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## 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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► **To cite this version:**

Anne Bristeau-Leprince, Véronique Mateo, Annick Lim, Aude Magerus-Chatinet, Eric Solary, et al.. 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.. *Journal of Immunology*, 2008, 181 (1), pp.440-8. 10.4049/jimmunol.181.1.440 . pasteur-00293060

**HAL Id: pasteur-00293060**

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Submitted on 3 Jan 2009

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**Human TCR  $\alpha/\beta^+$  CD4<sup>-</sup>CD8<sup>-</sup> double-negative T cells in patients with autoimmune lymphoproliferative syndrome (ALPS) express restricted V $\beta$  TCR diversity and are clonally related to CD8<sup>+</sup> T cells<sup>1</sup>**

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

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**Keywords** : Human, T cells, Autoimmunity, Immunodeficiency, Repertoire development

## **Abstract**

The peripheral expansion of  $\alpha/\beta^+$ -CD4<sup>-</sup>CD8<sup>-</sup> 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 TCRV $\beta$  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 V $\beta$  gene families that are used by their SP counterparts, though they dominantly use some V $\beta$  genes. Analysis of CDR3 length distribution revealed a diverse polyclonal TCR repertoire for sorted CD4<sup>+</sup> T cells, whereas both DN and CD8<sup>+</sup> T cells showed a skewed TCR repertoire with oligoclonal expansions throughout most of the V $\beta$  families. CDR3 sequencing of matching clonotypes revealed a significant sharing of CDR3 sequences from selected V $\beta$ -J $\beta$  transcripts between DN and CD8<sup>+</sup> T cells. Altogether, these data strongly argue for a CD8 origin of DN T cells in ALPS.

## Introduction

The autoimmune lymphoproliferative syndrome (ALPS)<sup>3</sup> (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)- $\alpha/\beta^+$  CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> 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  $\alpha\beta$  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 V $\beta$  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 TCR $\alpha\beta$ ) purchased from Becton Dickinson. DN T cells (CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>) were identified as shown in Fig. 1A. Sorting of DN T CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells from the three patients was performed with a FACSstar with 98% purity. Sorted DN T cells therefore contain a majority of  $\alpha\beta$ T cells. Possible presence of  $\gamma\delta$  T cells within sorted DN T cells does not skew the data since repertoire analyses were performed with V $\beta$ -specific primers.

### *Determination of V $\beta$ gene usage and Immunoscope analysis*

Determination of V $\beta$  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 0.5 $\mu$ g/ $\mu$ l 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 V $\beta$  family-specific primers, together with a TCR C $\beta$  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 C $\beta$  segment. In this reaction, all PCR products were copied into fluorescently labeled single-stranded DNA fragments, irrespective of their TCR J $\beta$  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 V $\beta$  repertoire.

#### *DNA cloning and sequencing analysis of TCR transcripts*

V $\beta$ /C $\beta$  PCR were carried out on cDNA with 5 U Pfu polymerase (Stratagene) for 25 cycles. PCR products were then cloned in pCR $\text{\textcircled{R}}$ 4Blunt-TOPO vector (Invitrogen Life Technologies). For sequencing purposes, PCR was carried out directly on LacZ $\text{\textcircled{r}}$  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  $\alpha/\beta$  T cells (Fig. 1A). 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 $^{\text{high}}$ , CD2 $^+$ , CD5 $^+$ , CD27 $^{++}$ , CD28 $^+$ , CD45RA $^+$ RO $^-$  CD31 $^+$ , CD62L $^{\text{dull}}$ , 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. 1B, 1C). 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. 1B, 1C). 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 V $\beta$ -gene usage of  $\alpha\beta$  DN T cells from ALPS patients quantitatively differs from that of SP cells*

The expression of 24 V $\beta$  gene families was examined by RT-PCR amplification of cDNA derived from sorted subpopulations. Representative data are shown for a control donor (Fig. 2A) 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 V $\beta$ -gene families that were used by CD4<sup>+</sup> and CD8<sup>+</sup> SP cells (Fig. 3). However, quantitative differences in the expression of several V $\beta$ s were apparent in each of the three patients, with preferential expression of some V $\beta$ s by DN T cells. For example, V $\beta$ 2 and V $\beta$ 7 families were preferentially expressed in DN T cells from P1 (V $\beta$ 2: 25% in DN T cells vs 12% and 8% in SP CD8 and CD4 SP T cells respectively; V $\beta$ 7: 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 V $\beta$ 17 and V $\beta$ 7 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 V $\beta$  repertoire, in contrast to CD4 T cells*

Analysis of CDR3 length distribution in sorted CD4 T cells from ALPS patients showed for all V $\beta$ -C $\beta$  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 V $\beta$  families (Fig. 4), such as V $\beta$ 2, V $\beta$ 8, V $\beta$ 13a, V $\beta$ 14, V $\beta$ 17, V $\beta$ 18, and V $\beta$ 20 for P1, V $\beta$ 1, V $\beta$ 2, V $\beta$ 5, V $\beta$ 8, V $\beta$ 13b, V $\beta$ 17, V $\beta$ 22, for P2, and V $\beta$ 1 and V $\beta$ 13b 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 V $\beta$  families (eg V $\beta$ 2, V $\beta$ 7, V $\beta$ 13a, V $\beta$ 13b, V $\beta$ 14, V $\beta$ 20 for P1, V $\beta$ 1, V $\beta$ 5 and V $\beta$ 22 for P2) while others showed more diverse patterns with specific

T cell expansions (for example VB1, VB21, VB22 and VB23 for P1, VB2, VB8, VB13a, VB22 for P2, VB1, VB13b, VB14, VB18 for P3 (Fig. 4). Oligoclonality of both CD8 SP and DN T cells was confirmed by sequence analysis selecting within V $\beta$  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 V $\beta$  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 VB2 TCR, 10 out of 72 VB14 TCR, 39 out of 230 VB18 TCR and 36 out of 233 VB23 TCR were identified. A total of 682 CDR3 sequences were generated from selected transcripts, among which only 124 were unique (18%), confirming V $\beta$  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 VB2 TCR and 78 out of 80 VB8 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 VB2 TCR, 21 out of 84 VB14 TCR, 94 out of 196 VB18 TCR and 103 out of 216 VB23 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. VB7 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 V $\beta$  and J $\beta$  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 V $\beta$  repertoire. For example, the V $\beta$ 7 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 V $\beta$ 13 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  $\alpha/\beta$ -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.  $ki67^+$ ,  $CD45RO^+CD45RA^+$  (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.  $TCR^{high}$ ,  $CD2^+$ ,  $CD5^+$ ,  $CD27^{++}$ ,  $CD28^+$ ,  $CD45RA^+RO^-$   $CD31^+$ ,  $CD62L^{dull}$ ,  $CXCR-5^-$ ,  $CD57^+$ ,  $CD11b^{16}$ . The uniform phenotype of  $\alpha/\beta$ -DN T cells from ALPS patients is similar to  $\alpha/\beta$ -DN T cells from mice with defective Fas (*lpr*) (7,16,28) and different from the minor  $\alpha/\beta$ -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 V $\beta$  repertoire diversity of sorted DN T cells and SP CD4 and CD8 T cells, and we performed sequence analyses of the CDR3 region within V $\beta$  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 V $\beta$  families, arguing for the CD8 origin of DN T cells.

V $\beta$  gene family usage was examined by quantitative RT-PCR amplification of cDNA derived from sorted DN and SP T cells. Though the V $\beta$ -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 V $\beta$  gene families that were used by the SP counterparts, preferential expression of some V $\beta$  was found in DN T cells as compared to autologous SP T cells. However, V $\beta$  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 V $\beta$ -C $\beta$  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-V $\beta$  Immunoscope profiles, with oligoclonal or monoclonal expansions throughout most of the V $\beta$  families, indicating a contracted TCR-V $\beta$  repertoire. Oligoclonality of both SP CD8 and DN T cells was confirmed by sequence analysis selecting, within V $\beta$  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 V $\beta$ -J $\beta$  transcripts. For example, the V $\beta$ 7 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 V $\beta$ 13 clonotype in P3, or V $\beta$ 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 V $\beta$  CDR3 sequences within several V $\beta$  families. Differences in V $\beta$  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 V $\beta$  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.

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## Footnotes

<sup>1</sup>This work was supported by Institutional grants from Institut Pasteur and Institut National de la Santé et de la Recherche Médicale (INSERM), grants from the Agence Nationale de la Recherche (ANR#05-MRAR-017), the Association pour la Recherche contre le Cancer (ARC), la Ligue Nationale contre le Cancer, la Ligue Parisienne contre le Cancer, and the European Union (6<sup>th</sup> FP STREP Autorome Consortium, “From Immune Responses in Rare Autoimmune Diseases to novel Therapeutic Intervention Strategies », LSHM-CT-2004-005264). ABL was supported by EU Autorome post-doctoral grant, VM was supported by a post-doctoral fellowship from European Molecular Biology Organization (EMBO), and AM was supported by a post-doctoral fellowship from ARC.

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<sup>3</sup> **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 TCR $\alpha\beta$ , CD3, CD4 and CD8 expression. Cells were next stained for GrzA, GrzB and perforin content.

A: surface CD4 and CD8 co-expression on gated TCR $\alpha\beta^+$  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 V $\beta$  repertoire analysis and Immunoscope profiles of SP T cells from a control donor.*

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

B: Immunoscope profiles of TCR V $\beta$  analysis on the same sorted SP T cells. Profiles for the 24 V $\beta$  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 V $\beta$  repertoire analysis on SP and DN T cells from ALPS patients.*

Quantitative V $\beta$  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 V $\beta$  families and the y axis their relative frequency of usage. Arrows point out the expansion of some V $\beta$  families detected in DN T cells as compared to SP T cells from the same individual.

**Figure 4.** *Immunoscope profiles of TCR V $\beta$  analysis of SP and DN T cells from ALPS patients.* The diversity of TCRV $\beta$  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 V $\beta$  families, indicative of a diverse repertoire, as observed in control donors (Fig. 2). Immunoscope profiles of CD8<sup>+</sup> and DN T cells are oligoclonal in most of the V $\beta$  families, indicative of a skewed restricted repertoire. **V $\beta$  families targeted for further CDR3 sequence analysis are pointed out with a star.**

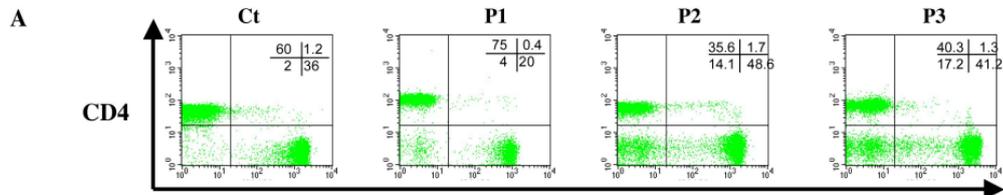
**Figure 5.** *Oligoclonality of both CD8<sup>+</sup> T cells and DN T cells for indicated V $\beta$  families confirmed by sequence analysis,* as shown in the upper part of the figure for each cell subset. For a given V $\beta$ , the number of sequences obtained for each CDR3 length was representative 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 V $\beta$ 1, 5, 7, 17, 20 24 families, as shown by the hatched lines.

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

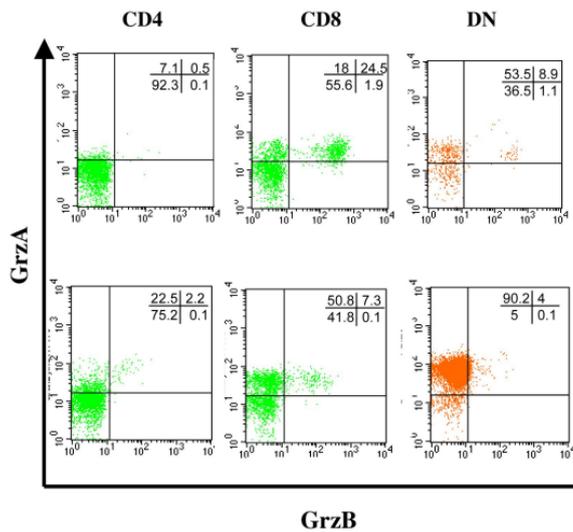
**Table 1 : Patients' clinical and immunological characteristics**

Patient No	Fas Mutation	Sex/ Age (yrs)	Age at presentat (yrs)	LA *	Spl <sup>†</sup>	Splenectomy Age (yrs)	AI <sup>‡</sup>	Serum Ig <sup>‡‡</sup>			Lympho <sup>¶</sup> (x10 <sup>3</sup> /ml)	Lymphocyte subsets <sup>¶</sup> (%)					
								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

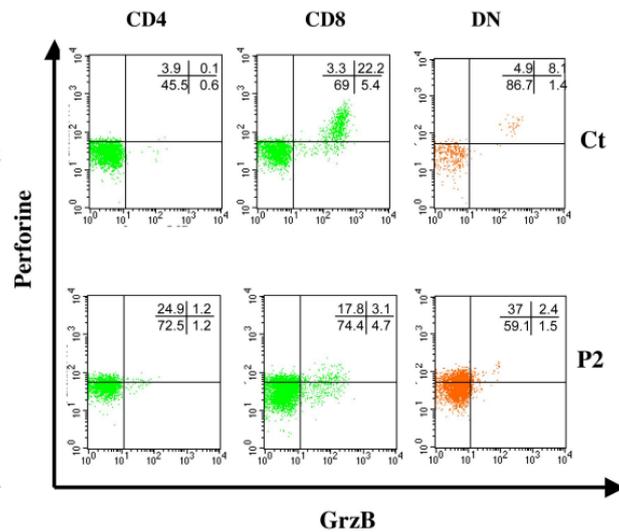
\*LA :Lymph-Adenopathy + : multiple nodes, size <2 cm, ++ : multiple nodes 2 cm <size < 5cm; †Spl : Splenomegaly + : above umbilicus,, ++ : below umbilicus, +++ : palpable in iliac fossa ; ‡AI : Auto Immunity 0 : absence of autoimmune manifestation ; UR : Urticarial Rash ; Ar : Arthralgia; Uv : Uveitis;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

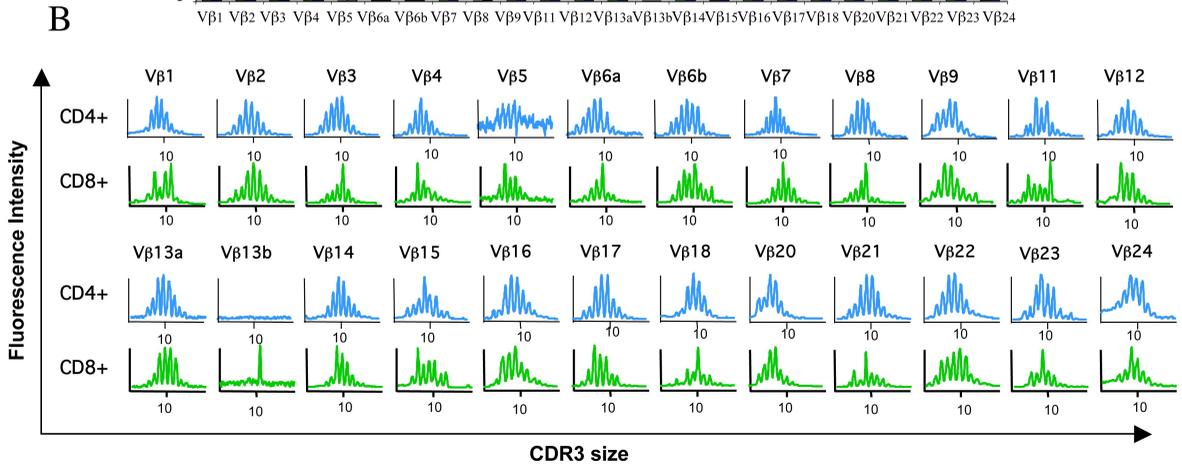
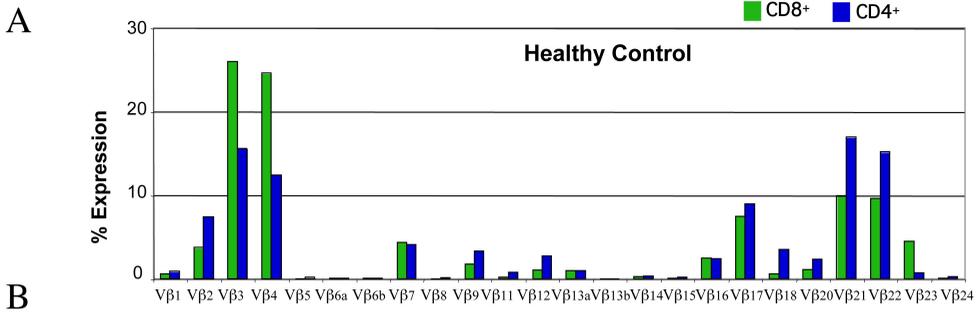


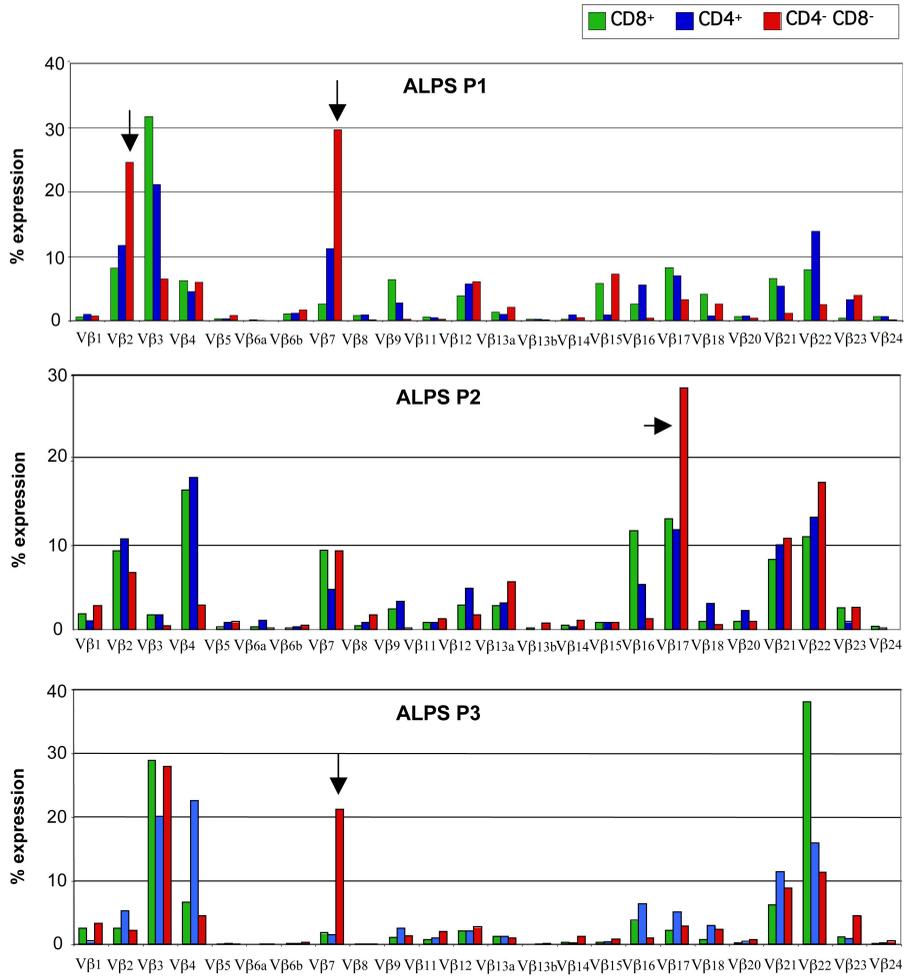
**B**



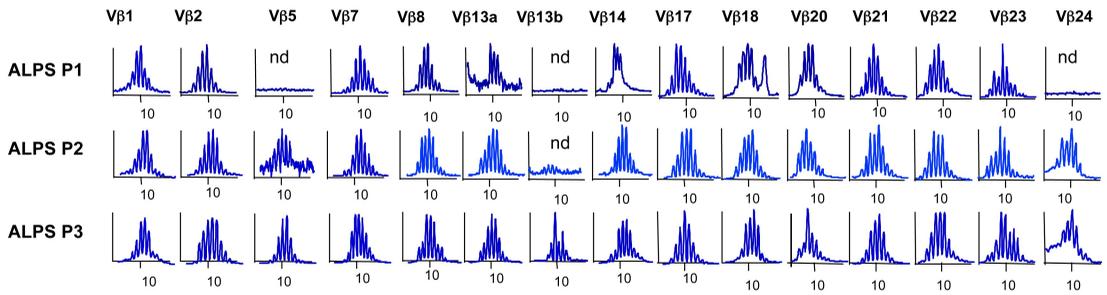
**C**



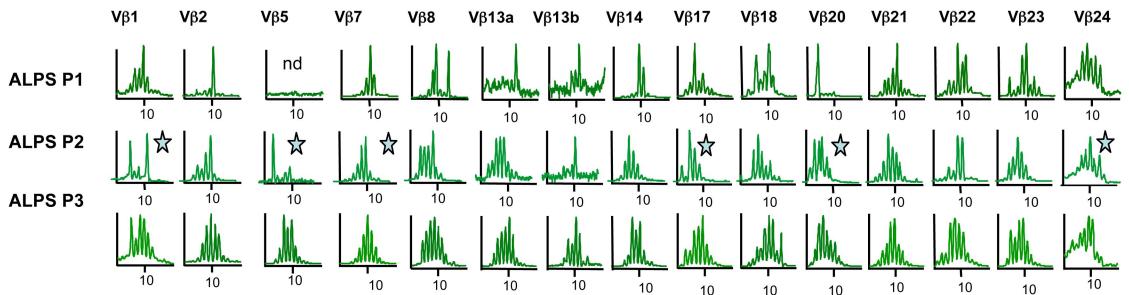




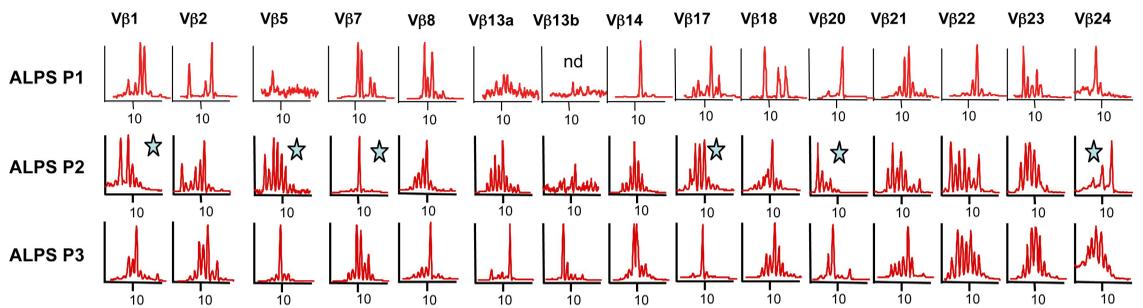
### CD4<sup>+</sup>



### CD8<sup>+</sup>

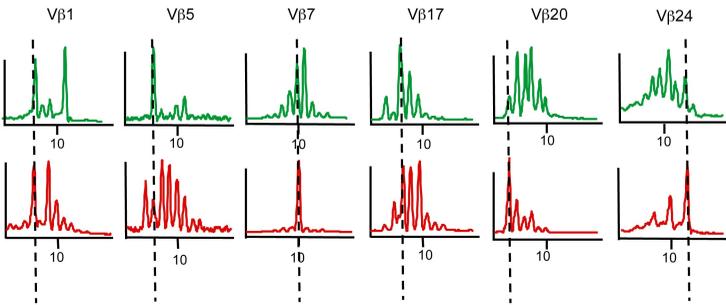
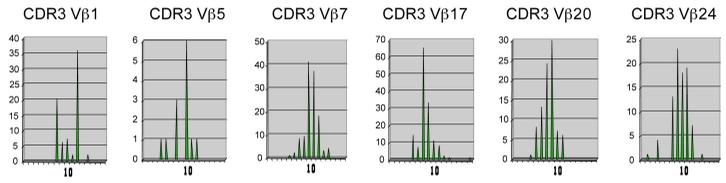


### CD4<sup>-</sup> CD8<sup>-</sup>

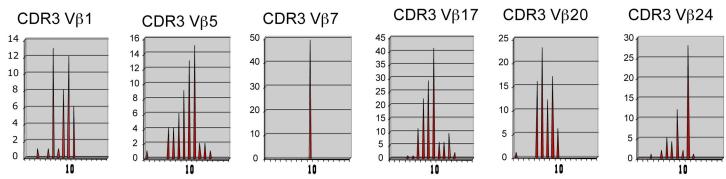


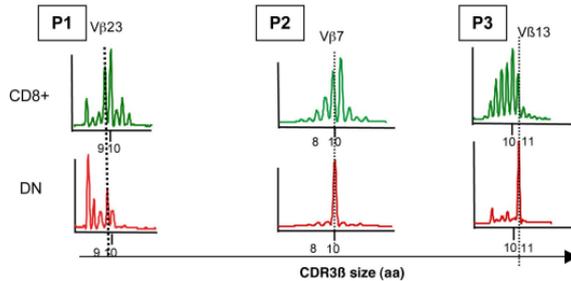
**ALPS P2**

**CD8<sup>+</sup>**



**CD4<sup>+</sup> CD8<sup>-</sup>**





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

**Table 2 : Frequency of unique CDR3 sequences**

Donor Cells		Frequency					Total	
		Vβ2	Vβ8	Vβ14	Vβ18	Vβ23		
P1	DN	14/88 (16%)	25/59 (42%)	10/72 (14%)	39/230 (17%)	36/233 (16%)	<b>124/682 (18%)</b>	
	CD8+	34/80 (42%)	23/55 (42%)	21/84 (25%)	94/196 (48%)	103/216 (48%)	<b>275/631 (44%)</b>	
	CD4+	66/75 (88%)	78/80 (97.5%)				<b>144/155 (93%)</b>	
		Vβ1	Vβ5	Vβ7	Vβ17	Vβ20	Vβ24	Total
P2	DN	40/42 (95%)	44/57 (77%)	25/186 (13%)	87/230 (38%)	43/75 (57%)	29/55 (53%)	<b>268/645 (41%)</b>
	CD8+	45/73 (62%)	9/13 (69%)	152/247 (61%)	91/142 (64%)	50/89 (56%)	66/86 (77%)	<b>413/650 (63%)</b>
		Vβ5	Vβ13a	Vβ13b	Total			
P3	DN	45/94 (48%)	62/156 (40%)	56/102 (55%)	<b>163/352 (46%)</b>			
	CD8+	57/69 (83%)	132/148 (89%)	35/64 (55%)	<b>224/281 (79%)</b>			