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PROMIDIS^α: a TCR^α signature associated with immunodeficiencies caused by V(D)J recombination defects

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CAPSULE SUMMARY

Immunodeficient patients with suboptimal V(D)J recombination activity generate a T cell repertoire that is biased in the TRAV/TRAJ usage. This bias led to the development of a robust biomarker, PROMIDIS α , which helps in the diagnosis of these patient at early stages.

CLINICAL IMPLICATIONS STATEMENT

- Suboptimal V(D)J recombination activity results in bias in TCR α usage.
- PROMIDIS α reveals specific TCR α signatures in patients with some Combined Immune Deficiency (CID), Ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS).
- PROMIDIS α may help identify patients with hypomorphic DNA repair deficiency.

ABSTRACT

- **Background:** V(D)J recombination ensures the diversity of the adaptive immune system. While its complete defect causes Severe Combined Immunodeficiency (T-B-SCID), its suboptimal activity, is associated with a broad spectrum of immune manifestations such as late onset combined immunodeficiency and autoimmunity. The earliest molecular diagnosis of these patients is required to adopt the best therapy strategy, in particular when it involves myelo-ablative conditioning regimen for hematopoietic stem cell transplantation (HSCT).
- **Objective:** We aimed at developing biomarkers based on the analysis of TCR α repertoire to assist in the diagnosis of primary immunodeficient patients (PID) with V(D)J recombination and DNA repair deficiencies.
- **Methods:** We used flow cytometry (FACS) analysis to quantify TCR-V α 7.2 expressing T lymphocytes in peripheral blood and developed PROMIDIS α , a multiplex RT-PCR/NGS assay, to evaluate a subset of the TCR α repertoire in T lymphocytes.
- **Results:** The combined FACS and PROMIDIS α analyses revealed specific signatures in patients with V(D)J recombination defective PIDs or Ataxia telangiectasia/Nijmegen breakage syndromes (AT/NBS).
- **Conclusion:** Analysis of the TCR α repertoire is particularly appropriate, in a prospective way, to identify patients with partial immune defects caused by suboptimal V(D)J recombination activity and/or DNA repair defect. It also constitutes a valuable tool for the retrospective *in vivo* functional validation of variants identified through exome or panel sequencing. Its broader implementation may be of interest to assist early diagnosis of patients presenting with hypomorphic DNA repair defects inclined to develop acute toxicity during pre-HSCT conditioning.

Key words: Primary immunodeficiency, V(D)J recombination, TCR α repertoire, Ataxia Telangiectasia, DNA repair, NGS.

Abbreviations:

TCR: T cell receptor
RAG: Recombination activating gene
DSB: DNA double strand break
SCID: Severe Combined Immunodeficiency
NBS: Nijmegen breakage syndrome
NBN: Nibrin
CVID: Common Variable Immunodeficiency
CID-G/A: Combined immunodeficiency with granuloma or autoimmunity
AT: Ataxia Telangiectasia
ATM: Ataxia Telangiectasia Mutated
XLF: XRCC4 like factor
NGS: Next Generation Sequencing
TRAV: T cell receptor alpha Variable gene
TRAJ: T cell receptor alpha Joining gene
SMARTtm□□switching mechanism at the 5'□end of the RNA transcript
PID: Primary immunodeficiency
PBL: Peripheral blood lymphocytes
PCA: Principal component analysis
HC: hierarchical clustering
MAIT: mucosal associated invariant T lymphocytes
CID: combined immunodeficiency disease
DCLRE1C: DNA Cross-Link Repair 1C/Artemis
PRKDC: Protein Kinase, DNA-Activated, Catalytic Polypeptide
RORC: RAR Related Orphan Receptor C
cDNA: complementary DNA
PGM: Personal Genome Machine
NHEJ: Non-Homologous DNA End Joining
HSCT: hematopoietic stem cell transplantation
FACS: Flow cytometry
RT-PCR: Reverse transcription-polymerase chain reaction
Ig: Immunoglobulin
OS: Omenn syndrome
APC: Allophycocyanin
PE: Phycoerythrin
EDTA: ethylenediamine tetra acetic acid
5'RACE: 5' Rapid amplification of cDNA ends
 δ Rec: TCR δ Recombination element
ROR γ T: RAR-related orphan receptor gamma T

HIV: human immunodeficiency virus

INTRODUCTION

The adaptive immune system is composed of B and T lymphocytes that express immune receptors, immunoglobulins (Ig) and T cell receptor (TCR) respectively, presenting a diversity allowing facing any antigens. This plasticity is ensured by the V(D)J recombination, a somatic DNA rearrangement process, which results in the combinatorial association of previously dispersed Variable (V), Diversity (D), and Joining (J) gene segments forming the immune recognition moiety of Igs and TCRs¹. Briefly, V(D)J recombination is initiated by the lymphoid specific factors encoded by *Recombination activating genes 1 and 2* (*RAG1/2*) through the introduction of a DNA double strand break (DSB) at the border of the rearranging segments (see Schatz et al. for review²). The DSB is then processed and repaired by the ubiquitously expressed DNA repair machinery of the cell, based on the Non Homologous DNA End joining (NHEJ) pathway (see Alt et al. for review³).

Ever since the cloning of the genes coding for the RAG1/2 factors, generation of deficient animal models and analyses of patients with Severe Combined Immune Deficiency (SCID) lacking T and B lymphocytes (T-B-NK+ SCID) clearly established the absolute requirement of these factors, and the V(D)J recombination machinery as a whole, for the proper maturation of the adaptive immune cells⁴. Nevertheless, a broad spectrum of immunodeficiencies was subsequently described associated with hypomorphic mutations in *RAG1/2* genes that spare some V(D)J recombination activity (for a recent review see Notarangelo et al.⁵). The phenotype of these patients extend from the very severe Omenn syndrome (OS) condition, characterized by an oligoclonal expansion of activated T lymphocytes that infiltrate the gut and skin, to milder conditions such as late-onset combined immune deficiency with granuloma and/or various autoimmune manifestations (CID-G/A).

The clinical severity appears as a function of the RAG residual activity, and hence, the extend of TCR repertoire diversity in these patients^{6, 7}. RAG deficiency was also identified as a possible cause of agammaglobulinemia, isolated T CD4 lymphopenia, common variable immunodeficiency (CVID), isolated IgA deficiency, hyper-IgM syndrome, and even sterile chronic multifocal osteomyelitis⁵. Beside mutations in *RAG* genes, defects in the DNA repair phase of the V(D)J recombination alter the generation of a diversified adaptive immune system. Consequences can be severe, ranging from T-B-NK+ SCID when associated with null mutations in *Artemis* or *Cernunnos* genes to almost asymptomatic in some cases of hypomorphic mutations in *DNA Ligase IV* for example⁴. Various degrees of immunodeficiency are also associated with other DNA repair defect conditions such as AT with mutations in the *Ataxia Telangiectasia Mutated (ATM)* gene⁸ or NBS harboring mutations in the *nibrin (NBN)* encoding gene⁹, the formal diagnosis of which is often difficult in young children, in the absence of fully developed clinical signs of the disease. It is thus crucial to benefit from robust experimental tools to help distinguish the various possible molecular causes underlying these immune deregulated conditions. The early diagnosis of DNA repair defects is also of critical importance as HSCT is often the only curative treatment and as these patients may suffer from acute toxicity following administration of DNA damage inducing drugs used for conditioning regimen¹⁰.

Based on our prior observations of a bias in the TCR α repertoire in patients with *Cernunnos* mutations and *XRCC4 like factor (Xlf)* KO mice¹¹, we aimed at developing biomarkers that would help in the diagnosis of suboptimal V(D)J recombination conditions as well as DNA repair defects. We herein present the combination of FACS analysis and PROMIDIS α , a RT-PCR/NGS based resource, to analyze a subset of the TCR α repertoire that distinctly reveals patients with V(D)J recombination anomalies and proved very robust in the early diagnosis of AT and NBS conditions.

METHODS

Patients and controls

For V α 7.2 analysis by FACS, healthy controls cohort was studied between January 2017 to March 2017. It included 16 children aged 0 to 18 years with no suspicion of PID or immunosuppressive treatment and 17 healthy adult individuals (aged 22 to 52 years). The healthy children were referred to the outpatient clinic of Necker-Enfants Malades Hospital, Paris, France, for diagnostic blood testing. The Immunodeficiency cohort analyzed by FACS consisted of 92 pediatric and adult patients assessed for the follow-up or the screening of a primary immunodeficiency or a common variable immunodeficiency. The AT cohort analyzed by PROMIDIS α consisted of 37 patients followed at the Necker Hospital. The genetic status of VDJ recombination deficient (VDJ) and AT patients enrolled in the PROMIDIS α analysis is presented in Suppl. Table 1. Peripheral venous blood samples were collected into ethylenediamine tetra acetic acid (EDTA) and processed within 24 hours. All genetic studies were performed after retrieving written consent from the patient or for minors from their legal guardian. The study was performed in accordance with the modified version of the Helsinki Declaration.

Whole TCR α repertoire determination by SMARTTM.

Determination of comprehensive TCR α repertoire by 5' Rapid amplification of complementary DNA (cDNA) ends (5'RACE) PCR/NGS (switching mechanism at the 5' end of the RNA transcript, SMART α) was performed, for the first time, on peripheral blood lymphocytes (PBL) from a DNA-LigaseIV¹² and a Cernunnos deficient¹³ patients using Clontech's SMARTTM technology¹⁴ as previously described¹¹. Sequencing on the Personal Genome Machine (PGM, IonTorrent) was performed according to manufacturer's

recommendations. Sequencing data were analyzed with LymAnalyzer¹⁵ to retrieve unique CDR3 clonotypes and determine T cells receptor alpha variable (TRAV) and T cell receptor alpha junction (TRAJ) gene segments. Output LymAnalyzer files were transformed with R to generate FlowJo9 compatible files with genomic coordinates of the various TRAV and TRAJ pairs. TRAV and TRAJ usage were determined using FlowJo (see Fig. 1). Gates were applied to score transcripts using either proximal (TRAV22-41/TRAJ61-J48) or distal (TRAV1-11/TRAJ17-1) TRAVJ combinations.

TRAV1 (V α 7.2) determination by flow cytometry.

For screening by FACS, 100 μ L of whole blood was incubated for 30 min at 4°C with 5 μ L of both indicated fluorochrome-labeled monoclonal antibodies: Allophycocyanin (APC) anti-CD3 (HIT3a, Sony) and Phycoerythrin (PE) anti-TCR V α 7.2 (3C10, Sony). After red cells lysis (FACS Lysing Solution, BD) and washing remaining cells twice with a cell wash buffer (BD), cells were fixed in a cell fixation solution (BD). CD3-positive cells were analyzed by flow cytometry within 24 hours (FACS Canto II, BD). Expression of TCR V α 7.2 on CD3-positive lymphocytes was assessed using FlowJo software (Tree Star Inc).

Sampling of TCR α repertoire by PROMIDIS α .

cDNA from PBL were amplified with a series of 8 primers specific for Hu-TRAV segments representing proximal (TRAV35, TRAV41), middle (TRAV20, TRAV21, TRAV23), and distal (TRAV1, TRAV5, TRAV10) TRAV segments together with TRAC3-REPF, a C α specific primer¹⁶. Primers include an anchor sequence (RepF and RepR) for subsequent NGS sequencing on the PGM. PCR bands were gel-purified and re-amplified using PGM sequencing primers A_BCx_REP_F 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGXXXXXXXXXXXXCGCCCCATTGACGCAA-3' where

xxx represent the barcode (BCx) sequence and P1_REP_R 5'-CCTCTCTATGGGCAGTCGGTGATCCCTCGCCGGACACGCTGAA-3'.

TRAV1 -RepR	ccctcgccggacacgctgaaAGGTCGTTTTTCTTCATTCCTTAGTC
TRAV5 -RepR	ccctcgccggacacgctgaaAAACAAGACCAAAGACTCACTGTTC
TRAV10-RepR	ccctcgccggacacgctgaaTACAGCAACTCTGGATGCAGACAC
TRAV20-RepR	ccctcgccggacacgctgaaGCCACATTAACAAAGAAGGAAAGCT
TRAV21-RepR	ccctcgccggacacgctgaaGCCTCGCTGGATAAATCATCAGGA
TRAV23-RepR	ccctcgccggacacgctgaaCACAATCTCCTTCAATAAAAGTGCCA
TRAV35-RepR	ccctcgccggacacgctgaaCTCAGTTTGGTATAACCAGAAAGGA
TRAV41-RepR	ccctcgccggacacgctgaaGATTAATTGCCACAATAAACATACAGG
TRAC3-RepF	cgccccattgacgcaaaGTCACTGGATTTAGAGTCTCTCAG

Sequencing on the PGM was performed according to manufacturer's recommendations, the metrics of which are presented in supl. Figure 1. Raw sequencing data were analyzed as for SMARTα. TRAV and TRAJ usage was quantified using FlowJo (see Fig. 3A). 9 parameters were extracted: frequencies of Vprox (TRAV35, 41), Vmid (TRAV20, 21, 23), Vdist (TRAV1, 5, 10), and frequencies of Jprox (TRAJ61-48) and Jdist (TRAJ17-1) within each TRAV categories. Principal component analysis (PCA) and hierarchical clustering (HC) were performed on these 9 parameters using the *PCA()* and *HCPC()* functions of the FactomineR package respectively [<http://factominer.free.fr/>¹⁷] and graphics were generated using the Factoextra R package [<http://www.sthda.com/english/rpkgs/factoextra>¹⁸]. Statistical analyses covering the 9 parameters were performed using Hotelling's T-square test (Real Statistics Resource Pack for Excel, (Release 4.3). Copyright (2013–2015) Charles Zaiontz, www.real-statistics.com). One-parameter representations were performed using PRISM6, with Statistical analysis performed using Mann-Whitney non-parametric t test.

RESULTS

TCR α repertoire bias in DNA repair deficient patients

One singularity of the TCR α locus, over the other TCR and Ig genes, consists of its multiple waves somatic rearrangement process. It initiates with the particular δ Rec/ Ψ j α recombination event, which associates the non-coding TCR δ Recombination element (δ Rec) upstream of the TCR δ locus to the most 5' J α segment (TRAJ61 or Ψ j α)¹⁹. Subsequent waves of recombination associate more upstream TRAV segments to more downstream TRAJ elements, until thymocyte positive selection arrests the process by down regulating RAG1/2 expression²⁰. This mechanism has two practical implications. First, a correlation exists between TRAV and TRAJ elements present in TCR α transcripts, i.e proximal 3' TRAV elements being preferentially associated with proximal 5' TRAJs and conversely, distal 5' TRAVs with distal 3' TRAJs²¹. Second, TCR α repertoire diversity is linked to the thymocyte lifespan. The TCR α repertoire is biased towards usage of more distal 5'TRAV/3'TRAJ when thymocyte lifespan is artificially increased by expression of a Bcl-x(L) transgene²². Conversely, reducing thymocyte lifespan (such as in RAR-related orphan receptor gamma T (ROR γ T) KO mice²²) results in a TCR α repertoire biased towards more proximal 3'TRAV/5'TRAJ associations. We previously noticed that T lymphocytes from XLF KO mice displayed a biased TCR α repertoire towards proximal TRAV/TRAJ recombination events, consistent with the reduced viability of their thymocytes as judged by their increased *ex vivo* apoptosis¹¹. We extended this observation in humans, by analyzing characteristics of T cells from two immunodeficient patients with *Cernunnos/XLF* and *DNA-Ligase IV* mutations respectively. As shown in Fig.1, the whole TCR α repertoire analyzed by SMART α was biased in these two patients since TCR α transcripts associating proximal TRAV and TRAJ elements (lower left quadrant) accounted for 44.6% and 38.8%

respectively, compared to 13.2% in a healthy control. Conversely, TCR α transcripts with distal TRAV/TRAJ (upper right quadrant) were barely detectable in these two patients (0.09% and 1.75% respectively compared to 11% in the healthy control individual). In light of these observations we considered that analyzing TCR α repertoire could indeed represent a valuable biomarker to assist the diagnosis of various pathologies affecting the development of the immune system.

Diagnosis of hypomorphic V(D)J recombination deficiencies through TCR-V α 7.2 determination by FACS

The easiest way to analyze the TCR α repertoire on a large series of patients would be to score TRAV expression by FACS. Unfortunately, very few anti-TRAV specific antibodies are available, and none to track proximal TRAV usage. Nevertheless, one antibody, 3C10, is directed towards TCR-V α 7.2, a TRAV segment expressed by human mucosal associated invariant T lymphocytes (MAIT)²³ and which represents the most distal (upstream) TCR α segment TRAV1. We used this antibody to analyze 92 blood samples from patients assessed for the screening or the follow-up of immunodeficiencies by FACS, excluding cases of “classical” T-B-NK+SCID (Fig. 2B). This series included 11 patients with PID related to V(D)J recombination deficiency (red category in Fig. 2B, Table1), 14 patients with AT (orange category in Fig. 2B) and 67 cases with various spectrum of PIDs. The frequency of TCR-V α 7.2 expressing CD3+ T lymphocytes was highly heterogeneous, with means ranging from 0.02 to 3.5%. Among the various categories of immune deficiencies, only the combined immune deficiency with V(D)J recombination defects (CID-VDJ) and the AT patients had a frequency of V α 7.2 expressing T cells statistically reduced (0.02% and 1.00% respectively, $p < 0.0001$) as compared to pediatric controls (grey dots in Fig. 2B, 4.31%). The frequency of TCR-V α 7.2 expressing T cells did not vary with age (Fig. 2C) since the difference between

mean values of pediatric and adult healthy controls was not significant ($4.31\pm 2.98\%$ and $3.80\pm 2.70\%$ respectively, $p=0.57$). The 11 patients with CID-VDJ (red category in Fig. 2B) were almost completely devoid of TCR-V α 7.2 expressing T cells (mean 0.02%), a situation never encountered in controls or in other tested patients. These patients ranged from newborn to 34 years of age and presented with combined CID mostly characterized by decreased numbers of both T and B lymphocytes, often associated with a sharp decrease in CD45RA⁺CD31⁺/CD4⁺ and CD8⁺ naïve T cells (Table1). These 11 patients harbored deleterious bi-allelic mutations in genes involved in the V(D)J recombination (*RAG1* (n=8), *DNA Cross-Link Repair 1C* (*DCLRE1C/Artemis*) (n=2), and *Protein Kinase, DNA-Activated, Catalytic Polypeptide* (*PRKDC*) (n=1)). For 5 of them the molecular defect was known prior to V α 7.2 analysis (R) while for the reminder 6, the absence of V α 7.2 expressing T cells indeed oriented the sequencing effort towards the V(D)J recombination candidate genes in a prospective manner (P). Importantly, apart from these 11 cases, none of the other 81 patients carried mutations in any of the classical V(D)J recombination associated genes. This first series of analysis thus demonstrated the usefulness of examining part of the TCR α repertoire as a tool to help the diagnosis of hypomorphic V(D)J recombination deficiencies.


We next extended this V α 7.2 FACS survey to a group of 14 patients with AT given the known implication of the DNA repair factor ATM to both cell survival and V(D)J recombination²⁴. The frequency of V α 7.2 expressing T cells was severely reduced in the AT group ($1.00\pm 0.63\%$ compared to $4.31\pm 2.98\%$ in pediatric controls) (orange category in Fig. 2B), a decrease that was highly statistically significant ($p<0.0001$). However, although the V α 7.2 frequency is clearly informative at the population level, it cannot be satisfactorily used as to assist diagnosis at the individual level given the overlap between V α 7.2 values observed in AT patients and controls. Several studies have shown that MAIT lymphocytes, which can be distinguished from conventional TCR-V α 7.2 positive T cells by their specific

expression of the cell surface marker CD161, account for the vast majority of total V α 7.2 positive T cells in peripheral blood of healthy individuals²⁵⁻²⁷. However, their frequency is highly fluctuant and is severely reduced in various conditions of immune deregulation such as human immunodeficiency virus (HIV) infection or severe bacterial infections²⁵⁻²⁷. As a consequence, the total number of V α 7.2 expressing lymphocytes decreases considerably in these situations. This may contribute to the wide distribution in the frequency of V α 7.2 positive T cells we observed in the 125 analyzed individuals presented in Fig. 2. Therefore, the sole scoring of V α 7.2 positive T cells cannot be reliably used to assist diagnosis in most AT patients. This prompted us to develop a more robust resource for analyzing possible bias in TCR α usage.

PROMIDIS α : A TCR α repertoire signature to identify primary immunodeficiency

SMART α (Fig. 1) and FACS analysis (Fig. 2) paved the way to using TCR α repertoire determination as an attractive tool to assist in the diagnosis of PIDs. Yet, none of these two experimental approaches can be envisioned as a biomarker for individual diagnosis purposes. SMART α allows the most comprehensive TCR α repertoire analysis but is technically very challenging for most routine laboratory. On the opposite, FACS analysis using anti-V α 7.2 antibody is simple and cost-effective but is not robust enough in many instances as discussed above. To combine robustness and simplicity, we setup to analyze the relative usage frequency of 8 different TRAV genes (and their associated TRAJ element) dispersed on the locus as PROximal, MIddle, and DIStal elements, hence the PROMIDIS α denomination, by RT-PCR and NGS in a multiplex PCR (Fig. 3A). Following RT-PCR/NGS, unique clonotypes expressed by TCR α transcripts were identified and scored using LymAnalyzer¹⁵ from raw sequencing files. We then calculated the relative proportions of proximal (TRAV35, TRAV41), middle (TRAV20, TRAV21, TRAV23), and distal (TRAV1,

TRAV5, TRAV10) TCR α transcripts and the frequency of proximal (TRAJ61 to TRAJ48) and distal TRAJ (TRAJ17 to TRAJ1) elements within each of these subgroups for a total of 9 parameters. As shown in Fig. 3A, this analysis faithfully reflected the TCR α peculiarity of TRAV/TRAJ correlation²¹. Indeed, TCR α transcripts containing proximal TRAV were predominantly associated with proximal TRAJ (36%) as opposed to distal ones (8.5%). Conversely, TCR α transcripts using distal TRAV were predominantly associated with distal TRAJ (49%) as opposed to proximal ones (2.6%). We next incorporated the values of these 9 parameters in PCA and hierarchical clustering analyses to survey 27 healthy controls, 18 PID patients with mutations in components of the V(D)J recombination machinery (*RAG1* (n=4), *DCLRE1C/Artemis* (n=5), *PRKDC/DNAPKcs* (n=2), *DNA-LigaseIV* (n=3), and *Cernunnos/XLF* (n=4)), 37 AT patients, and 3 NBS patients. The genotypes of the patients are recorded in suppl. Table 1. Six of the VDJ patients had their V α 7.2 expressing T cells scored by FACS (Table1, Fig. 2). Unsupervised hierarchical clustering analysis (Fig. 3B) revealed the partitioning of these 85 individuals into three groups, one comprising the 27 controls, one the 18 VDJ patients, and one the majority of the AT patients and the 3 NBS cases. Of note, none of the VDJ or AT/NBS cases were assigned to the Ctl group and, conversely, none of the 27 control individuals clustered with either the VDJ or the AT/NBS patients. A slight overlap between the VDJ and the AT/NBS was noticed for 3 AT patients (AT7, AT8, and AT12) clustering within the VDJ group. The clustering was highly statistically significant between the VDJs and the controls (Hotelling's square-T test, p=3.30E-16) as well as between the AT/NBSs and the VDJs (Hotelling's square-T test, p=1.28E-07). Consideration of the 9 variables contribution to the PCA (Fig. 3C) was clearly indicative of a bias in TCR α usage toward proximal TRAV and TRAJ in VDJ patients. One-parameter analysis (Fig. 3D and E) of the various variables demonstrated that TRAV/TRAJ usage in T cells from the VDJ patients was indeed severely skewed towards the usage of more proximal TRAV elements

(mostly to the expense of distal ones) with statistically significant differences in the three classes of TRAV between controls and patients (Fig. 3D). In addition, when considering the TRAJ usage (Fig. 3E), VDJ patients' repertoire was biased toward the usage of proximal TRAJ within the three classes of TRAVs. This was particularly striking within the few TCR α transcripts using distal TRAV elements, in which proximal TRAJ elements are usually largely underrepresented in T cells from control individuals. These observations outlined a specific TCR α repertoire signature in VDJ patients, which recapitulates the bias identified by SMART α ; *i.e.* a sharp decrease in distal TRAV/TRAJ usage within TCR α transcripts associated to an increase in more proximal TRAV and TRAJ elements. The PROMIDIS α signature is consistent with the reduced V(D)J recombination efficacy in thymocytes from these patients, but could as well reflect decreased thymocyte viability in patients harboring mutations in DNA repair encoding genes as previously observed in *xlf* KO mice¹¹. The PROMIDIS α signature in mature peripheral T lymphocytes therefore represents a reliable marker of the V(D)J recombination activity/Thymocyte lifespan during thymocytes development. This signature is very specific as none of 44 analyzed patients with non-VDJ associated PIDs clustered in the VDJ group (Supl. Fig. 1).

Early identification of ataxia telangiectasia patients through a specific PROMIDIS α signature

PROMIDIS α was used to analyze the TCR α repertoire in a series of 37 AT and 3NBS patients, 6 in a prospective manner based on the sole clinical presentation and prior to knowledge of the underlying ATM mutations, to extend the observation of a bias as suggested by FACS analysis using V α 7.2 staining in PBL (Fig. 2). As shown in Fig. 3B, most of the AT/NBS patients clustered in a separate group from the VDJs (Hotelling's square-T test, $p=1.28E-07$) and the controls. Three cases (AT7, AT8, and AT12) clustered within the VDJ

subgroup. The survey of more cases is now required to assess whether this “mis”-clustering reflects an meaningful structure/function relationship of the underlying mutations. The contributions of individual variables in the PCA (Fig. 3C) suggested that TCR α transcripts from AT and NBS patients were skewed toward the proximal TRAV usage also seen in the VDJ patients’ signature. However, in sharp contrast with VDJ patients, the TCR α transcripts from AT and NBS patients were biased towards the utilization of distal TRAJ elements, as opposed to expected proximal TRAJs. One parameter analysis confirmed both the increase in proximal TRAV usage (Fig. 3D) to the expense of distal TRAVs and the increase in distal TRAJ association in all TRAVs subgroups (Fig. 3F). The same bias towards usage of distal TRAJ elements was also observed in T lymphocytes and thymocytes from *Atm* KO mice (data not shown). The exact molecular mechanism causing the TRAJ bias specifically observed in *ATM* and *NBN* deficiencies is not presently understood. Anyhow, the striking difference between AT/NBS and VDJ patients’ PROMIDIS α signatures provides a specific marker that helps to identify AT/NBS patients confidently.

DISCUSSION

We herein described the development of a new immunological biomarker, based on the analysis of the TCR α repertoire designed to assist the diagnosis of patients with PID characterized by hypomorphic defects in the V(D)J recombination process and/or alteration of the DNA damage response. While most TCR and Ig genes rearrangements proceed through a unique wave of somatic recombination, the TCR α locus adopts a directional, multistep recombination process that is arrested by down regulation of RAG1/2 expression upon positive selection of thymocytes expressing sound TCR specificity on their surface²⁰. As a consequence, although slightly reduced V(D)J recombination efficacy may have no visible detrimental impact on most TCR and Ig loci, the persistent reduced recombinase activity over

the successive waves of TCR α rearrangement translates into a bias in TCR α usage, we herein describe. FACS analysis can readily highlights this bias through the sole quantification of TCR-V α 7.2 expressing T lymphocytes when V(D)J activity falls below a certain threshold as shown in a series of 11 patients. It can be more precisely characterized by analyzing 9 parameters related to the various TRAV and TRAJ gene usage among TCR α transcripts through PROMIDIS α , a multiplex RT-PCR followed by deep sequencing as shown here for 18 PID patients with variable V(D)J recombination deficiency and 37 AT/NBS patients. Human conditions with mutations in either *RAG1* or *RAG2* genes constitute the prototypical example of the broad spectrum of clinical consequences that patients may present depending on their specific *RAG1/2* gene mutation and, hence, the residual activity of the V(D)J recombination during T and B cell maturation⁵⁻⁷. At one end of the spectrum, complete *RAG1/2* loss of function leads to classical T-B-SCID. On the other hand, depending on the hypomorphic status of their mutations, patients may present various degrees of immune dysfunction up to mild autoimmunity. PROMIDIS α may in particular help identify cases of late onset immunodeficiency as presented by both adult patients (P1 and P5) included in our series. Overall, the almost absence of V α 7.2 expressing T cells together with the VDJ specific PROMIDIS α signature in 6 cases prospectively oriented the molecular study to direct Sanger DNA sequencing of VDJ recombination candidate genes, with the identification of underlying mutations in all cases, rather than sequencing using a PID specific gene panel or whole exome sequencing, which considerably accelerated the diagnosis and lowered its cost. These two combined assays are very robust and of high predictive value for PID with hypomorphic V(D)J recombination deficiency since no mutations in V(D)J recombination candidate genes could be found in the 92 TCR-V α 7.2 analyzed patients outside the 11 identified with almost complete absence of V α 7.2 expressing T cells. Likewise, all the 18 patients with mutations in V(D)J recombination related genes and none of the 27 controls or 44 PIDs with non-VDJ

presentation displayed the specific VDJ or AT/NBS PROMIDIS α signatures. PROMIDIS α should be very valuable also for the retrospective *in vivo* functional validation of variants identified through whole exome sequencing. With respect to RAG1/2 mutations, extensive studies from the Notarangelo's laboratory have linked the clinical status of VDJ defective patients to the residual level of recombinase activity tested through *in vitro* complementation of defective vAbl transformed proB-cell lines using retroviral expression of exogenous RAG1/2 mutants (for review see Notarangelo, L. et al.⁵). In some cases, results of these complementation studies do not concord with the observed clinical condition, and PROMIDIS α may thus represent an interesting alternative for *in vivo* validation of the identified variants.

In contrast to control individuals, the distribution of TCR-V α 7.2 positive T lymphocytes in PID patients is highly heterogeneous. More systematic studies using PROMIDIS α are thus required to provide an in-depth characterization of changes in TRAV and TRAJ usage characteristics and their possible defects in various PID. It is indeed known that, besides (or together with in some cases) the V(D)J recombination machinery *per se*, alterations of the thymocyte lifespan can exert a major impact on the TCR α repertoire generation. Mice with a targeted inactivation of the ROR γ T transcription factor are characterized by thymocytes with a reduced lifespan, which translates into a bias in V α J α usage toward the most proximal gene segments²². Likewise, T cells from patients with mutations in the *RAR Related Orphan Receptor C (RORC)* gene encoding the human ROR γ T counterpart exhibit a similar bias²⁸ which can be recapitulated by *in silico* PROMIDIS α (data not shown). One can therefore hypothesize that mutations in genes that encode factors involved in distinct aspects of thymocytes development could impact on TCR α repertoire, which may then be revealed in peripheral mature T lymphocytes by PROMIDIS α . Of note, study of the TCR α repertoire bias we describe here is the echo of a very specific window of T

cell maturation in the thymus, *i.e.* the CD4/CD8 double positive stage during which essentially non-cycling thymocytes undergo TCR α rearrangements. It does not apply to the upstream maturation events such as TCR β rearrangements and β -selection nor the downstream steps, such as the transition to CD4 and CD8 single positive mature thymocytes or egress from the thymus, or any event in the periphery. In that respect, one could hypothesize that conditions with impaired positive selection may translate into a specific bias in TCR α usage, but our preliminary experiments using MHC class I and II KO mice do not sustain this hypothesis (unpublished observations). Analyzing patients with HLA deficiency should be interesting in that regard.

In this study, we also established that PROMIDIS α constitutes a particular valuable biomarker to help identify AT and NBS patients, as we showed that mutations in the *ATM/NBN* genes result in a very specific TCR α signature. This should help in AT/NBN patients' care, particularly in the prevention of acute infections and the possibility of prenatal diagnosis in selected families. Likewise, PROMIDIS α could be of interest for the further investigation of patients with T cell lymphopenia identified through newborn screening²⁹. Finally, whether the altered signature identified in AT/NBS patients and patients with mutations in *Cernunnos/XLF*, *DNA-LigaseIV*, *DCLRE1C/Artemis*, and *PRKDC* can be found in other conditions of DNA repair deficiency will be of particular interest. Indeed, many of these patients are prone to cancer development for which treatments are often based on DNA damage-inducing drugs with a high risk of developing toxicity, as previously described in the first identified Lig4 patient who succumbed from cranial radiotherapy as part of his anti-leukemia treatment^{30, 31}.

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FIGURE LEGENDS

Figure 1: Comprehensive TCR α repertoire study.

2D heat map representation of TCR α transcripts determined by 5'RACE-PCR/NGS (SMART α) according to TRAJ (x-axis) and TRAV (y-axis) elements. Lower left and upper right quadrants represent proximal (TRAV22-41/TRAJ61-J48) and distal (TRAV1-11/TRAJ17-1) TRAV/TRAJ associations respectively. The clinical/genetic status, but not the TCR α repertoire, were previously described for the Cernunnos/XLF¹³ and Lig4¹² patients respectively.

Figure 2: Analysis of TCR-V α 7.2 expressing T lymphocytes by FACS.

A) Gating strategy to quantify TCR-V α 7.2 (V α 7.2-PE) expressing T lymphocytes among CD3 positive T cells (CD3-APC) in whole blood. **B)** Frequencies of V α 7.2 positive T cells in 92 PIDs of various etiology, 17 adult healthy controls, 16 pediatric (<18y.) healthy controls. **C)** Representation of V α 7.2 T cells frequency in healthy controls according to age. The limit between adult and pediatric is set at 18 years of age. Mann-Whitney statistical test: **** p<0.0001, ns: not significant.

Figure 3: TCR α repertoire analysis by PROMIDIS α

A) TCR α locus and primers used for PCR amplification. TRAV usage and TRAJ frequencies among Proximal, Medium, and distal TRAV in unique TCR α clonotypes. Proximal TRAJ segments include TRAJ1 to TRAJ48 and distal TRAJ segments include TRAJ17 to TRAJ1. **B)** Unsupervised hierarchical clustering of 85 tested individuals (27 controls, 18 “VDJ” patients, 37 AT patients, and 3 NBS case). The VDJ patients are labeled “NHEJ” or “RAG” depending on their gene defect as illustrated in Supl. Table 1. The three AT patients that

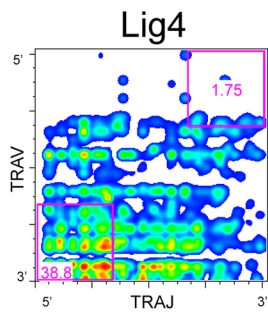
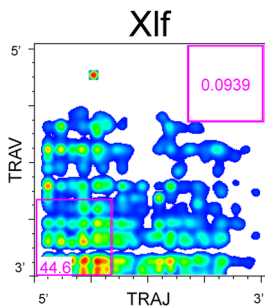
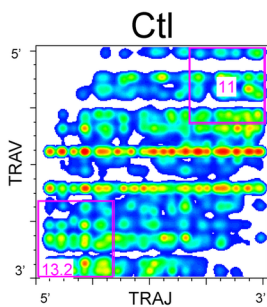
cluster with VDJ patients are underlined in blue. **C)** Contributions of the variables underlying the PCA and hierarchical clustering analysis, from dark blue (weak contribution) to light blue (high contribution). **D)** Single parameter analysis of TRAV usage between the controls (circles), V(D)J patients (triangles) and AT patients (squares) for each TRAV subgroup. **E)** Proximal TRAJ usage between controls (circles) and V(D)J patients (triangles) for each TRAV subgroup. **F)** Distal TRAJ usage in controls (circles) and AT patients (squares). Statistical analysis by Mann-Whitney non-parametric t test (**** $p < 0.0001$, *** $p < 0.001$).

Table 1: V(D)J recombination defective patients identified by absent Vα7.2 positive T cells

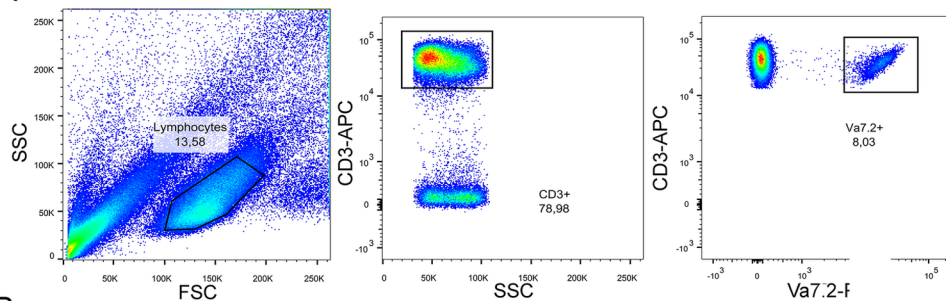
	P1/VDJ8	P2/VDJ18	P3/VDJ7 *	P4**	P5/VDJ17	P6	P7/VDJ9	P8	P9	P10	P11/VDJ1 0
Sex	F	M	F	M	F	F	M	M	M	M	M
Molecular diagnosis**	P	R	R	R	P	P	R	P	P	P	R
Age at diagnosis (y.)	28	9	9	15	34	2	8	7	1	0.2	1
Clinical phenotype	CID	CID	CID-G/AI	CID-G/AI	CID-G/AI	CID	CID-G/AI	CID with AIHA	CID	Omenn syndrome	****
Infections	LRTI with bronchiectasis Invasive aspergillosis HPV infection	LRTI with bronchiectas is Extended varicella	LRTI	LRTI with bronchiectasi s	Regional BCG-itis URTI and LRTI	Respirator y infections Recurrent diarrhoeae EBV and CMV replication	URTI and LRTI with bronchiectas is Molluscum contagiosum	URTI and LRTI with bronchiectasi s	Disseminate d infection to adenovirus		
Auto-immunity			Auto- immune myastheni a Alopecia, vitiligo	Cytopenia (ITP, neutropenia)	Auto-immune epidermolysis bullosa, vitiligo, premature ovarian failure, hypothyroidis m		AHAI Hepatopathy Chronic enteropathy	AHAI			
Other manifestations	EBV associated leiomyosarco ma			Systemic granulomatos is caused by rubella vaccine strain	Asthma						
Mutations	<i>DCLRE1C</i> p.D20H p.T167M	<i>DCLRE1C</i> c.1290- 1306del/ c.1290- 1306delb/	<i>RAG1</i> p.H375D/ p.R410W	<i>RAG1</i> p.H375D/ p.Y562C	<i>RAG1</i> p.R474C/ p.R918- F919delinsL	<i>RAG1</i> p.H612R/ p.H612R	<i>RAG1</i> p.G816R/ p.G816R	<i>RAG1</i> p.E174SfsX2 7/ p.P619L	<i>RAG1</i> p.R474H/ p.R559S	<i>RAG1</i> c.519del T/ c.519del T	<i>PRKDC</i> p.L3062R/ p.L3062R
Immunophenotyping											
CD3+ (number/mm3)	601 [807-1844]	176 [1200-2600]	273 [1200- 2600]	469 [1000-2200]	2125 [807-1844]	2248 [2100- 6200]	1423 [1200-2600]	1004 [1200-2600]	1372 [2100-6200]	6297 [2500- 5500]	ND
%Vα7.2/CD3+	0.2	0	0	0	0	0	0	0	0	0	0
T CD4+ cells (number/mm3)	313 [460-1232]	126 [650-1500]	169 [650- 1500]	418 [530-1300]	925 [460-1232]	519 [1300- 3400]	388 [650-1500]	414 [650-1500]	457 [1300-3400]	5634 [1600- 400]	ND
T CD8+ cells (number/mm3)	288 [187-844]	42 [370-1100]	42 [370- 1100]	20 [330-920]	875 [187-844]	303 [620-2000]	808 [370-1100]	430 [370-1100]	600 [620-2000]	663 [560- 1700]	ND
%CD45RA+CD31+/C D4	ND	1 [43-55]	3 [43-55]	ND	3 [30-48]	2 [57-65]	1 [43-55]	2 [43-55]	5 [57-65]	0.2 [60-72]	ND
%CCR7+CD45RA+/C D8	ND	4 [52-68]	19 [52-68]	ND	8 [37-50]	2 [52-68]	0.5 [52-68]	7 [52-68]	0.5 [52-68]	0.2 [52-68]	ND
%γδ/CD3+	ND	5	24	3	18	61	30	ND	22	1	ND
B cells (number/mm3)	5 [92-420]	4 [219-509]	56 [270-860]	20 [193-628]	150 [169-271]	1211 [319-1244]	2 [219-509]	143 [273-860]	114 [523-2149]	3 [712- 2059]	ND
NK cells (number/mm3)	564 [89-362]	160 [100-480]	150 [100-480]	20 [70-480]	200 [89-362]	130 [180-920]	49 [100-480]	383 [100-480]	1200 [180-920]	265 [170- 1100]	ND

URTI = upper respiratory tract infections; LRTI = low respiratory tract infections

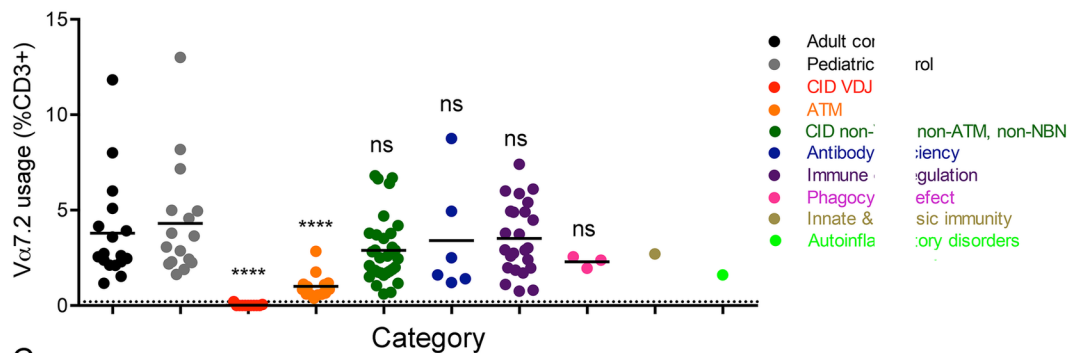
*reported as P4 in Rowe et al.³²; **reported as patient 5 in Neven et al.³³; *** Molecular diagnosis: prospective (P), retrospective (R);**** P9 is the brother of a previously identified CID patient with *PRKDC* mutatio



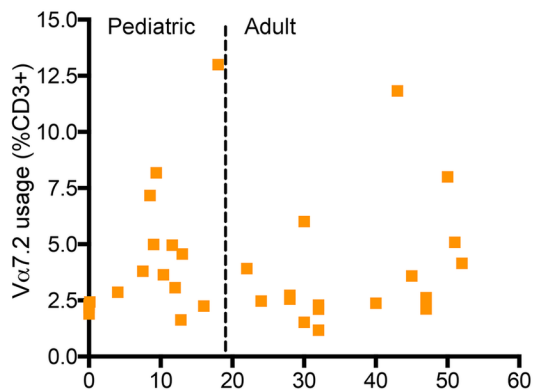
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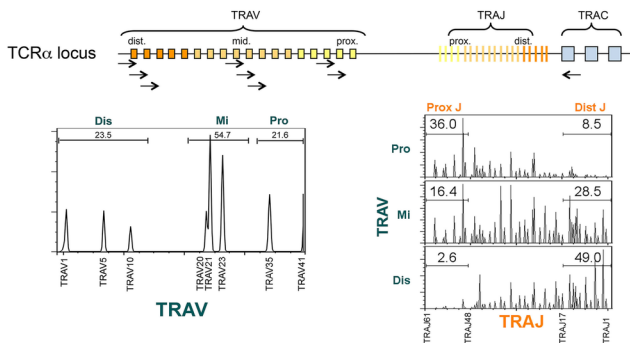
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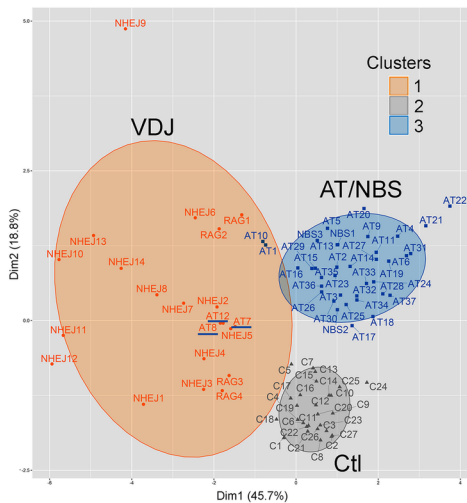
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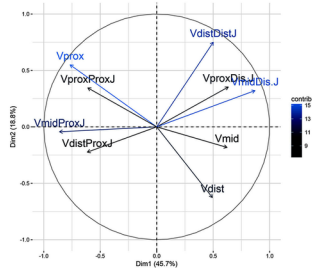
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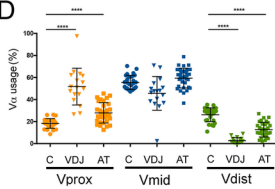
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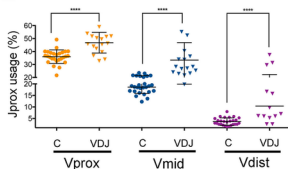
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E



F

