1187	M	Low	del13q	1	7.10#	NT	4.50	1.905	24.4
0936	M	High	NA	1	5.21#	NT	3.24	2.4	24.6
0879	M	Low	Normal	3	16.27#	NT	13.32	3.550	25.2
0784	U	High	NA	4	8.91#	NT	2.79	1.3	25.8
0230	U	Low	NA	1	6.82	NT	3.37	0.915	27.2
0781	M	High	NA	0	6.03#	NT	0.06	1	28
0424	U	High	NA	1	10.02#	NT	1.11	1.420	34.0
0724	M	Low	del13 q	1	7.49#	NT	NT	2.494	34.9
1396	U	Low	del17p	2	4.10#	T	1.81	1.5	39.4
0276	M	Low	del13q	1	11.91#	NT	NT	1.445	41.1
0439	U	Low	del6q,Trisomy 12	2	8.98	NT	6.48	1.3	99.9

*NA: Not available; NT: Not treated; +Percent of CD3+CD4+ cells containing intracellular IL-17A (Th17 cells) by flow cytometry. ++Absolute number of Th17 cells in blood (per mm³) for each patient was determined according to the formula: [ALC (per mm³)] x [% CD3 positive MNC] x [% CD4 positive CD3 cells] x [%IL-17 positive CD4 cells]. ALC means absolute lymphocyte count and MNC means mononuclear cells. Red text identifies patients in the Th17^{High} group (≥5.6). Cut off value of 5.6 is the median. *Probes used in FISH analysis were p53 for del17p, ATM for del11q and D13S319 for del13q. #Patients who are alive. **Time-to-first-treatment was measured as time of diagnosis until the time to start treatment in the patients who were treated and from the time of diagnosis until the time of patients' visit for the patients who were never treated.*

Online Supplementary Table S2. Clinical features of CLL patients whose spleens were analyzed for IL-17A-expressing cells.

CLL Spleen	M/U-CLL	CD38 Low/High	FISH report	Autoimmune associations (Yes/No)	Reason for splenectomy	G-CSF treatment	%Th17*
1	UM (0%)	High (53.4%)	Normal	No	Refractory pancytopenia	Multiple courses	2.3
2	UM (0%)	High (58.2%)	del14q,del11q, del17p,p53 mutated (by sequencing)	No	Refractory pancytopenia	Multiple courses	0.0
3	UM (0%)	Low (0.8%)	del17p alone	No	Massive splenomegaly in elderly patient	None	0.1
4	ND	ND	ND	Possible	Massive splenomegaly, hypogammaglobulinemia, thrombocytopenia	None	0.2
5	M (5.97%)	Low (0.8%)	Trisomy 12	No	Multiple times treated, spleen removed due to splenic infarcts	Possible	0.7
6	ND	ND	ND	Yes	Evan's Syndrome	None	0.4

**Percent of CD3+ cells coexpressing IL-17A (Th17) as determined by double immunofluorescence staining of formalin-fixed, paraffin-embedded spleen sections with anti-CD3 and anti-IL-17A antibodies. Numbers represent an average of results from 12 high power fields.*

Online Supplementary Table S3. Antibodies used for immunohistochemistry and immunofluorescence analyses.

Antibody	Clone	Source	Dilution
CD3	PS16	Invitrogen	Neat
CD4	1F6	Novacastra	Neat
CD13	Polyclonal	Novus Biologicals	1:50
CD14	7	Novacastra	1:100
CD15	Carb-3	Dako	Neat
CD20	L26	Dako	Neat
CD21	1F8	Dako	1:25
CD23	MHM6	Dako	1:10
CD31	JC/70A	Thermo Scientific	Neat
CD33	PWS44	Novacastra	1:100
CD38	SPC32	Novacastra	1:50
CD56	1B6	Vector Laboratories	1:25
CD68	PG-M1	Dako	Neat
CD117	c-kit	Dako	1:200
CD123	6H6	eBioscience	1:10
CD138	B-A38	AbDserotec	1:50
CD303	Polyclonal	Novus Biologicals	1:25
CD2AP	Polyclonal	SIGMA	1:50
IL17A	Polyclonal	Santa Cruz Biotechnology	1:25
IL17A	Polyclonal	R&D System	1:25
Pax5	24/Pax-5	BD Transduction Laboratories	1:25
Ki-67	MIB-1	Dako	Neat
Ki-67	Polyclonal	Abcam	1:25
Myeloperoxidase	Polyclonal	Thermo Scientific	Neat
Mast cell tryptase	AA1	Dako	1:200
Stat3	Polyclonal	Cell signaling	1:100
Glycophorin A	Monoclonal	Dako	1:100