

Letter to the Editor

Genetic variation in *schlafen* genes in a patient with a recapitulation of the murine *Elektra* phenotype*To the Editor:*

A 65-year-old female white patient with a previously unremarkable history of infections presented with a cutaneous Merkel cell carcinoma of the left thigh. This entity is commonly associated with Merkel cell polyoma virus (MCPyV).¹ Indeed, the excised carcinoma tissue tested positive for MCPyV DNA (Fig 1, A). Patient-derived T cells failed to proliferate and to produce IFN- γ when restimulated *in vitro* with MCPyV, while production of IL-10 appeared intact (see Fig E1 in this article's Online Repository at www.jacionline.org). The patient was moderately lymphopenic and mildly hypogammaglobulinemic with intact proportions of T, B, and natural killer (NK) cells (see Fig E2 in this article's Online Repository at www.jacionline.org). The patient developed lymphadenopathy, skin rash, weight loss, night sweats, and fatigue. EBV-DNA copy numbers in peripheral blood were progressively increasing (Fig 1, B). Lymph node histopathology revealed a disturbed follicular architecture and infiltration by atypical EBV-positive

(EBV-encoded small RNAs [EBER]⁺, latent membrane protein-1 [LMP-1]⁺) B-cell blasts (Fig 1, C). Treatment with anti-CD20 mAb (rituximab) resulted in a significant drop in EBV-DNA copy number in peripheral blood (Fig 1, B) and amelioration of symptoms. A few months later, the patient developed progressive lymphadenopathy, skin rash, and fever. EBV-DNA copy number was again elevated despite the absence of peripheral B lymphocytes. T-cell lymphoma was diagnosed, and the patient ultimately died despite chemotherapy. Moreover, an extremely high copy number (5×10^9 copies/mL) of Torque teno virus (TTV) DNA was detected in the patient's plasma, corresponding to the highest TTV titer measured in our experience (plasma samples of 33 healthy adult controls all harbored $<10^5$ copies/mL of TTV DNA). Characteristic for this nonenveloped small ssDNA virus is a lifelong low-level viremia in more than 90% of the healthy adult population.² There is evidence that TTV-DNA load correlates with the degree of immune dysfunction.³

Flow cytometry-based phenotyping revealed normal CD4/CD8 ratio and a normal distribution of naive (CCR7⁺CD45RA⁺) versus memory (CCR7⁺CD45RA⁻) CD4⁺ T cells. CD8⁺ T cells of the patient displayed a relatively increased CD45RA⁺CCR7⁻ terminally differentiated memory

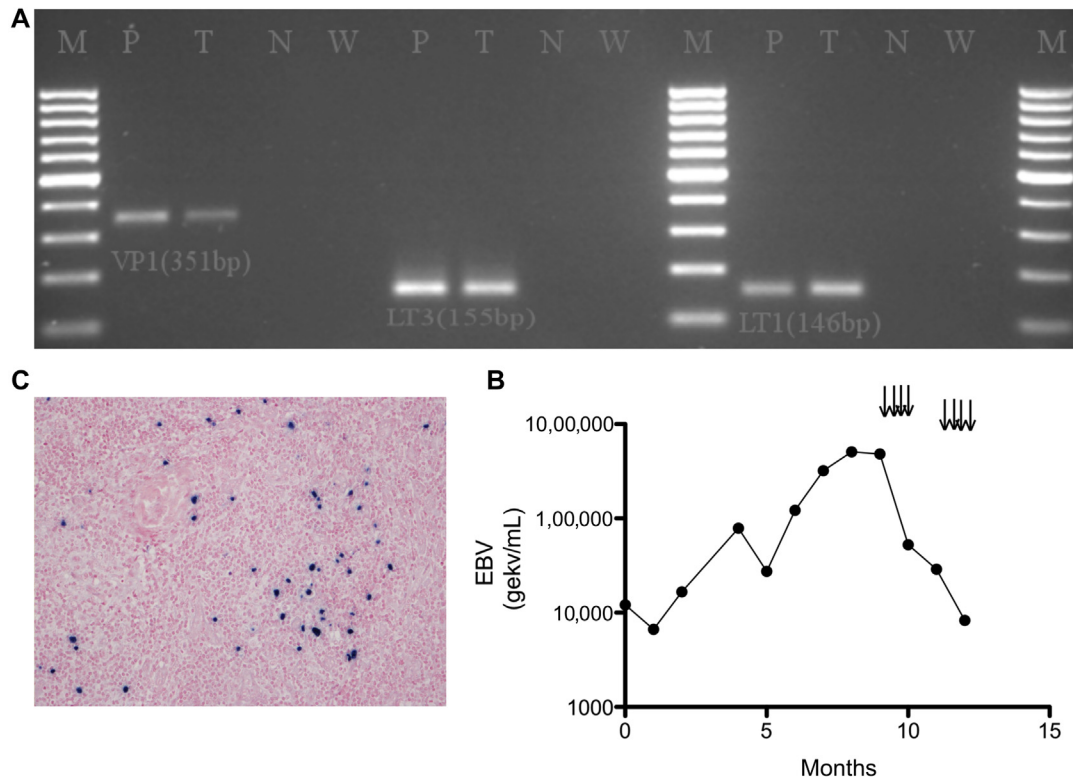


FIG 1. Clinical phenotype. **A**, DNA from Merkel cell carcinoma tissue (T) was extracted for the detection of MCPyV DNA by using 2 PCR primer sets specific for the MCPyV T-antigen (LT1 and LT3) and 1 for the VP1 gene. PCR products were separated on an agarose gel and visualized by ethidium bromide. A positive (P) and negative (N) control (tissues of previously diagnosed individuals), as well as water (W) control, were included. **B**, EBV DNA copy numbers (gekv: genomic equivalents) in plasma were assessed by using real-time PCR at the indicated time points. Arrows indicate the administration of anti-CD20 (rituximab). **C**, EBV-encoded small RNAs positivity was assessed by using *in situ* hybridization (visualized by a blue color).

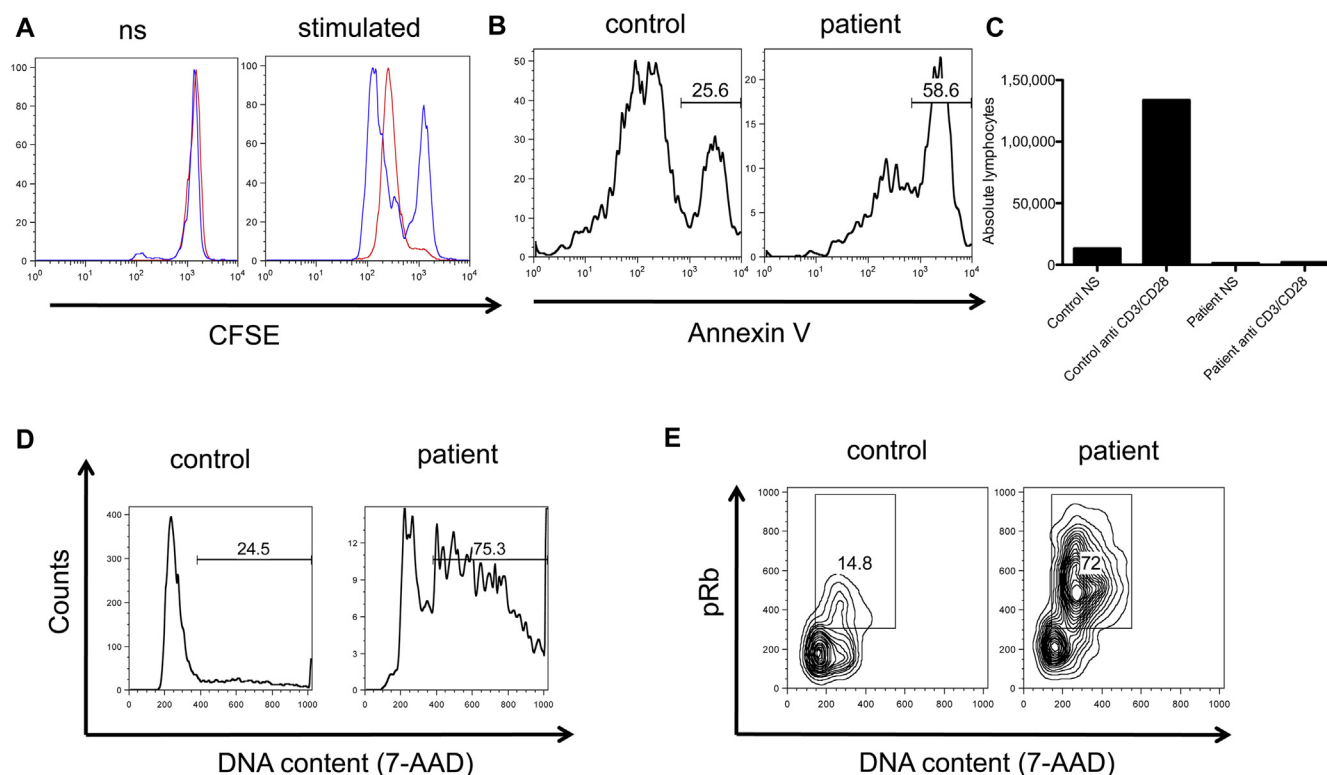


FIG 2. Immunologic phenotype. **A**, PBMCs from the patient (blue) and a healthy control (red) were CFSE labeled (5 μ M final concentration) and anti-CD3 (OKT3) and anti-CD28 (CD28.2) mAbs were treated or left unstimulated (ns). After 4 days, CFSE dilution among T cells was assessed by using flow cytometry. The experiment was repeated with similar results. At the time of assessment, the patient had been treated with B-cell-depleting antibody (rituximab) but was not treated with other known immunosuppressive medications. **B**, Apoptosis was determined after 24 hours of anti-CD3/CD28 mAb stimulation by testing for Annexin V expression on T cells by using flow cytometry. The experiment was repeated with similar results. **C**, T-cell proliferation was assessed in a flow cytometry-based assay. Absolute T-cell numbers were measured following 5 days of *in vitro* stimulation of PBMCs by agonistic anti-CD3 (OKT3) and anti-CD28 (CD28.2) mAbs or in the absence of stimulation (nonstimulated, NS) and compared between the patient and an age-matched control. The experiment was repeated with similar results. **D**, PBMCs were stimulated *in vitro* with PMA/ionomycin (5 ng/mL, 50 ng/mL) for 16 hours. Cellular DNA content of T cells was measured by using 7-AAD binding and flow-cytometric analysis. The experiment was repeated with similar results. **E**, PBMCs were stimulated with PMA/ionomycin (5 ng/mL, 50 ng/mL). After 16 hours, phosphorylated retinoblastoma protein (pRb) was assessed by using phospho-specific mAb and flow cytometry. DNA content was measured in parallel by using 7-AAD binding. The experiment was repeated with similar results. CFSE, Carboxyfluorescein-succinimidyl-ester; PMA, phorbol 12-myristate 13-acetate.

subset (see Fig E3 in this article's Online Repository at www.jacionline.org). When peripheral blood mononuclear cells were stimulated *in vitro* via CD3/CD28, an aberrant carboxyfluorescein-succinimidyl-ester dilution pattern was observed. A proportion of the patient's T cells failed to proliferate, whereas others underwent a higher number of cell divisions than did control cells (Fig 2, A). This phenomenon was observed in both the CD8⁺ and CD4⁺ T-cell compartment (see Fig E4 in this article's Online Repository at www.jacionline.org). Moreover, excessive T-cell apoptosis was detected in the patient's T cells, as assessed by Annexin V staining (Fig 2, B). Dysregulated proliferation and excessive apoptosis of the patient's T cells resulted in an overall failure to increase the absolute T-cell numbers in response to CD3/CD28 stimulation (Fig 2, C). It is unlikely that chronic EBV infection *per se* accounted for the abnormalities of T-cell proliferation and apoptosis observed in our patient because normal expansion of CD8⁺ T cells has been reported in chronic EBV viremia.⁴ Upregulation of CD25

expression on TCR crosslinking and IL-2-induced phosphorylation of Signal transducer and activator of transcription 5 were comparable in patient's and control's T cells, indicating that early events of T-cell activation were intact (see Fig E5 in this article's Online Repository at www.jacionline.org).

Initial investigation of large-scale genomic deletion and duplications by comparative genome hybridization revealed reduced copy number of 84 kb of genomic DNA corresponding to markers located on chromosomal band 17q12, encompassing the *schlafen* (*SLFN*) 11, *SLFN*12, and *SLFN*13 genes. The observed heterozygous deletion of the *SLFN* gene region at chromosome 17q12 is found in 2.2% (10 of 450) of healthy persons in the Finnish population (data not shown), suggesting that this deletion alone is not responsible for the clinical phenotype. Direct sequencing of the coding region of the hemizygous *SLFN*-11-13 allele and in the homologous genes *SLFN* 5-12L-14 confirmed the wild-type sequence. It may be noteworthy that our index patient carries the C allele at an

intragenic single nucleotide polymorphism (rs9916629) located between *SLFN12L* and *SLFN13* that has recently been associated with altered NK cell frequencies and NK function.⁵ The C allele is specifically associated with lower NK cell frequencies and higher expression of the inhibitor C-type lectin *NKG2A*.⁵

To further evaluate potential pathogenic genetic variation, whole exome sequencing was performed. These data were used to directly interrogate the coding regions of genes known to cause failure of EBV immune control (*AP3B1*, *BIRC4*, *FCGR3A*, *CD27*, *CORO1A*, *GATA2*, *HPLH1*, *ITK*, *LIG4*, *LRBA*, *LYST*, *MAGT1*, *PIK3CD*, *PRKCD*, *PRF1*, *RAB27A*, *SH2D1A*, *STX11*, *STXBP2*, *TRAC*, *UNC13D*, *WAS*). No rare (minor allele frequency < 0.001) alleles that could be considered as potential candidate mutations were observed in these genes. Variation within the extended *SLFN* gene family was also assessed from the exome sequencing data, revealing homozygosity for a missense variant in the *schlafen*-like gene 1 predicted to be damaging (exon2:c.G431C:p.R144T, SIFT score 0.01, genotype G/G has a population frequency of 0.067 in Europeans) and a previously unobserved heterozygous missense substitution in the same gene (c.C1047A:p.D349E). In contrast to the other *SLFN* family members, *schlafen*-like gene 1 is located at chromosome 1p34.2, shows limited sequence homology to other *SLFN* genes, but contains the same ATP-binding homology-divergent AAA domain.

The *SLFN* genes control cell-cycle progression and act as virulence factors in orthopoxviruses.^{6,7} Intriguingly, along with the clinical phenotype of virus-associated disease, this case is mimicking the murine *Elektra* phenotype described recently in *Slfn2* mutated mice⁸—in which lymphocytic-choriomeningitis-virus infection results in hyperproliferating T cells that undergo excessive apoptosis, linked to an overall failure of T-cell expansion. A unique feature of T cells in the *Elektra* mouse is premature entry into cell-cycle S-phase on activation.⁸ Similarly, 18 hours after *in vitro* activation with phorbol 12-myristate 13-acetate/ionomycin, more than 75% of the patient's T cells versus less than 25% of the control's T lymphocytes displayed hyperdiploid DNA content, indicative of increased S-phase transition (Fig 2, D). Moreover, phosphorylation of the retinoblastoma protein, a major orchestrator of S-phase entry, was augmented in the patient's T cells (Fig 2, E).

To our knowledge, this is the first case in which aberrant T-cell responses associated with susceptibility to MCPyV and possibly TTV have been observed and genetic variation evaluated. Although the full extent of the genotype-phenotype relationship of the identified alleles still needs to be investigated in detail, the genetic variants found in *schlafen* family genes are notable, especially in conjunction with the *Elektra* mouse model.

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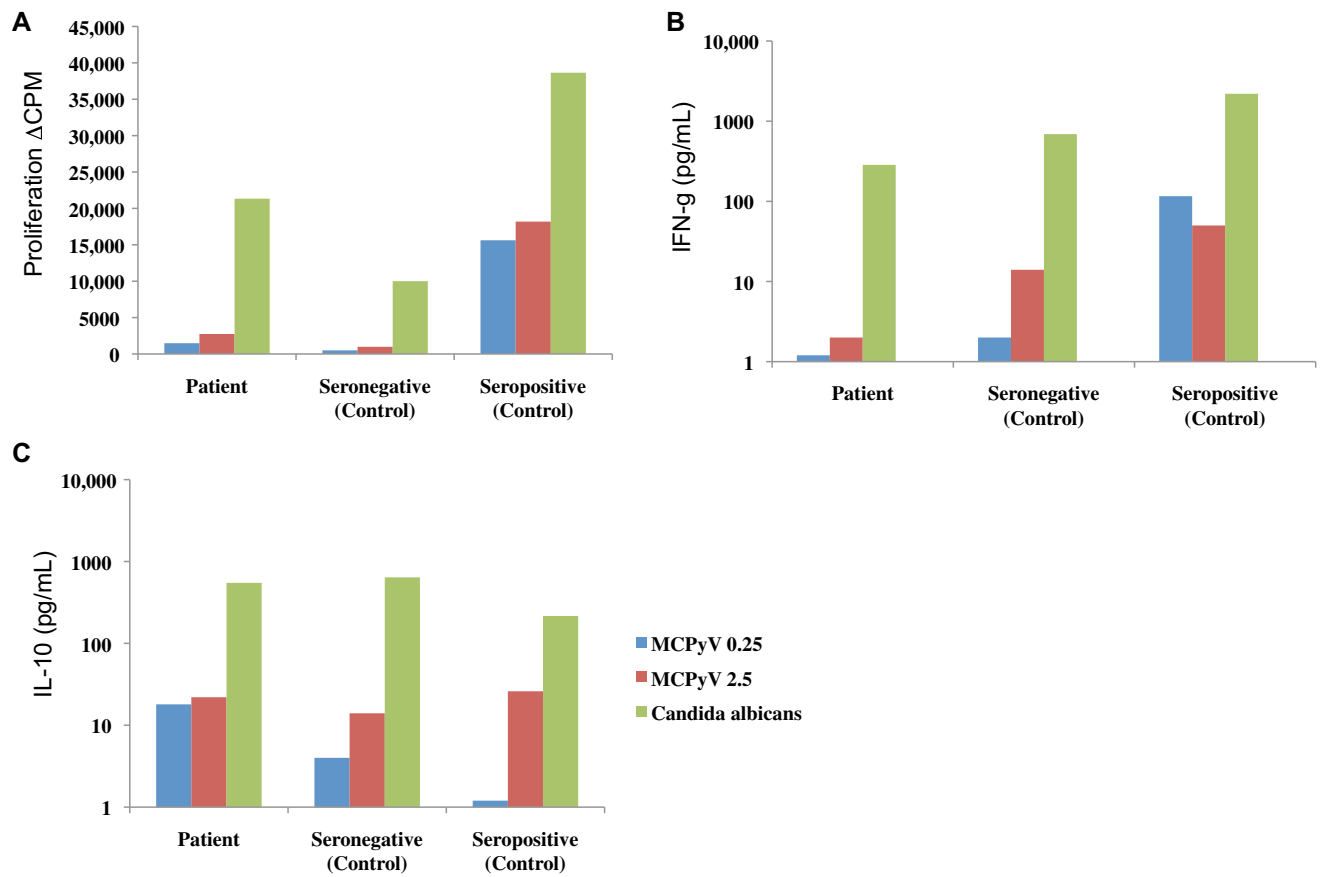


FIG E1. MCPyV-specific T-cell proliferation (H_3 -thymidine incorporation) (**A**) and T-cell-derived cytokine secretion (ELISA) (**B, C**) were assessed in the patient and MCPyV-seronegative and seropositive controls as described (blue and red bars). Responses to *Candida albicans* (green bars) served as an internal control. The experiment was repeated with similar results.

Parameter analyzed	Patient data	Normal range
Whole white blood cells	5.7x 10 ⁹ /l	3.5-10x10 ⁹ /l
Absolute neutrophil count	4.73 x 10 ⁹ /l	1.3-6.7 x 10 ⁹ /l
Absolute lymphocyte count	0.55 x 10 ⁹ /l	1-3x10 ⁹ /l
% T cells (CD3 ⁺)	83%	55-86%
% B cells (CD19 ⁺)	7%	5-22%
% NK cells (CD3 ⁺ CD56 ⁺)	6%	5-26%
Serum IgG	5.80 g/l	7-16g/l
Serum IgA	0.52 g/l	0.7-4.0g/l
Serum IgM	0.51 g/l	0.4-2.3g/l

FIG E2. Basic immunologic data. The numbers of the indicated white blood cell subsets and serum concentration of the indicated immunoglobulin isotypes are shown in relation to age-matched reference values (results of B-cell frequencies were measured before treatment with anti-CD20 mAb).

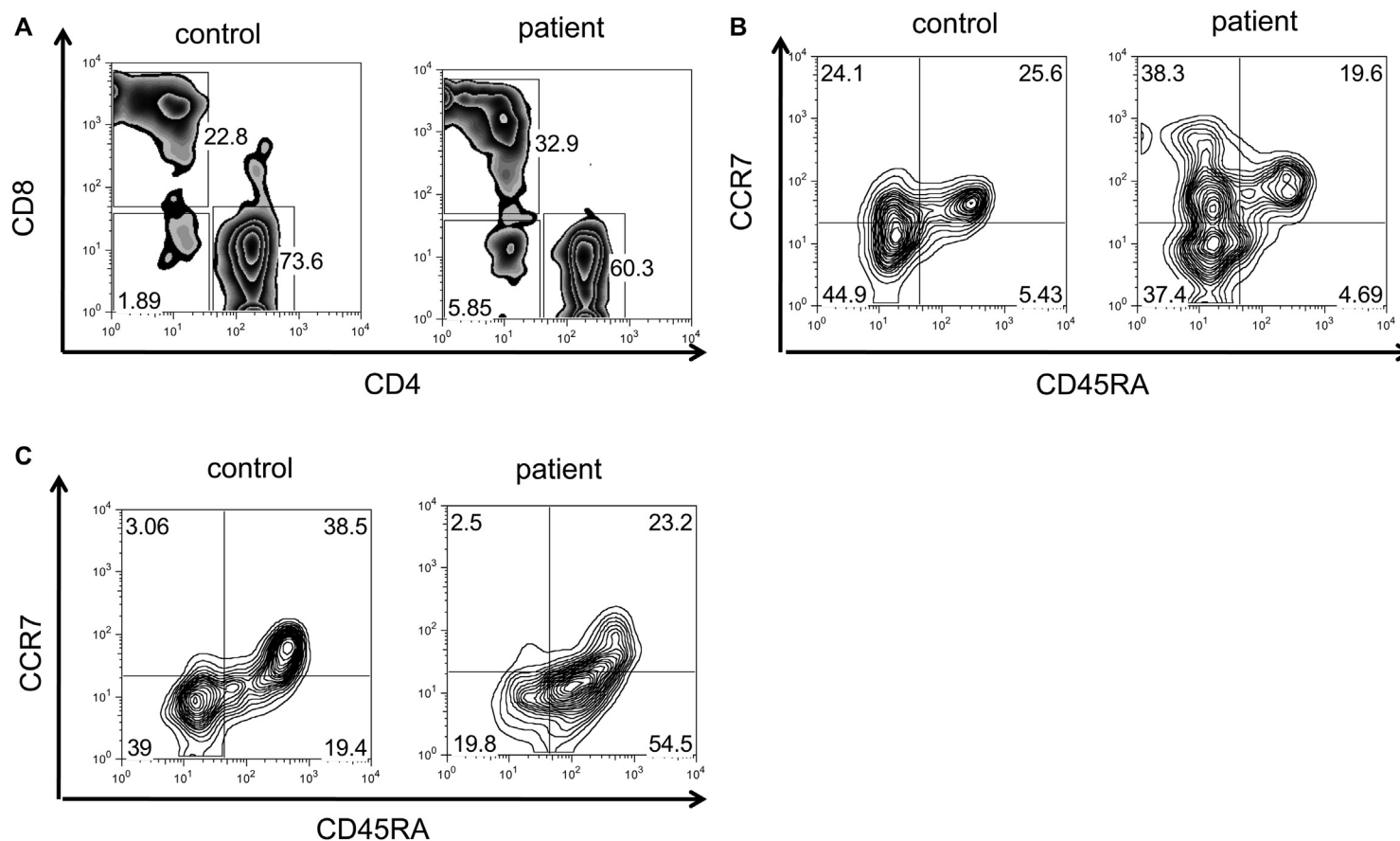


FIG E3. T-cell phenotype. **A**, PBMC-derived T cells (CD3⁺) were analyzed for the expression of CD4 (T helper cells) versus CD8 (cytotoxic lymphocytes). **B** and **C**, PBMC-derived T cells (CD3⁺) were analyzed for the expression of the memory markers CCR7 and CD45RA gated on CD4⁺ (**B**) and CD8⁺ (**C**) T-cell subsets.

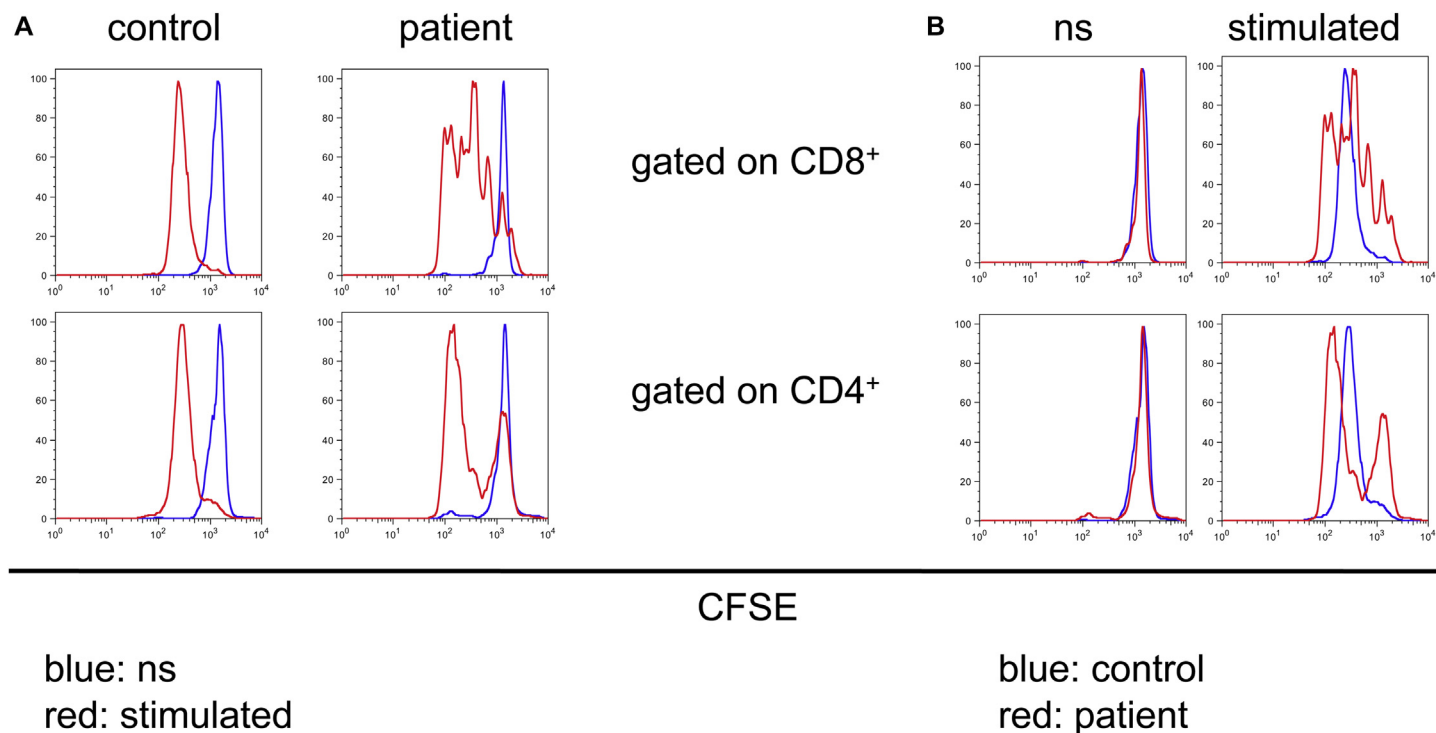


FIG E4. Dysregulated T-cell proliferation. **A** and **B**, PBMC-derived T cells of patient or age-matched control were CFSE labeled (5 μ M final concentration) and then *in vitro* stimulated with agonistic CD3/CD28 antibodies or left unstimulated as indicated. Ninety-six hours later, CFSE profiles gated on CD4⁺ or CD8⁺ T-cell subsets were measured by using flow cytometry. Comparison of CFSE profiles of nonstimulated (*blue*) versus stimulated (*red*) T cells (**A**) and control (*blue*) versus patient-derived (*red*) T cells (**B**). The experiment was repeated with similar results. *CFSE*, Carboxyfluorescein-succinimidyl-ester.

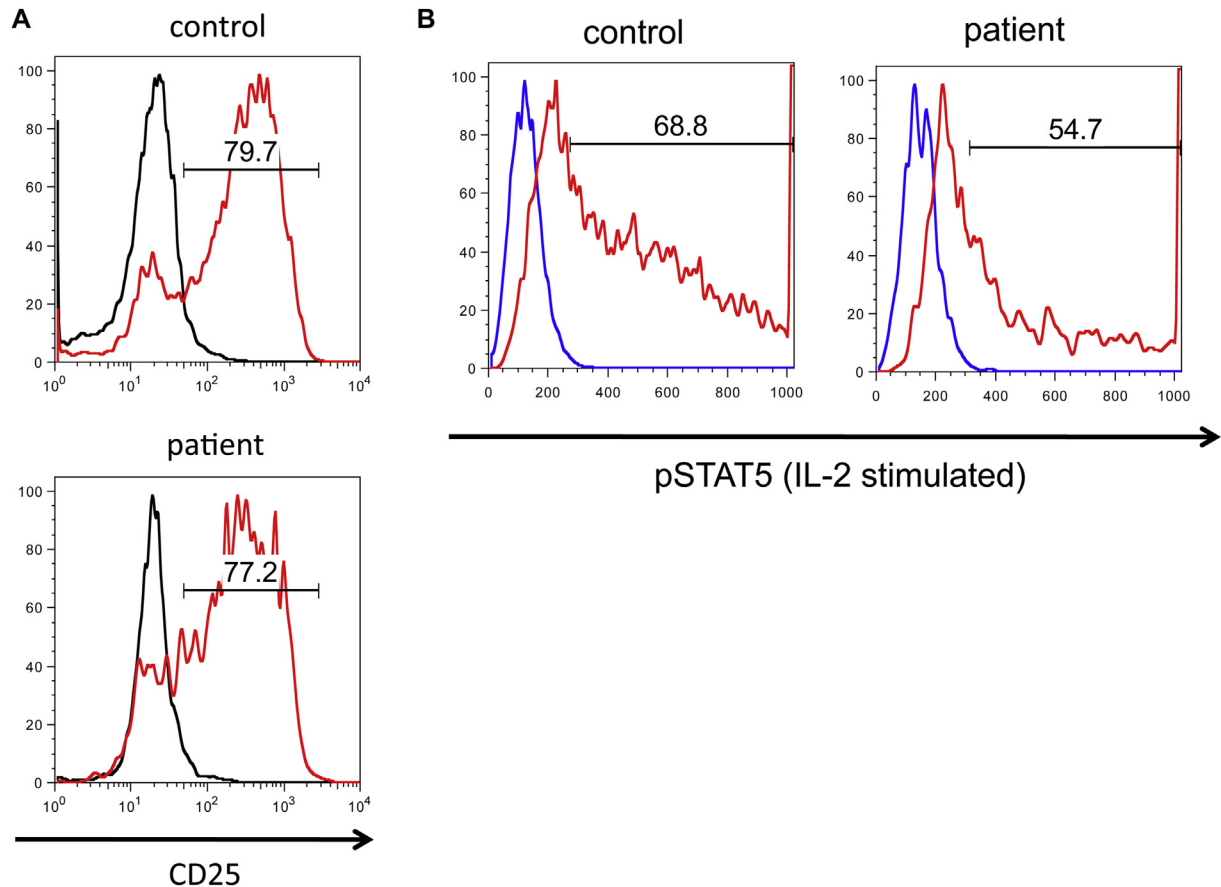


FIG E5. Normal upregulation of CD25 and normal STAT5 phosphorylation. **A**, PBMCs of the patient and an age-matched healthy control—as indicated—were stimulated *in vitro* by anti-CD3/CD28 antibodies (red histograms) or left nonstimulated (black histograms). Eighteen hours later, the expression of CD25 on T cells was analyzed by using flow cytometry. **B**, PBMC-derived T cells of the patient or an age-matched healthy control—as indicated—were stimulated with 200 ng/mL recombinant IL-2 for 10 minutes. Phosphorylated STAT5 was assessed by using flow cytometry (red histograms) and compared with staining with isotype control antibody (blue histograms). The gate was set at 1% using the isotype control. *STAT5*, Signal transducer and activator of transcription 5.