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Somatic *STAT3* mutations in Felty syndrome: an implication for a common pathogenesis with large granular lymphocyte leukemia

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ABSTRACT

Felty syndrome is a rare disease defined by neutropenia, splenomegaly, and rheumatoid arthritis. Sometimes the differential diagnosis between Felty syndrome and large granular lymphocyte leukemia is problematic. Recently, somatic *STAT3* and *STAT5B* mutations were discovered in 30-40% of patients with large granular lymphocyte leukemia. Herein, we aimed to study whether these mutations can also be detected in Felty syndrome, which would imply the existence of a common pathogenic mechanism between these two disease entities. We collected samples and clinical information from 14 Felty syndrome patients who were monitored at the rheumatology outpatient clinic for Felty syndrome. Somatic *STAT3* mutations were discovered in 43% (6/14) of Felty syndrome patients with deep amplicon sequencing targeting all *STAT3* exons. Mutations were located in the SH2 domain of *STAT3*, which is a known mutational hotspot. No *STAT5B* mutations were found. In blood smears, overrepresentation of large granular lymphocytes was observed, and in the majority of cases the CD8⁺ T-cell receptor repertoire was skewed when analyzed by flow cytometry. In bone marrow biopsies, an increased amount of phospho-*STAT3* positive cells was discovered. Plasma cytokine profiling showed that ten of the 92 assayed cytokines were elevated both in Felty syndrome and large granular lymphocyte leukemia, and three of these cytokines were also increased in patients with uncomplicated rheumatoid arthritis. In conclusion, somatic *STAT3* mutations and *STAT3* activation are as frequent in Felty syndrome as they are in large granular lymphocyte leukemia. Considering that the symptoms and treatment modalities are also similar, a unified reclassification of these two syndromes is warranted.

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Introduction

Felty syndrome (FS) is a disease defined by neutropenia, splenomegaly, and rheumatoid arthritis (RA). It affects less than 1% of RA patients.¹ A classical FS patient has long-standing RA, unexplained neutropenia (causes such as antirheumatic drugs must be ruled out), and splenomegaly. Splenomegaly is not an absolute criterion for diagnosis.¹ The disease has much in common with T-cell large granular lymphocyte (T-LGL) leukemia, an indolent chronic hematological malignancy in which patients have persistent monoclonal LGL lymphocytosis >0.5x10⁹/l, which in the majority of cases consists of CD8⁺ T cells. Concomitant autoimmune manifestations such as neutropenia (70-80% of cases), RA (11-36%), and splenomegaly (20-60%) are also observed.²

Recent studies have unveiled the molecular pathogenesis of LGL leukemia; somatic *STAT3* mutations in lymphocytes leading to constitutive *STAT3* activation occur in 30-40% of LGL leukemia patients.³⁻⁶ Similar mutations have also been dis-

covered in the *STAT5B* gene in 2% of cases.⁷ *STAT3* mutation status is closely linked to RA in LGL leukemia; patients with multiple *STAT3* mutations have RA (43%) more frequently than patients without mutations (6%).⁶

Due to the similarity of clinical and laboratory findings, the differential diagnosis of LGL leukemia with RA and FS may sometimes be difficult. Past studies have shown a resemblance in patient phenotypes, responses to treatment, high HLA-DR4 prevalence, and analogous findings in splenectomy samples.² Furthermore, both diseases are treated with immunosuppressive agents.² Some authors have suggested that these two diseases represent the same entity,^{2,8,9} but this hypothesis currently lacks molecular evidence. Herein, we utilized deep next-generation sequencing (NGS) of *STAT3* and *STAT5B* genes and cytokine profiling in order to examine if these two diseases are part of the same disease continuum.

Methods

All methods are described in more detail in *Online Supplementary Methods*.

Patient recruitment

The diagnosis of FS is defined by RA, neutropenia, and splenomegaly. We included patients with an established Felty syndrome diagnosis (n=14) stated in patient records. In addition, samples from healthy controls (n=8), RA patients without Felty syn-

drome (n=20) and LGL leukemia patients (n=9) were used. The ethical boards of our institutions approved the study, and the Declaration of Helsinki guidelines were followed. Patients gave written informed consent.

Sample preparation

DNA samples from different cell types were obtained (*Online Supplementary Table S1* and *Online Supplementary Figure S1*). For six FS patients, only archived samples were available (*Online Supplementary Table S1*). Eight FS patients gave fresh peripheral blood samples. Mononuclear cells were extracted *via* Ficoll gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare) from fresh blood samples and CD4⁺ and CD8⁺ cells with magnetic bead selection (Miltenyi Biotec). DNA was extracted according to manufacturer's instructions using the NucleoSpin Tissue DNA extraction kit (Macherey-Nagel).

T-cell clones were investigated with flow cytometry using a panel of antibodies against the different variable β regions of the T-cell receptor (IO Test V β Mark Kit, Beckman Coulter), accompanied by anti-CD3 (SK7), anti-CD4 (SK3), and anti-CD8 (SK-1) (Becton Dickinson) antibodies. The IO Test V β mark kit covers approximately 70% of the V β T-cell repertoire. Sorting of expanded CD8⁺ T-cell populations was performed using the same antibodies.

STAT3 and *STAT5B* sequencing

Amplicon sequencing of 23 exons of the *STAT3* gene, *STAT5B* exon 16, and *STAT5A* exon 17 was performed on the DNA samples. Amplicon sequencing is a polymerase chain reaction (PCR)-

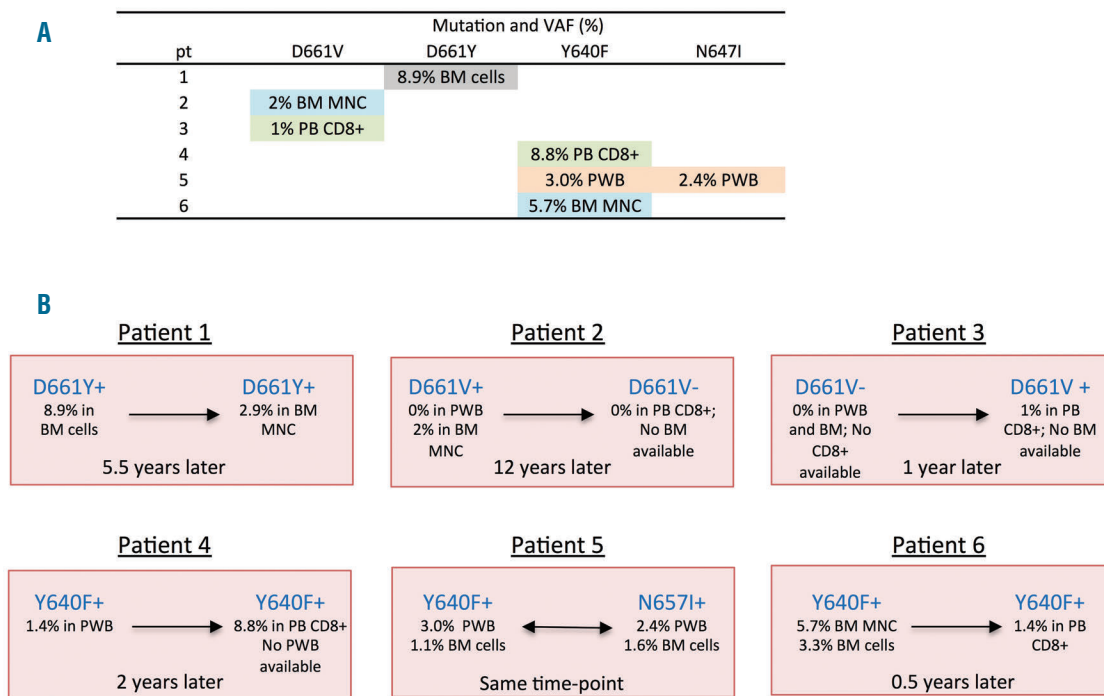


Figure 1. Felty syndrome patients harbor *STAT3* mutations. (A) *STAT3* mutations (presented as protein level amino acid changes) identified in study patients. Mutation variant-allele frequencies (VAFs) in the sample with the highest VAF are shown in the table. All samples and mutations detected are shown in *Online Supplementary Table S1*. (B) Schematic of sequencing results of all six patients with *STAT3* mutations. Each patient is presented separately, and the presence of a mutation is indicated by -/+ . Presented in smaller font are the data on the VAF and the sample type that was available for sequencing from the time-points. DNA sample types in A-B: Bone marrow (BM) cells, cultured cells from the BM (originally obtained for chromosome analysis); BM MNC: bone marrow mononuclear cells; PWB: peripheral whole blood; PB CD8⁺: peripheral blood CD8⁺ cells.

based NGS method that enables very high sequencing depths (up to over 100,000x). It detects variants with low variant-allele frequencies (VAF), locally reaching a sensitivity of 0.5%.⁶ Sequencing was performed using the Illumina MiSeq System, and the data analysis pipeline has been described in more detail previously.⁶ Herein, we considered the mutation to be true if it occurred in the sequenced sample with a VAF >1% and absent in control samples. All primers are listed in *Online Supplementary Table S2*.

Cytokine profiling

Ethylenediaminetetraacetic acid (EDTA) plasma samples from eight healthy controls, nine RA patients, seven FS patients, and nine LGL leukemia patients were analyzed with the Proseek Multiplex Inflammation I (Olink Biosciences) immunoassay which detects 92 different biomarkers simultaneously in one sample *via* oligonucleotide-labeled antibodies. The target-antibody complexes are detected with real-time PCR. A list of the analyzed biomarkers is presented in *Online Supplementary Table S3*. The assay does not report protein concentrations, and the results are reported as normalized protein expression units (NPX).

Immunohistochemistry

To study phosphorylation of STAT3, we stained bone marrow biopsy samples from seven FS patients with anti-phospho-STAT3 (Tyr705) and anti-CD57 antibodies. We also used 14 control bone marrow samples, which had been taken to examine abnormal blood counts, but no specific diagnosis had been achieved.

Statistics

Multiplex cytokine data was analyzed as NPX values with Qlucore Omics Explorer (Qlucore). Cytokines with a false discov-

ery rate <0.1 (Benjamini-Hochberg method) were reported, and further *post hoc* testing was performed using Dunn's multiple comparison tests. Statistical analyses for the clinical data were performed with GraphPad Prism software. Statistical tests included the Kruskal-Wallis omnibus test, Dunn's multiple comparison test, Mann-Whitney test and Fisher's exact test.

Results

Patient characteristics

The clinical characteristics of the 14 FS patients included herein are presented in Table 1 and Table 2, and *Online Supplementary Table S4* and *Online Supplementary Table S5*. None of the patients represented classical LGL leukemia with marked LGL lymphocytosis and T-cell receptor (TCR) γ gene rearrangement. Thirteen patients had long-standing RA prior to Felty syndrome diagnosis, and they had been treated with immunosuppressive drugs at the time of sample collection. The first sample of patient 9 was taken before initiating immunosuppressive treatment.

As LGL lymphocytosis is the hallmark of LGL leukemia, peripheral blood smears were re-examined for this study. FS patients showed an overrepresentation of LGL lymphocytes (Table 2; normal range up to 25% of lymphocytes), but only two patients (patients 3 and 6) had absolute LGL lymphocytosis. Patients' bone marrow exams did not show dysplasia, but they often showed a slight left shift in granulopoiesis, and in some cases, overrepresentation of lymphocytes.

Large CD8⁺ T-cell expansions are characteristic for

Table 1. Basic clinical characteristics of Felty syndrome patients.

Patient ID	Sex	Age at sample collection	RA duration	Felty dg	Infections	Serostatus	Erosions	Active arthritis at sample collection	Splenomegaly	Other extra-articular symptoms
1	M	76	15	6	3	pos	no	no	yes 15cm	rheumatoid nodules
2	F	49	17	5	0	pos	yes	no	yes	no
3	F	71	10	0	1	pos	yes	no	yes 16cm	weight loss
4	F	63	10	0	0	pos	yes	no (1 s)	yes 13cm	rheumatoid nodules
5	F	72	23	2	5	pos	yes	no	no 11cm	weight loss, rheumatoid nodules
6	F	71	24	0	0	pos	no	yes (1s, 1t)	no 10cm	no
7	F	68	54	22	1	neg*	yes	yes (1s, 1t)	yes 17cm	leg ulcer
8	M	71	21	21	0	pos	yes	no	yes 13cm	no
9	F	65	0	0	1	pos	no	yes (>5s, >2t)	yes 17cm	fever, chills, cough
10	F	64	10	0	5	pos	yes	Yes >3s	yes	weight loss, maculopapular rash
11	F	57	24	0	1	pos	yes	no	no	fever
12	M	40	3	0	0	unknown	no	no	no	persistent rash
13	F	70	42	7	10	pos	yes	no	yes 14cm	leg ulcer, weight loss
14	F	38	5	0	0	pos	yes	yes	no	no
Summary	79% F, 21% M	66.5 (55.0-71.0)	16.0 (8.8-24.0)	0 (0.0-6.3)	1 (0.0-3.5)	92% pos, 8% neg	71% yes, 29% no	36% yes, 64% no	64% yes, 36% no	

Clinical characteristics of the patient cohort. Summaries of the parameters are shown on the bottom row. Age, RA duration, Felty diagnosis (dg), and infections are summarized as medians with interquartile ranges in parenthesis. The patients' ages are shown as ages at the time of the first sample collection. RA duration is shown as years of RA prior to collecting the first sample for the study. Felty dg is shown as years, starting from FS diagnosis to the collection of the first sample. The number of infections requiring hospitalization is shown. Serostatus was defined as either elevated rheumatoid factor and/or anti-citrullinated protein antibodies (ACPAs). Erosions were defined *via* hand and feet x-rays. The number of swollen (s) and tender (t) joints at sample collection is shown in parenthesis. Spleen size was measured with ultrasonography. *Rheumatoid factor normal, however, ACPAs were not determined. ID: identity; ND, not determined; RA: rheumatoid arthritis.

T-LGL leukemia.⁴ In FS patients, flow cytometry screening revealed skewed CD8⁺ TCR V β usage (Table 2 and *Online Supplementary Figure S2*). In 40% (4/10) of the examined cases, CD8⁺ expansions comprised over 20% of all CD8⁺ T cells.

Most of our patients' clinical features were well in accordance with the FS criteria (Table 1 and Table 2). Only three patients did not show the classical phenotype. No neutropenia was recorded in the clinical registry of patient 8. Patient 9 had cytopenias during tuberculosis therapy (although neutropenia continued years after the completion of the therapy). In addition, patient 3 showed lymphocytosis, but her bone marrow and peripheral blood TCR γ rearrangement tests were negative, and flow cytometry did not show any excess of natural killer (NK) cells.

Somatic *STAT3* mutations were identified in 43% of patients

For many patients, several sample types and samples from multiple time-points were available for sequencing (*Online Supplementary Table S1*). *STAT3* mutations occurred in 43% (6/14) of FS patients (Figure 1A,B). One patient had two different *STAT3* mutations (N647I and Y640F). In all assessable cases, *STAT3* mutations occurred in CD8⁺ cells but not in CD4⁺ cells. In 4/6 *STAT3*-mutated cases, the mutations were detected in multiple sample types, whereas in 2/6 patients the mutation was detected only in one sample (Figure 1B and *Online Supplementary Table S1*). Five *STAT3*-mutated patients had follow-up samples available,

and in three cases the mutations were also detected during the follow-up (Figure 1B). None of the patients had *STAT5B* mutations.

When patients with *STAT3* mutations were compared with patients with no *STAT3* mutations, no differences emerged in age, sex, LGL counts, CD8⁺V β clone size, neutropenia duration (adjusted for laboratory follow-up time), lymphopenia duration (adjusted for laboratory follow-up time), highest and lowest lymphocyte counts, duration of RA, or the number of infections (compared with Mann-Whitney test). No statistically significant differences occurred in TCR γ rearrangement status, existence of erosions, splenomegaly, or active arthritis status (compared with Fisher's test).

Sorting of expanded CD8⁺ T cells with flow cytometry using V β antibodies showed that patient 6 harbored the Y640F mutation in the major expanded CD8⁺ T-cell population (V β 3) (Figure 2), but no *STAT3* mutations were found in CD4⁺ cells or other CD8⁺ cells lacking V β 3. A similar analysis was also performed with the sample of patient 4, but the mutation was not located in the largest CD8⁺ T-cell expansion (V β 22) which was detectable by flow cytometry (Figure 2). The sum of all of the detected CD8⁺ T-cell expansions made up only 38% of all CD8⁺ T cells, which is clearly less than the anticipated level of 70% of the TCR repertoire (*Online Supplementary Figure S3*). This suggests that the sensitivity of flow cytometry is insufficient to detect all clones. An 'undetected' clone is likely to harbor the identified mutation, since the VAF of

Table 2. Laboratory findings of Felty syndrome patients.

Patient ID	LGL count	TCR γ assay positive?	<i>STAT3</i> mutation	CD8 ⁺ expansion (% of CD8 ⁺)	Lab follow-up	Neutropenia duration	Lowest neutrophil count	Lymphopenia duration	Lowest lymphocyte count	Highest lymphocyte count	Thrombocytopenia duration
1	33%/0.20	no	yes	ND	163	94	0.17	135	0.28	1.52	18
2	ND	no	yes	7.6	210	73	0.4	191	0.5	1.74	0
3	62%/0.86	no	yes	13.9	156	14	0	0.07	1.37	4.96	0.2*
4	30%/0.42	yes: PB	yes	10.6	115	3	<0.05	4	1.03	2.93	2.8
5	28%/0.34	no	yes	ND	52	18	<0.05	38	0.41	0.46	2
6	69%/0.57	yes: BM	yes	21.7	189	103	0.66	63	0.82	2.42	0
7	ND	ND	no	ND	207	32	1.22	194	0.34	0.64	204
8	ND	ND	no	25.5	180	ND	1.58	173	0.7	1.33	394**
9	28%/0.29	no	no	ND	144	33	0.08	61	0.79	1.82	0
10	ND	yes	no	52.4	4	4	1.14	4	0.75	1.25	4
11	ND	equivocal	no	17.2	11	11	1	2	0.9	1.6	11
12	ND	no	no	6.3	5	3	1.1	3	0.8	1.5	2
13	36%/0.26	ND	no	27.8	119	33	0	88	0.4	1.82	0
14	ND	ND	no	8.8	62	16	0.6	57	0.4	1.3	0
Summary	33%/0.34 (28-62)/ (0.26-0.57)	33% yes 67% no	43% yes, 57% no	15.6 (8.5-26.1)	131.5 (41.8-182.3)	18 (7.5-53)	0.5 (0-1.1)	59 (3.8-144.5)	0.73 (0.4-0.84)	1.6 (1.3-2.0)	2 (0-9.3)

The table shows the laboratory findings of Felty syndrome patients. The bottom row shows summary statistics for all patients (when applicable): median and interquartile range in parenthesis. All blood cell counts are shown as 10⁹/l. The patients' peripheral blood (PB) smears were, where possible, re-examined for this study, and LGL cells were counted as percentage of lymphocytes (counting 300 lymphocytes). Absolute LGL counts (10⁹/l) were calculated from lymphocyte counts. The sizes of CD8⁺ T-cell expansions were studied *via* flow cytometry. Laboratory follow-up time in months (Lab follow-up) was calculated. The cumulative durations of neutropenia (<1.5x10⁹/l), lymphopenia (<1.3x10⁹/l), and thrombocytopenia (<100x10⁹/l) were calculated as months from laboratory records. Of note, patients 10 and 12 have laboratory follow-up only for the period before hematology referral, but oral history indicates a history of years (patient 10) and decades (patient 12) of leukopenia. *HIT: heparin-induced thrombopenia, not included in summary statistic calculations. **Thrombocytes 100-150, not included in summary statistic calculations. ND: not determined; LGL: large granular lymphocyte; TCR γ : T-cell receptor γ ; BM: bone marrow.

the mutation was high in the CD8⁺ Vβ22-negative fraction (18.8%).

Previously, we have studied the presence of *STAT3* mutations in 82 newly diagnosed RA patients, and no mutations were detected.¹⁰ Herein, we sequenced the *STAT3* hotspot exon 21 from RA patients who had been treated for several years with no evidence of FS (n=14). As in newly diagnosed RA, no *STAT3* mutations were detected in this patient cohort. The patients are described in more detail in *Online Supplementary Table S6*.

FS patients have increased phosphorylation of STAT3 in bone marrow samples

We had access to seven bone marrow biopsy samples from FS patients 1-6 and 9. Patients 1-6 harbored somatic *STAT3* mutations (Figure 1). Immunohistochemical staining of the samples showed increased amounts of phospho-STAT3 positive cells in FS patients when compared to the controls (Figure 3). The degree of phosphorylation was not associated with mutation VAFs: patient 3 did not have mutations in bone marrow samples, nonetheless the

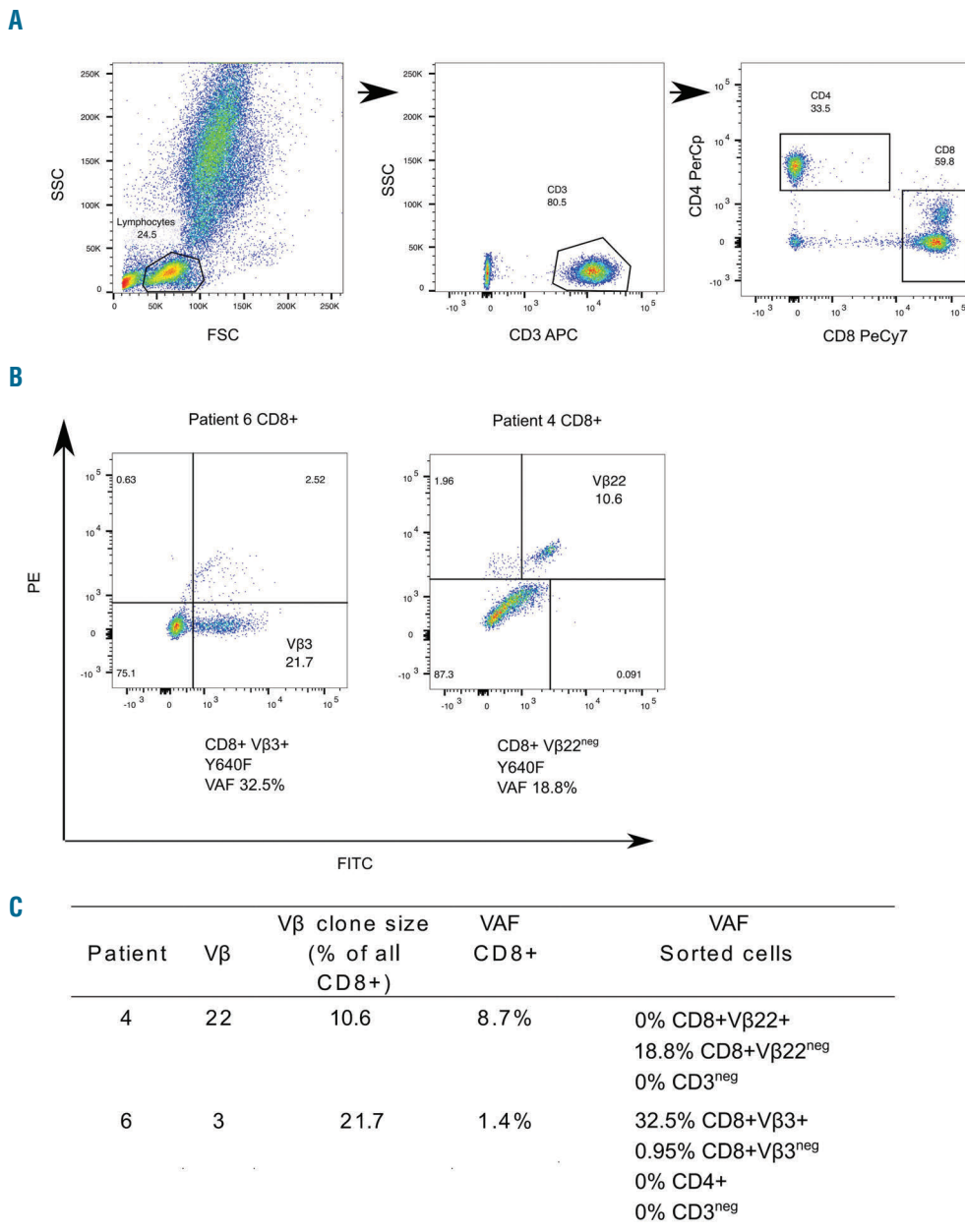


Figure 2. Sequencing of sorted, expanded CD8⁺ T-cell populations. The expanded CD8⁺ T-cell populations that were detected by the initial Vβ flow cytometry screen were sorted via flow cytometry and sequenced with Amplicon sequencing (n=2; patients 4 and 6). (A) Gating strategy for Vβ analysis. The subpopulation of interest was included in subsequent gates and other populations were excluded. (B) The expanded CD8⁺ clones in patients 6 and 4. The expanded clones expressing the Vβ in question, as well as all other CD8⁺ cells were collected and sequenced. (C) The summarized sequencing results of sorted cells. The small number of mutation-positive cells present in the CD8⁺Vβ3^{neg} cells of patient 6 are likely due to sorting impurities. APC: allophycocyanin; FSC: forward scatter; FITC: fluorescein isothiocyanate; VAF: variant-allele frequencies.

bone marrow biopsy showed marked *STAT3* phosphorylation. Additionally, patient 9 showed *STAT3* phosphorylation in the bone marrow despite the absence of *STAT3* mutations. Overall, *CD57*⁺ cells were less abundant than phospho-*STAT3*⁺ cells (Figure 3).

Felty syndrome patients shared similar plasma cytokine profiles with LGL leukemia and RA patients

Previous reports have shown that LGL leukemia patients have aberrant levels of several cytokines in their plasma.¹¹⁻¹⁷ Analysis *via* a multiplex cytokine panel

showed significant differences between healthy controls and patients with immune-mediated disease (RA, Felty syndrome, LGL leukemia). Only minor differences emerged between individual disease conditions (Figure 4). Many plasma cytokines (10/92; colony stimulating factor 1 (CSF1), C-X-C motif chemokine 10 (CXCL10), interleukin (IL)-15RA, macrophage inflammatory protein 1- α (MIP-1- α), oncostatin-M (OSM), tumor necrosis factor receptor superfamily member 9 (TNFRSF9), programmed cell death 1 ligand 1 (PD-L1), CUB domain-containing protein 1 (CDCP1), IL-6, and hepatocyte growth factor

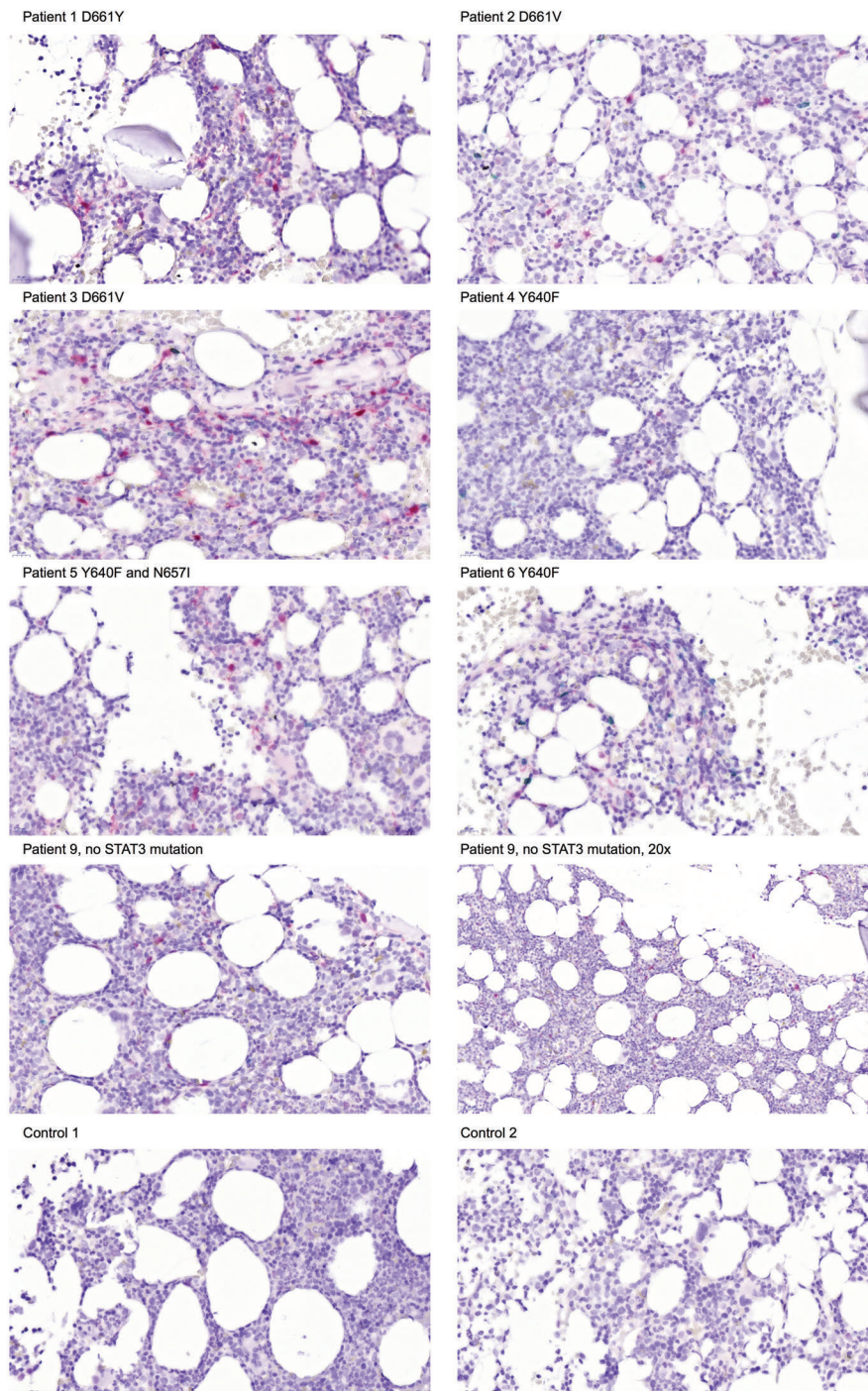


Figure 3. Felty syndrome patients show *STAT3* phosphorylation in bone marrow. Representative immunohistochemical analysis of phospho-Tyr705-*STAT3* (red) and *CD57* (green) in bone marrow tissue samples of Felty syndrome (n=7) and control samples (n=14) counterstained with hematoxylin. All images presented in the figure are 40x magnified unless otherwise specified. Felty syndrome patients show more phosphorylated *STAT3* than controls, but the amount of phosphorylation was not related to the *STAT3* mutation VAFs. Patient numbers and mutation status are presented in the figure.

(HGF) were elevated in both FS and LGL leukemia (*Online Supplementary Figure S4*). CDCP1, IL-6, and HGF were elevated in all three diseases. RA patients shared some characteristics with FS and LGL leukemia patients, but seven cytokines were statistically significantly elevated only in RA patients and not in other patients (*Online Supplementary Figure S4*). Clinical information on the LGL leukemia patients, RA patients, and healthy controls used in this assay is shown in *Online Supplementary Table S7* and *Online Supplementary Table S8*.

When *STAT3*-mutated patients were compared with patients without *STAT3* mutations, only CXCL1 was statistically significantly elevated in plasma samples of *STAT3*-mutated patients (*Online Supplementary Figure S5*). However, there was no significant difference when compared to healthy controls.

Discussion

Previous studies have shown that Felty syndrome and LGL leukemia share many clinical features.² The study herein confirms that in addition to similar clinical aspects,

these two disease entities also share analogous pathogenetic molecular markers. In a cohort of 14 FS patients, *STAT3* mutations were discovered in 43% of the cases. This is comparable with the *STAT3* mutation frequency in LGL leukemia.^{4,6}

The diagnostic criteria for FS are not specific. Therefore, our study patients are a heterogeneous group, which may be subject to criticism. However, these patients represent the clinical spectrum of the FS patients who are monitored at rheumatology outpatient clinics. Further, most of our patients' clinical features were in accordance with the FS diagnostic criteria.

All detected mutations occurred in the hotspot exon 21 in the SH2 domain of *STAT3*. Likewise, as in LGL leukemia, Y640F and D661 mutations were the most common.^{4,6} Further, *STAT3* mutations occurred in CD8⁺ cells and not in CD4⁺ cells in the cases in which this could be assessed. Interestingly, we also observed increased *STAT3* activation in the bone marrow samples of FS patients. The presence of phospho-*STAT3*⁺ cells was not related to *STAT3* mutation status. Similarly, it has been shown in LGL leukemia that *STAT3* is activated in the majority of patients,^{5,18,19} although *STAT3* mutations only occur in 30-40% of patients.^{4,6} In

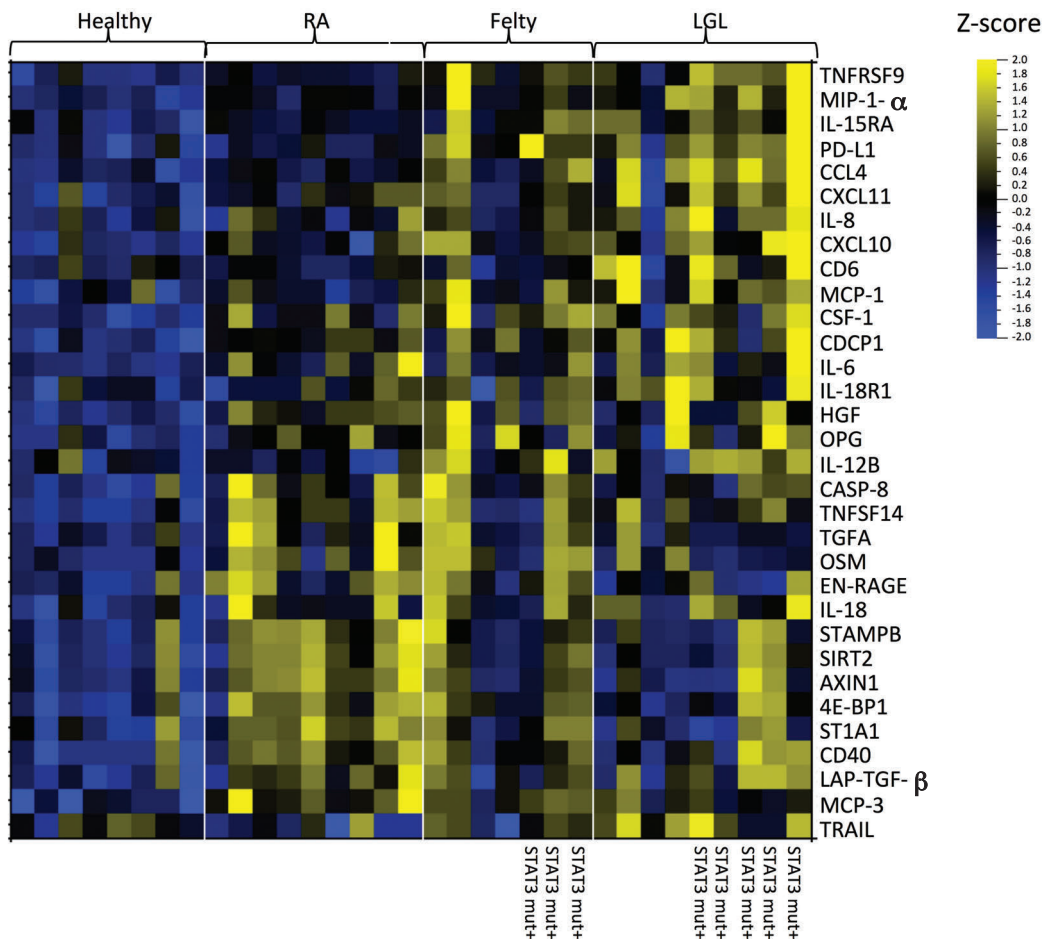


Figure 4. Felty syndrome patients have a similar cytokine profile as that of LGL leukemia. The heatmap compares the plasma cytokine profiles of healthy controls, RA patients, Felty syndrome patients, and LGL leukemia patients. Data was analyzed as NPX units, and were normalized to allow for coloring on the same scale for the heatmap. Bright blue represents smaller protein concentration, while bright yellow represents higher protein concentration. The heatmap shows all cytokines for which the Q-value (false discovery rate) was less than 0.1. RA: rheumatoid arthritis; LGL: large granular lymphocyte.

addition to LGL leukemia, somatic *STAT3* mutations have been described in other disease conditions, such as aplastic anemia, myelodysplastic syndrome, T-cell lymphomas, and inflammatory hepatocellular adenoma.²⁰ In addition, one case report has shown *STAT3* mutations in Felty syndrome.²¹ Of note, we did not detect any *STAT3* mutations in patients with chronic RA who had no evidence of FS. This is concordant with our previous findings showing that in a cohort of 82 newly diagnosed RA patients, no *STAT3* mutations were detected.¹⁰

LGL lymphocytosis is a hallmark of LGL leukemia. Peripheral blood smear re-examinations revealed overrepresentation of LGL cells in FS patients. Despite the high percentages of LGL cells, only two of our patients had LGL lymphocytosis ($>0.5 \times 10^9/l$; patients 3 and 6). Absolute lymphocyte counts were low, excepting one patient. A previous study, which defined LGL cells with flow cytometry, reported increased percentages of LGL cells in FS.²² This is concordant with our data. Our patients' skewed TCR V β usage also indicates that large T-cell clones often exist in FS, but the expansions were smaller when compared with results obtained with the same antibody panel in T-LGL leukemia.⁴ More detailed clonality analysis would require TCR deep sequencing.

The smaller T-cell clone sizes in FS patients when compared to LGL leukemia patients could also explain why the *STAT3* mutation VAFs were generally lower in FS patients. The VAF is highly dependent on the proportion of cells harboring mutations in the sequenced sample, and smaller clone sizes result in smaller detected VAFs. We had CD8⁺ cells available from 4/6 patients with *STAT3* mutations, and even these cell fractions had low VAFs (1-1.4%), barring one case (8.8%). However, some LGL leukemia patients also harbor clones with small (0.8%) VAFs.⁶ Thus, our results do not differ from LGL leukemia cases with oligoclonal TCR expansions. Therefore, we suggest that LGL leukemia and FS form a disease continuum, which could explain the differences in VAFs and clone sizes.

Many plasma cytokines (10/92) were elevated both in FS and LGL leukemia when compared to healthy controls (such as IL-15RA, CXCL10 and PD-L1). Importantly, only three of them were also elevated in RA patients (CDCP1, IL-6, and HGF). Furthermore, our results confirm the previous findings of increased IL-15RA, IL-8, C-C motif chemokine ligand 4 (CCL4=MIP1- β), and CXCL10 in LGL leukemia.^{11,12,14} Of these cytokines, IL-15RA and CXCL10 were also elevated in FS. Although some differences emerged between the diseases, we discovered that the three immune-mediated diseases are not strikingly different in terms of cytokine profiles. This is not unexpected; many LGL leukemia patients have RA, comparative to the other patient groups.

In this small study, patients with *STAT3* mutations did not differ from patients without *STAT3* mutations in terms of clinical or biochemical characteristics. In LGL leukemia, neutropenia and RA are more common in patients with

mutations.^{4,5} Similarly, germline gain-of-function *STAT3* mutations lead to early-onset multiorgan autoimmunity, including cytopenias.²³⁻²⁵ However, the exact mechanism of neutropenia in LGL leukemia and FS is not clear. Based on the current hypothesis, *STAT3* mutations lead to clonal outgrowth.²⁶ Depending on the antigen target, the lymphocyte clones can attack bone marrow and joints. In addition, disease manifestations may also result from the production of proinflammatory cytokines mediated by hyperactive *STAT3* signaling.²⁷ Thus, the activation of *STAT3* is a likely causative factor for autoimmunity, but future research needs to address the exact mechanisms in more detail.

Of note, four of the FS patients had celiac disease, despite the fact that RA (without FS) does not have an established connection with celiac disease. Although the observed overrepresentation may occur by chance, celiac disease could also be a result of chronic systemic immune dysregulation. This is strengthened by recent findings showing that activating *STAT3* mutations can be detected in refractory celiac disease patients' duodenal biopsy-derived intraepithelial lymphocyte cell lines.²⁸ The constitutive *STAT3* activation of immune cells may contribute to the persistent autoimmune inflammation that occurs in these patients. A similar mechanism is possible in FS patients, but this issue requires further investigation.

In conclusion, our results supplement the evidence that FS and LGL leukemia belong to the same disease continuum and diagnostic group. These diseases share similar molecular pathogenetic features and phenotypes, and are treated similarly with immunosuppressive agents. Thus, unified re-classification of these two diseases should be considered.

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