Human TCR alpha/beta+ CD4-CD8- Double-Negative T Cells in Patients with Autoimmune Lymphoproliferative Syndrome Express Restricted Vbeta TCR Diversity and Are Clonally Related to CD8+ T Cells.

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Human TCR α/ß$^+$ CD4$^-$CD8$^-$ double-negative T cells in patients with autoimmune lymphoproliferative syndrome (ALPS) express restricted Vß TCR diversity and are clonally related to CD8+ T cells[1]

[Running title: Repertoire Diversity and CD8 origin of DN T cells in ALPS]

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Abstract

The peripheral expansion of $\alpha/ß^{+}$-$CD4^{-}CD8^{-}$ double negative (DN) T cells in patients with autoimmune lymphoproliferative syndrome (ALPS) is a consistent feature of this disease, and part of the diagnostic criteria of ALPS. The origin of these cells remains undetermined. They could derive from mature T cells that have lost co-receptor expression, or represent a special minor cell lineage. To investigate relationship of DN and single positive (SP) T cells in ALPS, we used Immunoscope technology to analyze the TCRVß repertoire diversity of sorted DN and SP T cells, and we performed CDR3 sequence analyses of matching clonotypes. We show that DN T cells express all the Vß gene families that are used by their SP counterparts, though they dominantly use some Vß genes. Analysis of CDR3 length distribution revealed a diverse polyclonal TCR repertoire for sorted $CD4^{+}$ T cells, whereas both DN and $CD8^{+}$ T cells showed a skewed TCR repertoire with oligoclonal expansions throughout most of the Vß families. CDR3 sequencing of matching clonotypes revealed a significant sharing of CDR3 sequences from selected Vß-Jß transcripts between DN and $CD8^{+}$ T cells. Altogether, these data strongly argue for a CD8 origin of DN T cells in ALPS.

Introduction

The autoimmune lymphoproliferative syndrome (ALPS)[3] (also known as Canale-Smith syndrome) is one of the first human inherited disorders of apoptosis to be described (1,2). It is a rare disorder of disrupted lymphocyte homeostasis caused by an impaired Fas/CD95-mediated apoptosis, resulting in chronic lymphoproliferation and a breakdown in immunologic tolerance (3-7). The main features of this disease are chronic splenomegaly and lymphadenopathy of early onset, hypergammaglobulinemia (IgG and IgA), autoimmune manifestations, and expanded populations of T-cell receptor (TCR)-$\alpha/ß^+$ $CD3^+CD4^-CD8^-$ T cells, called double negative T cells (DN T cells) (3-7). The genetic defect responsible for this syndrome is in most cases a mutation in the *Fas* gene. Fas, a member of the tumor necrosis factor (TNF) receptor superfamily, induces apoptosis of lymphocytes when triggered by its ligand (FasL). The interaction between Fas and FasL leads to the formation of the death-inducing signaling complex, which initiates a cascade of caspases that culminates in apoptosis (8). The Fas apoptotic pathway is crucial for the downregulation of the immune response, and loss-of-function mutations in the *Fas* (TNFRSF6) or *FasL* gene results in chronic lymphoid hyperplasia and autoimmunity, as seen in mice with *lpr* and *gld* mutations (9) and in patients with ALPS (2). ALPS is classified according to underlying genetic defect (10). In ALPS type I, heterozygous *Fas* mutations (ALPS type Ia) (11,12) or heterozygous *FasL* mutation (ALPS type Ib) (13) are usually associated with a partial defect in apoptosis mediated by Fas and its ligand. ALPS type II, which is characterized by resistance to Fas-mediated apoptosis, despite the presence of normal Fas and FasL, has been found in patients with *caspase-10* mutations (14). Somatic heterozygous mutations of *Fas* were found to cause a sporadic form of ALPS (ALPS type III) by allowing lymphoid precursors to resist to normal process of cell death(15).

Lymphoproliferation results from the gradual accumulation of lymphocytes that have not undergone normal programmed cell death, and expansion of an unusual population of peripheral DN T cells is striking. In the blood, DN T cells constitute less than 1% in normal individuals but can reach up to

40% in patients with ALPS (4,16). These DN T cells express αß TCR chains, they exhibit an unusual phenotype including expression of the CD45RA isoform (B220) and altered cell surface O-glycans (16), and they are high producers of IL-10, in contrast to DN T cells from normal individuals (17,18). Besides elevated IL-10, ALPS patients exhibit a Th2 cytokine profile that may favor autoantibody production (19). The origin of DN T cells remains elusive. They could be either previously activated mature T cells that have lost CD8 or CD4 co-receptor expression, or a special minor cell lineage selectively expanded owing to defect in Fas signaling. To investigate the relationships of DN T cells and the single positive (SP) T cell populations (CD4+ and CD8+), we have analyzed the TCR diversity and characterized complementarity determining regions 3 (CDR3) of TCR Vß chains in sorted DN and SP CD4+ and SP CD8+ T cells in patients with ALPS type 1a.

Materials and methods

Patients and samples

Blood samples were obtained from three patients with ALPS type Ia. All patients, or their parents, provided written informed consent, validated by the Ethics Committee (*Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale*) from the Necker Hospital. Table 1 summarizes the clinical features of the patients.

Flow cytometry and sorting of double-negative T cells

Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparin-treated blood by means of Ficoll-hypaque density gradient centrifugation. The proportion of DN T cells was determined by flow cytometry on PBMC following staining with monoclonal antibodies (CD3-APC, CD4-PE, CD8-FITC and TCRαß) purchased from Becton Dickinson. DN T cells ($CD4^-CD8^-CD3^+TCR\alpha\beta^+$) were identified as shown in Fig. 1*A*. Sorting of DN T $CD3^+CD4^-CD8^-$ cells from the three patients was performed with a FACSstar with 98% purity. Sorted DN T cells therefore contain a majority of αβT cells. Possible presence of γδ T cells within sorted DN T cells does not skew the data since repertoire analyses were performed with Vß-specific primers.

Determination of Vß gene usage and Immunoscope analysis

Determination of Vß gene usage and Immunoscope analysis was performed as described in A. Lim et al. (20). Briefly, total RNA was extracted using the RNeasy mini-kit (Qiagen, Courtaboeuf, France) according to the manufacturers specifications. cDNA was then prepared by RNA reverse-transcription with 0.5µg/µl oligo (dT)17 and 200 U of Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). An aliquot of cDNA synthesis reaction was amplified with each of the 24 TCR Vβ family-specific primers, together with a TCR Cβ primer and a Minor Groove Binder (MGB)–TaqMan probe (Applied Biosystems). Real-time quantitative PCR was carried out in an ABI7300 device (Applied Biosystems, Foster City, CA). In a second approach, two microliters of each of these amplification reactions were used as template in run-off reactions

using a nested fluorescent primer specific for the Cß segment. In this reaction, all PCR products were copied into fluorescently labeled single-stranded DNA fragments, irrespective of their TCR Jβ usage or CDR3 sequence. These fluorescent products were separated on an ABI-PRISM 3730 DNA analyzer (Applied Biosystems). The size and intensity of each band were analyzed with Immunoscope software (21,22). Fluorescence intensity was plotted in arbitrary units on the y-axis, whereas the x-axis corresponds to CDR3 length in amino acids. The Gaussian distribution of the different CDR3 lengths is characteristic of normal Vß repertoire.

DNA cloning and sequencing analysis of TCR transcripts

Vß/Cß PCR were carried out on cDNA with 5 U Pfu polymerase (Stratagene) for 25 cycles. PCR products were then cloned in pCR®4Blunt-TOPO vector (Invitrogen Life Technologies). For sequencing purposes, PCR was carried out directly on $LacZ^-$ colonies with Taq polymerase (Promega, Madison, MI) (23). Sequencing was carried out with the Big Dye Terminator Reaction Kit (Applied Biosystem) and analyzed on an ABI PRISM 3730 DNA Analyzer.

Results

Clinical Features

Demographic and clinical features of the study patients are shown in Table 1**.** Patients presented with ALPS, characterized by a lymphoproliferative syndrome consisting in massive splenomegaly (splenectomy was performed at age 12 years in P1 because of hypersplenism), and lymphadenopathy consisting in multiple lymph nodes sometimes larger than 2 cm (P1). Autoimmune manifestations, were detected in P1 and P3, consisting in urticarial rash and arthralgia in P1, uveitis, glomerulopathy and autoimmune thrombocytopenia in P3. Hypergammaglobulinemia was found in all three patients, associated with hyper IgA in P1 and P3, and normal or low (P3) serum IgM level. This was associated with a heterozygous mutation in the *Fas* gene, and a defect in anti-CD95 monoclonal antibody induced-apoptosis, as previously described for such patients (12). In all cases, an elevated proportion of $TCR\alpha\beta^{+}/CD4^{-}/CD8^{-}$ DN T cells was detected in peripheral blood, the size of this population varying from one patient to another (Table 1) (12).

Purification and immunophenotypic characteristics of DN T cells in ALPS patients

The proportion of DN T cells in the study patients ranged from 4.4% to 17.2% of peripheral blood a/ß T cells (Fig. 1*A*). SP and DN T cells were purified from patients' PBMC by positive selection of $CD3^{+}$ T cells with magnetic beads, followed by FACS sorting of three subsets: $CD3^{+}CD4^{+}$ SP, $CD3^{+}CD8^{+}$ SP, $CD3^{+}CD4^{-}CD8^{-}$ DN T cells. DN T cells from these patients exhibited the phenotype of mature "end stage" cells i.e. TCR^{high}, $CD2^{+}$, $CD5^{+}$, $CD27^{++}$, $CD28^{+}$, $CD45RA^{+}RO^{-}$ $CD31^{+}$, $CD62L^{dull}$, CXCR-5^{-}, $CD57^{+}$, CD11b (data not shown). Furthermore, analysis of the cytotoxic granule content showed that *ex vivo* DN T-cells from ALPS patients were mostly GrzA positive (for example 90.2% for P2) while GrzB was barely detected (Fig. 1*B*, 1*C*). In contrast, $CD8^{+}$ T-cells from the same individual significantly over-expressed GrzA and GrzB, as compared to control, and granzymes were detected in $CD4^{+}$ lymphocytes too (Fig. 1*B*, 1*C*). These data suggest that DN T cells are unable to exert a cytolytic granule-dependent activity. In addition, as it was demonstrated recently (24), cytotoxic granules-dependent cell-death in SP T cells may compensate for Fas-

deficiency in ALPS.

The TCR Vβ*-gene usage of α/ß DN T cells from ALPS patients quantitatively differs from that of SP cells*

The expression of 24 Vß gene families was examined by RT-PCR amplification of cDNA derived from sorted subpopulations. Representative data are shown for a control donor (Fig. 2*A*) and three patients (Fig. 3). Qualitatively, repertoire diversity was conserved in all three subsets from ALPS patients, and in particular DN T cells expressed all Vß-gene families that were used by $CD4^+$ and $CD8^+$ SP cells (Fig. 3). However, quantitative differences in the expression of several Vßs were apparent in each of the three patients, with preferential expression of some Vßs by DN T cells. For example, Vß2 and Vß7 families were preferentially expressed in DN T cells from P1 (Vß2: 25% in DN T cells vs 12% and 8% in SP CD8 and CD4 SP T cells respectively; Vß7: 30% in DN T cells vs 12.1% and 2.5% in SP CD8 and CD4 SP T cells respectively). Similarly, a preferential usage of Vß17 and Vß7 genes was found in DN T cells from P2 and P3 respectively (Fig. 3).

CD8 SP and DN T cells from ALPS patients express a skewed TCR Vß repertoire, in contrast to CD4 T cells

Analysis of CDR3 length distribution in sorted CD4 T cells from ALPS patients showed for all Vß-Cß rearrangements Gaussian Immunoscope profiles (representative profiles are shown in Fig. 4), which is the hallmark of a polyclonal T cell repertoire observed in control donors (Fig. 2) (20). In sharp contrast, SP CD8 T cells from ALPS patients displayed skewed Immunoscope profiles with oligoclonal expansions for some Vß families (Fig. 4), such as Vß2, Vß8, Vß13a, Vß14, Vß17, Vß18, and Vß20 for P1, Vß1, Vß2, Vß5, Vß8, Vß13b, Vß17, Vß22, for P2, and Vß1 and Vß13b for P3 (Fig. 4). This restricted diversity was not observed in sorted SP CD8 T cells from healthy donors (Fig. 2), as previously reported (20). DN T cells from ALPS patients displayed highly limited clonality with characteristic clonotypes for some Vß families (eg Vß2, Vß7, Vß13a, Vß13b, Vß14, Vß20 for P1, Vß1, Vß5 and Vß22 for P2) while others showed more diverse patterns with specific

T cell expansions (for example Vß1, Vß21, Vß22 and Vß23 for P1, Vß2, Vß8, Vß13a, Vß22 for P2, Vß1, Vß13b, Vß14, Vß18 for P3 (Fig. 4). Oligoclonality of both CD8 SP and DN T cells was confirmed by sequence analysis selecting within Vß families matching clonotypes that had the same CDR3 length in TCR transcripts from CD8 SP and DN T cells (as exemplified for patient 2 in Fig. 5). Table 2 indicates the frequency of unique in-frame CDR3 sequences within the total analyzed selected Vß TCR clonotypes from CD4 SP, CD8 SP and DN T cells from the three patients. In DN T cells from P1, as few as 14 unique CDR3 sequences out of 88 Vß2 TCR, 10 out of 72 Vß14 TCR, 39 out of 230 Vß18 TCR and 36 out of 233 Vß23 TCR were identified. A total of 682 CDR3 sequences were generated from selected transcripts, among which only 124 were unique (18%), confirming Vß gene skewing among DN T cells. In contrast, paired CD4 SP T cells revealed polyclonal frequency of CDR3 sequences with almost no repeat (66 unique CDR3 sequences out of 75 Vß2 TCR and 78 out of 80 Vß8 TCR), as found on a total of 155 CDR3 sequences among which 144 were unique (93%) (Table 2). Oligoclonality of SP CD8 T cells suggested by Immunoscope profiles was confirmed by sequence analysis, the frequency of unique CDR3 sequences being intermediate between SP CD4 and DN T cells, 34 unique CDR3 sequences out of 80 Vß2 TCR, 21 out of 84 Vß14 TCR, 94 out of 196 Vß18 TCR and 103 out of 216 Vß23 TCR. Overall, a total of 631 CDR3 sequences were generated from selected transcripts, among which 275 were unique (44%). A similar skewed oligoclonal frequency of CDR3 sequences was found in patients 2 and 3 DN T cells (Table 2).

CDR3 sequence analysis of TCR transcripts containing common clonotypes in DN T and SP CD8 T cells

To address whether DN T cells may originate from SP CD8 T cells, we identified dominant clonotypes in DN T cells and selected clonotypes with similar CDR3 size in SP CD8 T cells for sequence analysis (e.g. Vß7 in P2, Fig. 5). Selected TCR transcripts of common clonotypes were cloned and the resulting DNA clones were subsequently analyzed for CDR3 sequences using corresponding Vß and Jß primers. Fig. 6 shows that for each selected clonotype, CDR3 sequences

were detected at very high frequency in DN T cells, consistent with the oligoclonality of the Vß repertoire. For example, the Vß7 clonotype in P2 included two major CDR3 sequences, SLRQGLSTEA and SQGTGGNQPQ (76% and 6.4% of total analyzed sequences respectively). Importantly, both CDR3 sequences were also detected in SP CD8 T cells, but with a much lower frequency (4.8% and 0.4% of total analyzed sequences respectively) (Fig. 6). Similarly, the Vß13 clonotype in P3 was dominated by an 11aa CDR3 sequence (SSDSGGSYNEQ), which represented 53% of the clones in that gene family. This CDR3 sequence was also detected in SP CD8 T cells, though at a lower frequency, as expected. In P1, The 9 aa CDR3 sequence RPGLPNPEA was detected both in DN and SP CD8 T cells. Overall, the Immunoscope analysis and CDR3 sequencing experiments argue for the hypothesis that DN T cells from ALPS patients may represent, at least in part, originally SP CD8 T cells that down-modulated the co-receptor CD8. In addition, the extent of oligoclonality together with phenotypic features of DN T cells suggest recent antigenic activation by a limited set of antigens, possibly self antigens.

Discussion

The expansion of α/ß-DN T cells in peripheral blood of ALPS patients is a consistent feature of this disease, and part of the diagnostic criteria of ALPS (1,10). DN T cell expansion is also evident histologically in secondary lymphoid organs, particularly in the paracortical areas of lymph nodes, where they accumulate and express markers of activated T cells, i.e. $ki67^{+}$, $CD45R0^{-}CD45RA^{+}$ (25). Lymphoproliferation in ALPS is associated with chronic generalized activation, as demonstrated by upregulation of HLA-DR expression on peripheral CD3 T cells as well as high levels of activation markers such as soluble interleukine-2 receptor, soluble CD30 and IL10 in sera of ALPS patients (7,26,27). Consistent with their unregulated peripheral proliferation and possible recognition of self antigens, DN T cells exhibit the phenotype of mature antigen-experienced cytotoxic T cells, i.e. TCR^{high}, $CD2^{+}$, $CD5^{+}$, $CD27^{++}$, $CD28^{+}$, $CD45RA^{+}RO^{-}$ $CD31^{+}$, $CD62L^{dull}$, $CXCR\text{-}5^{-}$, $CD57^{+}$, $CD11b^{16}$. The uniform phenotype of a/ß-DN T cells from ALPS patients is similar to a/ß-DN T cells from mice with defective Fas *(lpr)* (7,16,28) and different from the minor a/ß-DN T cell compartment in healthy subjects that contains multiple subpopulations (29,30). To investigate the origin of DN T cells from ALPS patients, we analyzed with Immunoscope technology the TCR Vß repertoire diversity of sorted DN T cells and SP CD4 and CD8 T cells, and we performed sequence analyses of the CDR3 region within Vß families showing, on Immunoscope profiles, common clonotypes between DN and SP T cells. This study shows for the first time that DN T cells from ALPS patients share with CD8 T cells unique CDR3 sequences across several TCR Vß families, arguing for the CD8 origin of DN T cells.

Vß gene family usage was examined by quantitative RT-PCR amplification of cDNA derived from sorted DN and SP T cells. Though the Vß-gene usage of DN T cells did not dramatically differ from that of SP T cells in ALPS patients – i.e. DN T cells expressed all the Vß gene families that were used by the SP counterparts, preferential expression of some Vß was found in DN T cells as compared to autologous SP T cells. However, Vß usage skewing in DN T cells varied from one patient to the other. Analysis of CDR3 length distribution revealed dramatic differences between all

three subsets. A Gaussian distribution of CDR3 peaks for all Vß-Cß rearrangements, the hallmark of a polyclonal TCR repertoire, was obtained in sorted SP CD4 T cells from the ALPS patients. In contrast, DN and SP CD8 T cells displayed skewed TCR-Vß Immunoscope profiles, with oligoclonal or monoclonal expansions throughout most of the Vß families, indicating a contracted TCR-Vβ repertoire. Oligoclonality of both SP CD8 and DN T cells was confirmed by sequence analysis selecting, within Vß families, matching clonotypes that had the same CDR3 length in TCR transcripts. For example, in DN T cells from P1, a total of 682 CDR3 sequences were generated from selected transcripts, among which only 18% were unique, in contrast to SP CD4 T cells that showed 93% of unique CDR3 sequences. The frequency of unique CDR3 sequences was intermediate (44%) in SP CD8 T cells from P1. Similar data were found for patients 2 and 3. That expanded DN T cells originate from SP CD8 T cells is suggested by several instances of shared CDR3 sequences from selected Vß-Jß transcripts. For example, the Vß7 clonotype in DN T cells from P2 was dominated by two major CDR3 sequences (SLRQGLSTEA and SQGTGGNQPQ) that were both detected in SP CD8 T cells. We report similar findings for Vß13 clonotype in P3, or Vß 23 clonotype in P1. Interestingly, CDR3 sequences that are shared between DN and CD8 T cells could not be detected in CD4 T cells. Therefore, from this study, we conclude that DN T cells from ALPS patients are oligoclonally expanded, and they originate from SP CD8 T cells, as suggested by shared Vß CDR3 sequences within several Vß families. Differences in Vß gene usage by DN as compared to CD8 T cells suggest that the former originate from the expansion of a minor CD8 subset. Overall, this conclusion differs from the one proposed in a recent study, focusing on CDR3 spectratyping profiles of sorted DN and SP T cells from a single ALPS patient. The authors concluded that DN are not clonally related to SP T cells because of the lack of CDR3 size matching of the oligoclonal expansions detected on spectratype profiles from the three subsets (31). However, the lack of CDR3 sequence analysis in this study precludes any valid conclusion on the origin of DN T cells.

The accumulation of DN T cells in murine models of ALPS, i.e. mice lacking expression of Fas

(lpr) or Fas-ligand (gld), suggests that this subset might represent a subpopulation destined for apoptosis in normal mice (9). It would arise from high affinity interactions of TCR with peptide/MHC, and would accumulate to vast numbers in the absence of Fas-induced apoptosis (32). The SP T cell origin of DN T cells has been suggested by several findings, including their bearing of a demethylated CD8 gene (33,34), and their absence in mice lacking expression of class I MHC (35). In ALPS patients, the homogenous phenotype of DN T cells, that characterizes mature antigen-experienced cytotoxic T cells, suggest their CD8 T cell origin (16). Interestingly, the peculiar expression pattern of cytolytic proteins by DN T cells makes them unable to exert a cytolytic granule-dependent activity. Indeed, they strongly express granzyme A, whereas granzyme B and perforin are barely detected, as opposed to granule expression in SP T cells (24) (and this study). As recently discussed (24) the repressor mechanism that down-regulates expression of granzyme B and perforin while sparing granzyme A in DN T cells may involve IL-10 [highly produced by DN T-cells (17,36)] previously shown to repress granzyme B mRNA expression in cytotoxic lymphocytes (37). This repressor mechanism could be involved in the modulation of the CD4 or CD8 co-receptors (38).

The mechanisms involved in peripheral expansion of DN T cells in ALPS patients are not fully understood. Holzelova et al. have shown that resistance to Fas-mediated apoptosis would confer a selective advantage to mutant cells, leading to their accumulation and evolution towards the phenotype of DN T cells (15). The recent observation that *in vivo* triggering of mitochondrial apoptosis by rapamycin in murine ALPS induces a dramatic decrease in DN T cells (39) supports this hypothesis. The lack of co-receptors in DN T cells from ALPS patients may also contribute to their accumulation. Indeed, it has been reported in a TCR transgenic murine model that co-receptor engagement controls expansion of normal T cells, and in the absence of co-receptor T cells survive chronic stimulation and express B220, as seen in ALPS disease (40). In the present study, DN T cells appeared to be oligoclonally expanded, as evidenced by CDR3 length distribution. This extent of oligoclonality together with phenotypic features suggesting historical antigen encounter and

recent activation of large proportions of these cells may reflect stimulation of DN T cells by a limited yet ubiquitous set of antigens. The antigen specificities recognized by the TCR of DN T cells have not been defined, but the general assumption is that they will include self-antigens. Interestingly, this study shows for the first time that CD8 T cells from ALPS patients (children and young adults) exhibit a restricted TCR diversity, with oligoclonal and monoclonal expansions throughout most of the Vß families, and redundant CDR3 sequences. This is reminiscent of repertoire contraction and oligoclonal expansions seen in aged individuals, and supposed to be a heterogeneous phenomenon including impaired homeostasis and antigen-driven stimulation (41). The conserved CDR3 sequences between CD8 T cells and DN T cells reported here suggest that they may recognize identical peptide sequences, and argue for their filiation. It has been proposed that the defect in killing of *lpr* CD8 T cells following stimulation by self-antigen, would lead to the modulation of CD8 co-receptor and accumulation as IL-10 secreting anergic cells (42,43). The comparative molecular analysis of CDR3 distribution and sequencing of CD8 T cells and DN T cells in the present study extends for the first time this hypothesis to the human disease.

References

1. Bidere, N., H. C. Su, and M. J. Lenardo. 2006. Genetic disorders of programmed cell death in the immune system. *Annu. Rev. Immunol.* 24:321-352

2. Rieux-Laucat, F., F. Le Deist and A. Fischer. 2003. Autoimmune lymphoproliferative syndrome : genetic defects of apoptosis pathways. *Cell Death Diff.* 10:124-133

3. Sneller, M. C., S. E. Strauss, J. S Jaffe, T. A Fleisher, M. Stetler-Stevenson, and W. Strober. 1992. A novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld disease. *J. Clin. Invest.* 90:334-341

4. Rieux-Laucat, F., F. Le Deist, C. Hivroz, M. C. Sneller, S. E. Straus, E. S. Jaffe, T. A. Jaffe, T. A. Fleisher, M. Stetler-Stevenson, and W. Strober. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268:1347-1349.

5. Fisher, G. H., F. J. Rosenberg, S. E. Straus, J. K. Dale, L. A. Middleton, A. Y. Lin, W. Strober, M. J. Lenardo, and J. M Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 81:935-946.

6. Drappa, J., A. K. Vaishnaw, K. E. Sullivan, J. L. Chu, and K. B. Elkon. 1996. Fas gene mutation in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. *N. Eng. J. Med.* 335:1643-1649

7. Bettinardi, A., D. Brugnoni, E. Quiros-Roldan, A. Malagoli, S. La Grutta, A. Correra, and L. D. Notarangelo. 1997. Missense mutations in the Fas gene resulting in autoimmune lymphoproliferative syndrome : a molecular and immunological analysis. *Blood* 89:902-909

8. Krammer, P.H. 2000. CD95's deadly mission in the immune system. *Nature* 407 :789-795

9. Nagata, S. and T. Suda. 1995. Fas and Fas ligand: lpr and gld mutations. *Immunol. Today* 16:39-43.

10. Rieux-Laucat, F., A. Fischer, and F. Le Deist. 2003. Cell-death signaling and human disease. *Curr. Opin. Immunol.* 15:325-331.

11. Jackson, C. E., R. E. Fischer, A. P. Hsu, S. M Anderson, Y. Choi, J Wang, J. K Dale, T. A.

Fleisher, L. A. Middleton, M. C. Sneller, M. J. Lenardo, S. E Straus, and J. M Puck. 1999. Autoimmune lymphoproliferative syndrome with defective Fas : genotype influences penetrance. *Am. J. Hum. Genet.* 64 :1002-1014

12. Rieux-Laucat, F., S. Blachere, S. Danielan, J. P. De Villartay, M. Oleastro, E. Solary, B. Bader-Meunier, P. Arkwright, C. Pondare, F. Bernaudin, H. Chapel, S. Nielsen, M. Berrah, A. Fischer and F. Le Deist, 1999. Lymphoproliferative syndrome with autoimmunity: A possible genetic basis for dominant expression of the clinical manifestations. *Blood* 94:2575-2582.

13. Wu, J., J. Wilson, J. He, L. Xiang, P. H. Schur, and J. D. Mountz, 1996. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J. Clin. Invest.* 98 :1107-1113

14. Wang, J., L. Zheng, A. Lobito, F. K. Chan, J. Dale, M. Sneller, X. Yao, J. M. Puck, S. E. Straus, and M. J. Lenardo. 1999. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell* 98:47-58.

15. Holzelova, E., C. Vonarbourg, M. C. Stolzenberg, P. D. Arkwright, F. Selz, A. M. Prieur, S. Blanche, J. Bartunkova, E. Vilmer, A. Fischer, F. Le Deist, and F. Rieux-Laucat. 2004. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *N. Engl. J. Med.* 351:1409-1418.

16. Bleesing, J. J., M. R Brown, J. K Dale, S. E Straus, M. J. Lenardo, J. M. Puck, T. P. Atkinson, and T. A. Fleisher, 2001. TcR-alpha/beta(+) CD4(-)CD8(-) T cells in humans with the autoimmune lymphoproliferative syndrome express a novel CD45 isoform that is analogous to murine B220 and represents a marker of altered O-glycan biosynthesis. *Clin. Immunol.* 100:314-324.

17. Lopatin, U., X. Yao, R. K. Williams, J. J. Bleesing, J. K Dale, D. Wong, J. Teruya-Feldstein, J. S. Fritz, M. R. Morrow, I. Fuss, M. C Sneller, M. Raffeld, T. A. Fleisher, J. M. Puck, W. Strober, and S. E. Straus. 2001. Increases in circulating and lymphoid tissue interleukin-10 in autoimmune lymphoproliferative syndrome are associated with disease expression. *Blood* 97:3161-

3170.

18. Ohga S, A. Nomura, Y. Takahata , K. Ihara, H. Takada, H. Wakiguchi, Y. Kudo, and T. Hara. 2002. Dominant expression of interleukin 10 but not interferon gamma in CD4(-)CD8(-)alpha betaT cells of autoimmune lymphoproliferative syndrome. *Br. J. Haematol.* 119:535-538.

19. Fuss, I.J., W. Strober, J.K. Dale, S. Fritz, G. R. Pearlstein, J. M. Puck, M. J. Lenardo, and S.E. Straus. 1997. Characteristic T helper 2 T cell cytokine abnormalities in autoimmune lymphoproliferative syndrome, a syndrome marked by defective apoptosis and humoral autoimmunity. *J. Immunol*.158:1912-1918.

20. Lim, A., V. Baron, L. Ferradini, M. Bonneville, P. Kourilsky, and C. Pannetier, 2002. Combination of MHC-peptide multimer-based T cell sorting with the Immunoscope permits sensitive *ex vivo* quantitation and follow-up of human CD8+ T cell immune responses. *J. Immunol. Methods* 261:177-194.

21. Pannetier, C., M. Cochet, S. Darche, A. Casrouge, M. Zöller, P. Kourilsky. 1993. The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. *Proc. Natl. Acad. Sci. U S A*. 90:4319–4323.

22. Pannetier, C., J. Even, and P. Kourilsky. 1995. T-cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol. Today* 16:176–181.

23. Bousso, P., A. Casrouge, J. D. Altman, M. Haury, J. Kanellopoulos, J. P. Abastado, and P. Kourilsky. 1998. Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. *Immunity* 9:169-78.

24. Mateo, V., M. Ménager, G. de Saint-Basile, M. C. Stolzenberg, B. Roquelaure, N. Andre, B. Florkin, F. le Deist, C. Picard, A. Fischer, and F. Rieux-Laucat, 2007. Perforin-dependent apoptosis functionally compensates Fas-deficiency in activation-induced cell-death of human T-lymphocytes. *Blood* 110 :4285-4292

25. Rieux-Laucat, F. 2006. Inherited and acquired death receptor defects in human autoimmune lymphoproliferative syndrome. *Curr. Dir. Autoimmun*. 9:18-36.

26. Le Deist, F., J. F Emile, F. Rieux-Laucat, M. Benkerrou, I. Roberts, N. Brousse, and A.

Fischer. 1996. Clinical, immunological, and pathological consequences of Fas-deficient conditions. *Lancet* 348 :719-723

27. Sneller, M. C., J. Wang, J. K. Dale, W. Strober, L. A. Middelton, Y. Choi, T. A. Fleisher, M. S. Lim, E. S. Jaffe, J. M. Puck, M. J. Lenardo, and S. E. Straus, 1997. Clinical, immunologic, and genetic features of an autoimmune lymphoproliferative syndrome associated with abnormal lymphocyte apoptosis. *Blood* 89:1341-1348

28. Morse, H.C., W. F. Davidson, R. A. Yetter, E. D. Murphy, J. B. Roth, and R. L. Coffman. 1982. Abnormalities induced by the mutant gene lpr: expansion of a unique lymphocyte subset. *J. Immunol. 129*:2612-2615

29. Reimann, J. 1991. Double-negative (CD4-CD8-), TCR alpha beta-expressing, peripheral T cells. *Scand. J. Immunol.* 34:679-688

30. Fischer, K., S. Voelkl, J. Heymann, G. K. Przybylski, K. Mondal, M. Laumer, L. Kunz-Schughart, C. A. Schmidt, R. Andreesen, and A. Mackensen. 2005. Isolation and characterization of human antigen-specific TCRαß+CD4-CD8- double negative regulatory T cells. *Blood* 105:2828-2835

31. Marlies, A., G. Udo, B. Juergen, S. Bernd, M. Herrmann, and J. P. Haas. 2007. The expanded double negative T cell populations of a patient with ALPS are not clonally related to CD4+ or to CD8+ T cells. *Autoimmunity* 40:299-301

32. Mixter, P. F., J. Q. Russell, G. J. Morrissette, C. Charland, D. Aleman-Hoey, and R. C. Budd. 1999. A model for the origin of TCR-alphabeta+ CD4-CD8- B220+ cells based on high affinity TCR signals. *J. Immunol.* 162:5747-56

33. Landolfi, M. M., N. Van Houten, J. Q. Russell, R. Scollay, J.R. Parnes, and R.C. Budd, 1993. $CD2^{-}CD4^{-}CD8^{-}$ lymph node T lymphocytes in MRL *lpr/lpr* mice are derived from a $CD2^{+}CD4^{+}CD8^{+}$ thymic precursor. *J. Immunol.* 151:1086-1091

34. Wu, L., M. Pearse, M. Egerton, H. Petrie, and R. Scollay. 1990. $CD4^{-}CD8^{-}$ thymocytes that express the T cell receptor may have previously expressed CD8. *Int. Immunol.* 2:51-56.

35. Mixter, P.F., J. Q Russell, F. H. Durie, and R. C. Budd. 1995. Decreased $CD4^{-}CD8^{-}$ TCR-

$\alpha\beta^+$ cells in *lpr/lpr* mice lacking b2-microglobulin. *J. Immunol. 154*:2063-2067

36. Ohga, S., A. Nomura, Y. Takahata, K. Ihara, H. Takada, H. Wakiguchi, Y. Kudo, and T. Hara. 2002. Dominant expression of interleukin 10 but not interferon gamma in CD4(-) CD8(-) alpha beta T cells of autoimmune lymphoproliferative syndrome. *Br. J. Haematol.* 119:535-538.

37. Fitzpatrick, L., A. P. Makrigiannis, M. Kaiser, and D. W. Hoskin, 1996. Anti-CD3-activated killer T cells: interferon-gamma and interleukin-10 cross-regulate granzyme B expression and the induction of major histocompatibility complex-unrestricted cytotoxicity. *J. Interferon Cytokine Res.* 16:537-546.

38. Pestano, G. A., Y. Zhou, L. A. Trimble, J. Daley, G. F Weber, and H. Cantor. 1999. Inactivation of misselected CD8 T cells by CD8 gene methylation and cell death. *Science* 284:1187-1191.

39. Teachey, D. T., D. A. Obzut, K. Axsom, J. K. Choi, K. C. Goldsmith, J. Hall, J. Hulitt, C. S. Manno, J. M. Maris, N. Rhodin, K. E. Sullivan, V. I. Brown, and S. A. Grupp. 2006. Rapamycin improves lymphoproliferative disease in murine autoimmune lymphoproliferative syndrome (ALPS). *Blood* 108:1965-1971.

40. Hamad, A. R., A. Srikrishnan, P. Mirmonsef, C. P. Broeren, C. H. June, D. Pardoll, and J. P. Schneck. 2001. Lack of coreceptor allows survival of chronically stimulated double-negative alpha/beta T cells: implications for autoimmunity. *J. Exp. Med.* 193:1113-1121.

41. Clambey, E. T., J. W. Kappler, and P. Marrach. CD8 T cell clonal expansions and aging: a heterogeneous phenomenon with a common outcome. *Exp. Gerontology* 42:407-411

42. Ford, M. S., K. J. Young, Z. Zhang, P.S. Ohashi, and L. Zhang. 2002. The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. *J. Exp. Med.* 196:261–267.

43. Watanabe, N., K. Ikuta, S. Nisitani, T. Chiba, and T. Honjo. 2002. Activation and differentiation of autoreactive B-1 cells by interleukin 10 induce autoimmune hemolytic anemia in Fas-deficient antierythrocyte immunoglobulin transgenic mice. *J. Exp. Med.* 196:141–146.

Footnotes

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[3] **Abbreviations used**: ALPS, Autoimmune Lymphoproliferative Syndrome; SP, Single Positive; DN, Double Negative

Figure legend

Figure 1. *FACS analysis of cytotoxic granule content of peripheral T cells in control and ALPS patients.* Freshly drawn heparinized peripheral blood samples were stained for TCRαß, CD3, CD4 and CD8 expression. Cells were next stained for GrzA, GrzB and perforin content.

A: surface CD4 and CD8 co-expression on gated TCRαß$^+$ CD3$^+$ T cells from a control donor and the three ALPS patients. The percentage of cells within each quadrant among gated cells is indicated.

B: Intracellular GrzA and GrzB content in gated CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, and CD3$^+$CD4$^-$CD8$^-$DN T cells from a control donor and P2. C: Intracellular perforin and GrzB content in gated CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, and CD3$^+$CD4$^-$CD8$^-$DN T cells from a control donor and P2.

Figure 2. *Quantitative Vß repertoire analysis and Immunoscope profiles of SP T cells from a control donor*.

A: Quantitative Vß repertoire was determined by real-time PCR analysis on sorted CD4$^+$ and CD8$^+$ T cells from a representative healthy donor. The *x* axis indicates Vß families and the *y* axis their relative frequency of usage.

B: Immunoscope profiles of TCR Vß analysis on the same sorted SP T cells. Profiles for the 24 Vß families are shown. The x-axis indicates CDR3 length (amino-acid), and the y-axis displays arbitrary fluorescence intensity of the run-off products

Figure 3. *Quantitative Vß repertoire analysis on SP and DN T cells from ALPS patients.* Quantitative Vß repertoire was determined by real-time PCR analysis on sorted SP CD4$^+$ and CD8$^+$ T cells, and DN T cells from the three ALPS patients. The *x* axis indicates Vß families and the *y* axis their relative frequency of usage. Arrows point out the expansion of some Vß families detected in DN T cells as compared to SP T cells from the same individual.

Figure 4. *Immunoscope profiles of TCR Vß analysis of SP and DN T cells from ALPS patients.* The diversity of TCRVß repertoire from sorted SP and DN T cells from ALPS patients was analyzed and Immunoscope profiles are shown. The x-axis indicates CDR3 length (amino-acid), and the y-axis displays arbitrary fluorescence intensity of the run-off products. Immunoscope profiles of CD4 T cells are Gaussian for all Vß families, indicative of a diverse repertoire, as observed in control donors (Fig. 2). Immunoscope profiles of $CD8^+$ and DN T cells are oligoclonal in most of the Vß families, indicative of a skewed restricted repertoire. Vß families targeted for further CDR3 sequence analysis are pointed out with a star.

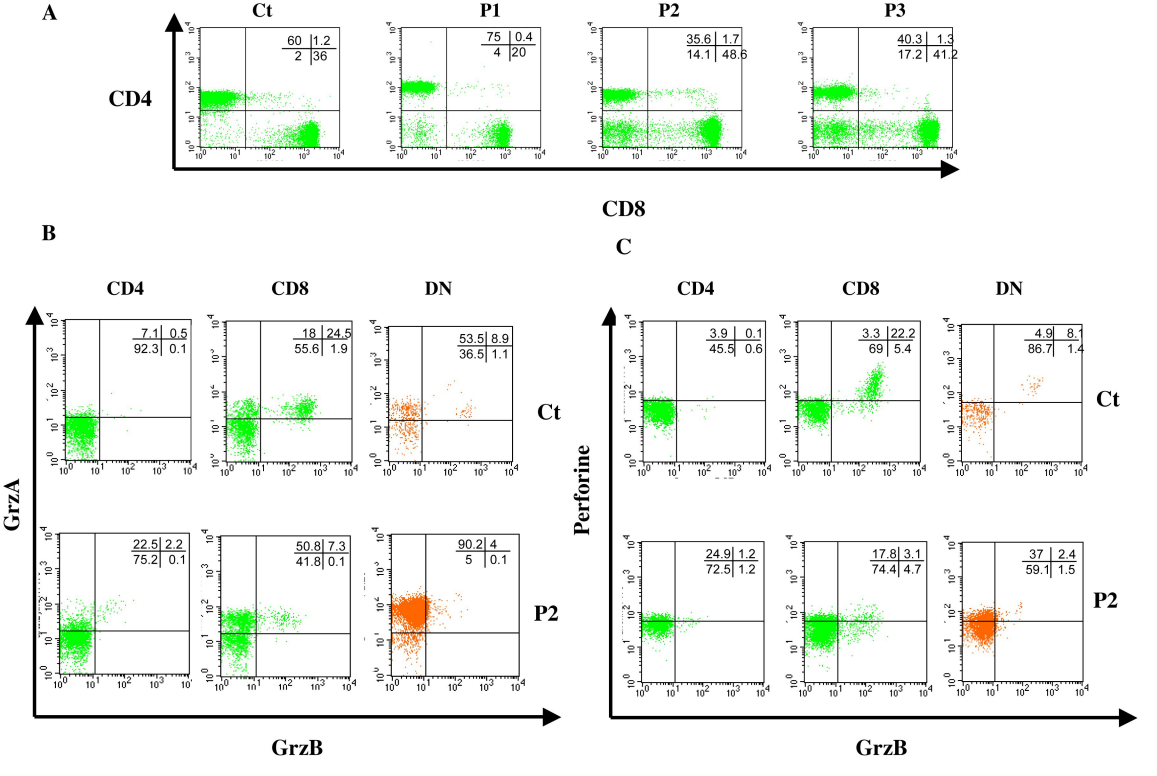
Figure 5. *Oligoclonality of both $CD8^+$ T cells and DN T cells for indicated Vß families confirmed by sequence analysis*, as shown in the upper part of the figure for each cell subset. For a given Vß, the number of sequences obtained for each CDR3 length was representative of the Immunoscope profile described in Fig. 4. Identification of matching clonotypes with same CDR3 length in TCR transcripts from CD8 SP and DN T cells from ALPS P2 was performed within Vß1, 5, 7, 17, 20 24 families, as shown by the hatched lines.

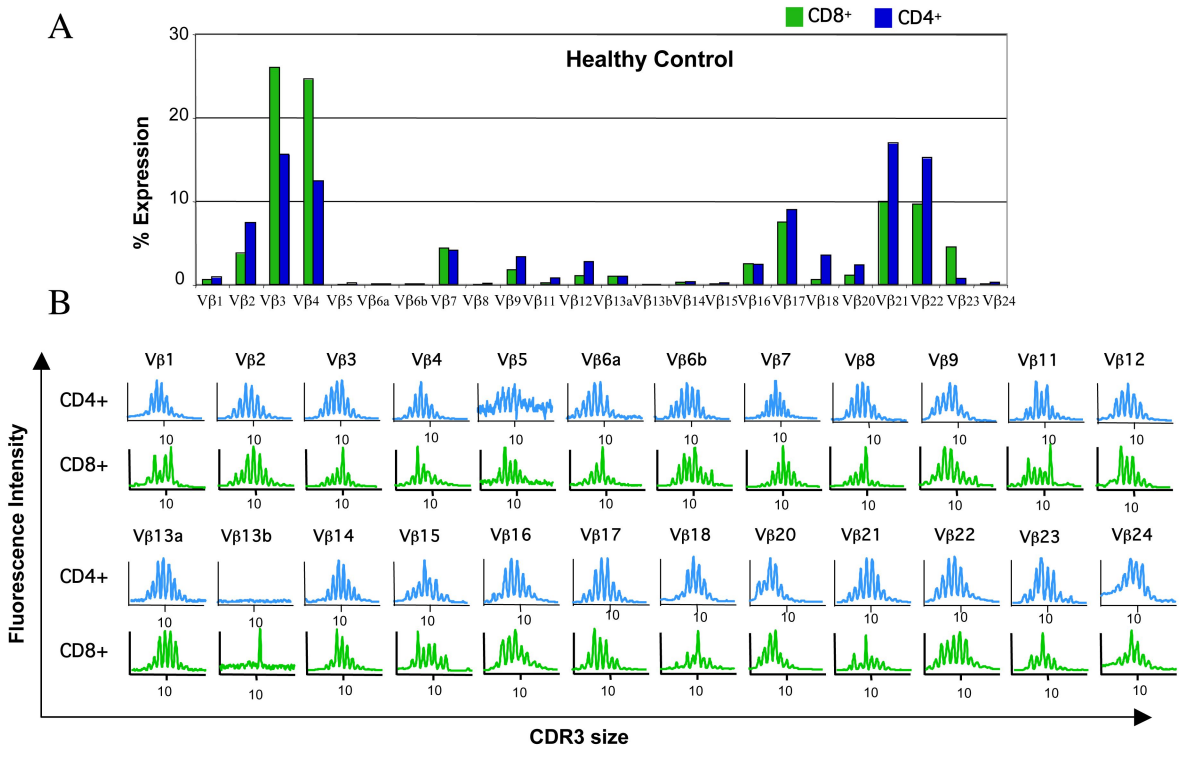
Figure 6. *CDR3 sequence analysis of selected TCR Vß transcripts of dominant clonotypes between $CD8^+$ T cells and DN T cells*. Selected TCR transcripts were cloned and the resulting DNA clones were subsequently analyzed for CDR3 sequences using corresponding Vß and Jß primers. For each selected clonotype, the frequency of CDR3 sequences in DN and SP $CD8^+$ T cells is indicated.

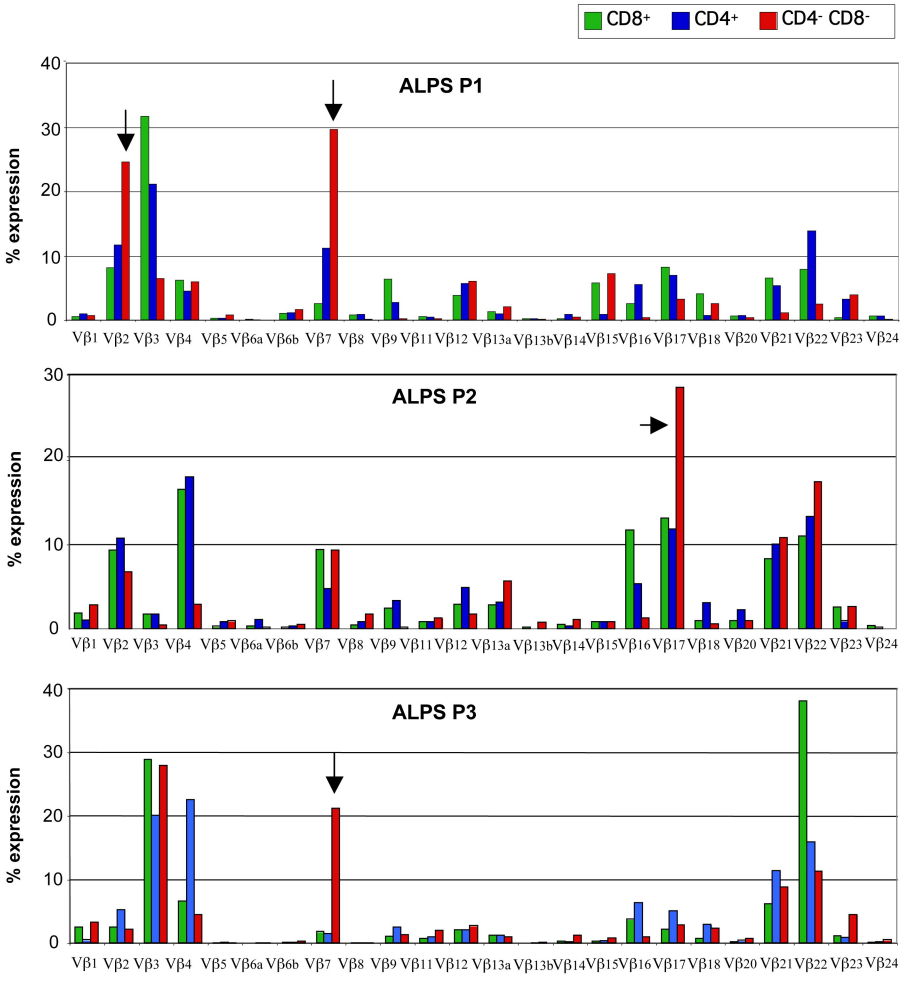
Table 1 : Patients' clinical and immunological characteristics

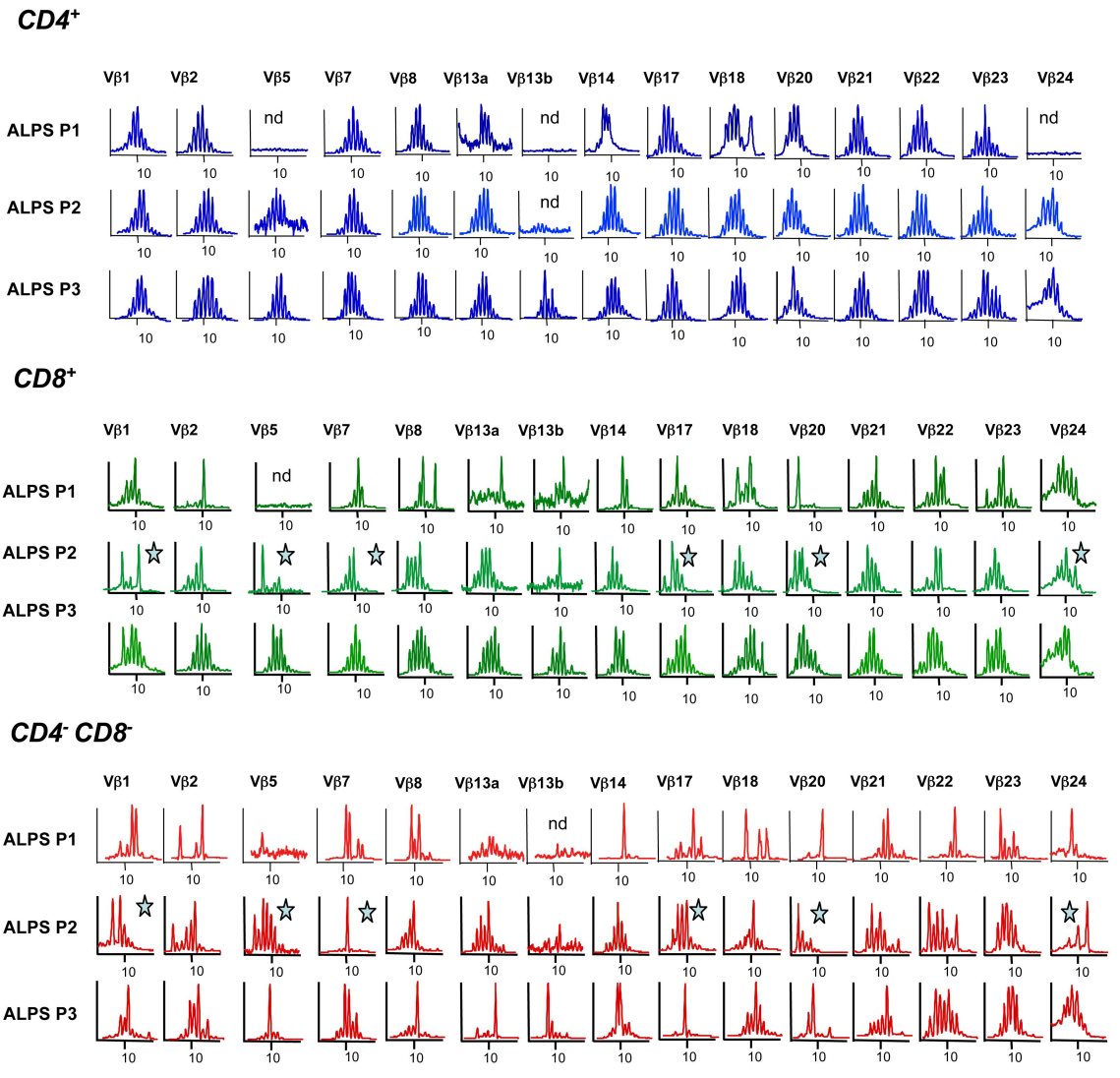
Patient No	Fas Mutation	Sex/ Age (yrs)	Age at presentat (yrs)	LA*	Spl†	Splenectomy Age (yrs)	AI‡	Serum Ig‡‡			Lympho¶ (x10³/ml)	Lymphocyte subsets¶ (%)					
								IgG	IgA	IgM		CD3	CD4	CD8	DNT	CD19	CD16+ CD56+
1	K280fs	M/32	6	++	+++	+ (12)	UR, Ar	↗↗ -> N	↗↗ -> N	N	2728	66	40	19	6	4	24
2	S214fs	M/10	0.3	+	+++	-	0	↗↗	N	N	3 400	90	24	41	20	7	2
3	S227fs	F/22	4	+	++	-	Uv, G,T	↗↗	↗	↘	1600	80	25	27	12	12	na

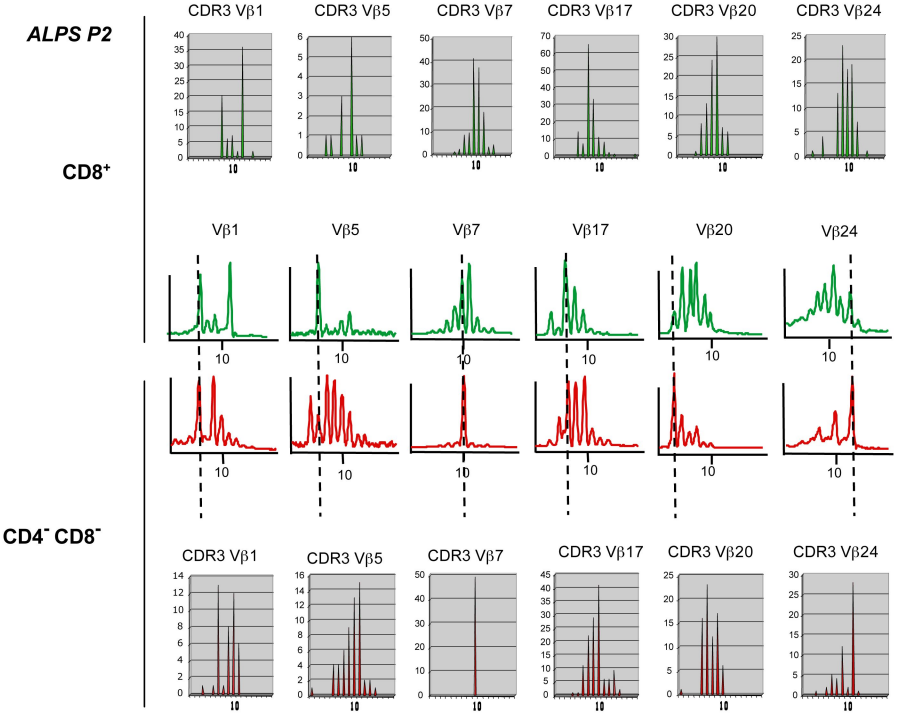
*LA :Lymph-Adenopathy + : multiple nodes, size <2 cm, ++ : multiple nodes 2 cm <size < 5cm; †Spl : Splenomegaly + : above umbilicus,, ++ : below umbilicus, +++ : palpable in iliac fossa ; ‡AI : Auto Immunity 0 : absence of autoimmune manifestation ; UR : Urticarial Rash ; Ar : Arthralgia; Uv : Uveitis; G : Glomerulopathy; T : Thrombocytopenia; ‡‡Serum Immunoglobulin level: N: within the normal range, ↗ : > +2SD <+4SD, ↗↗ :> +4SD ; ¶ Lymphocyte counts in controls are 2000-4000 $x10^3$/ml, with 70-80% $CD3^+$ cells, 40-50% CD4+ cells, 20-30% CD8+ cells, 10-20% CD19+ cells and 10-20% of CD16+CD56+ cells. ** DN T cells : % $CD4^-CD8^-$ among TCR α/β T cells (normal limit : 2%); na : not available


A
Ct
P1
P2
P3
CD4
CD8
60 1.2
2 36
75 0.4
4 20
35.6 1.7
14.1 48.6
40.3 1.3
17.2 41.2
B
CD4
CD8
DN
Ct
P2
GrzA
GrzB
7.1 0.5
92.3 0.1
18 24.5
55.6 1.9
53.5 8.9
36.5 1.1
22.5 2.2
75.2 0.1
50.8 7.3
41.8 0.1
90.2 4
5 0.1
C
CD4
CD8
DN
Ct
P2
Perforine
GrzB
3.9 0.1
45.5 0.6
3.3 22.2
69 5.4
4.9 8.1
86.7 1.4
24.9 1.2
72.5 1.2
17.8 3.1
74.4 4.7
37 2.4
59.1 1.5


A
CD8+
CD4+
Healthy Control
% Expression
30
20
10
0
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5 Vβ6a Vβ6b Vβ7 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13a Vβ13b Vβ14 Vβ15 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21 Vβ22 Vβ23 Vβ24
B
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5 Vβ6a Vβ6b Vβ7 Vβ8 Vβ9 Vβ11 Vβ12
CD4+
CD8+
Vβ13a Vβ13b Vβ14 Vβ15 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21 Vβ22 Vβ23 Vβ24
CD4+
CD8+
Fluorescence Intensity
CDR3 size


CD8+
CD4+
CD4- CD8-
ALPS P1
ALPS P2
ALPS P3
% expression
0
10
20
30
40
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5 Vβ6a Vβ6b Vβ7 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13a Vβ13b Vβ14 Vβ15 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21 Vβ22 Vβ23 Vβ24


CD4+
Vβ1
Vβ2
Vβ5
Vβ7
Vβ8
Vβ13a
Vβ13b
Vβ14
Vβ17
Vβ18
Vβ20
Vβ21
Vβ22
Vβ23
Vβ24
ALPS P1
ALPS P2
ALPS P3
nd
10
CD8+
CD4- CD8-


ALPS P2
CD8+
CD4- CD8-
CDR3 Vβ1
CDR3 Vβ5
CDR3 Vβ7
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CDR3 Vβ24
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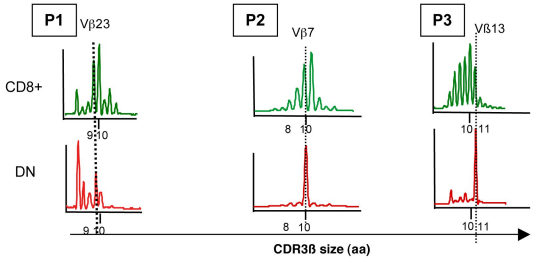
Vβ5
Vβ7
Vβ17
Vβ20
Vβ24
10



#P	Vß	CDR3	Jß		DN	CD8+
	C A S	R P G L P N P E A	F F G Q G T R L T V V E	1.1	14/233 (6%)	2/216 (0.9%)
1	23					
	TGCGCCAGC	AGACCGGGACTCCCGAACCCTGAAGCT	TTCTTTGGACAAGGCACCAGACTCACAGTTGTAGAG			
2	7 C A S	S L R Q G L S T E A	F F G Q G T R L T V V E	1.1	141/186 (76%)	12/247 (4.8%)
	TGCGCCAGC	AGCCTCAGACAGGGTCTATCGACTGAAGCT	TTCTTTGGACAAGGCACCAGACTCACAGTTGTAGAG			
	7 C A S	S Q G T G G N Q P Q	H F G D G T R L S I L E	1.5	12/186 (6.4%)	1/247 (0.4%)
	TGCGCCAGC	AGCCAAGGGACAGGGGGGAACCAGCCCCAG	CATTTTGGTGATGGGACTCGACTCTCCATCCTAGAG			
	7 C A S	S Q G N T G E L	F F G E G S R L T V L E	2.2	1/186 (0.5%)	1/247 (0.4%)
	TGCGCCAGC	AGCCAAGGGAACACCGGGGAGCTG	TTTTTTGGAGAAGGCTCTAGGCTGACCGTACTGGAG			
3	13a C A S	S S D S G G S Y N E Q	F F G P G T R L T V L E	2.1	83/156 (53%)	2/148 (1.3%)
	TGCGCCAGC	AGTTCCGATAGCGGGGGCTCCTACAATGAGCAG	TTCTTCGGGCCAGGGACACGGCTCACCGTGCTAGAG			

Table 2 : Frequency of unique CDR3 sequences

		Frequency					
Donor	Cells	Vß2	Vß8	Vß14	Vß18	Vß23	**Total**
P1	DN	14/88 (16%)	25/59 (42%)	10/72 (14%)	39/230 (17%)	36/233 (16%)	**124/682 (18%)**
	CD8+	34/80 (42%)	23/55 (42%)	21/84 (25%)	94/196 (48%)	103/216 (48%)	**275/631 (44%)**
	CD4+	66/75 (88%)	78/80 (97.5%)				**144/155 (93%)**

		Vß1	Vß5	Vß7	Vß17	Vß20	Vß24	**Total**
P2	DN	40/42 (95%)	44/57 (77%)	25/186 (13%)	87/230 (38%)	43/75 (57%)	29/55 (53%)	**268/645 (41%)**
	CD8+	45/73 (62%)	9/13 (69%)	152/247 (61%)	91/142 (64%)	50/89 (56%)	66/86 (77%)	**413/650 (63%)**

		Vß5	Vß13a	Vß13b	**Total**
P3	DN	45/94 (48%)	62/156 (40%)	56/102 (55%)	**163/352 (46%)**
	CD8+	57/69 (83%)	132/148 (89%)	35/64 (55%)	**224/281 (79%)**