Basic and Clinical Aspects of the T-cell Receptor in Mature T-cell Malignancies

Basale en klinische aspecten van de T-celreceptor in rijpe T-celmaligniteiten

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

During normal human T-cell development, T cells undergo several immunogenotypic and immunophenotypic changes, with the final aim to express a functional antigen-specific T-cell receptor (TCR)/CD3 complex on the membrane. During early T-cell development in the thymus, T-cell progenitors start to rearrange their TCR genes via a process called V(D)J recombination, finally resulting in expression of a TCR molecule. This molecule is a unique transmembrane heterodimer composed of two generally disulfide-linked chains (Figure 1). Two types of TCR are known: the "classical" TCRαβ receptor, and the "alternative" TCRγδ receptor. T cells that have successively rearranged their TCR genes to express a membrane-bound TCR molecule that is not autoreactive, leave the thymus and migrate to the periphery where they can encounter antigen (Ag).

T cells can undergo malignant transformation in the various stages of T-cell development. The various types of T-cell leukemias and lymphomas are generally regarded as malignant counterparts of immature (thymic) or more mature (post-thymic) lymphoid cells. T-cell malignancies can thus be classified according to their corresponding normal T-cell differentiation stage. Immature acute lymphoblastic leukemias (ALLs) are of T-cell origin in 15-20% of patients, but in the group of mature lymphoid leukemias and in non-Hodgkin lymphomas (NHLs) T-cell malignancies are relatively rare except for primary cutaneous lymphomas where the T-cell type predominates. Classification of T-cell malignancies includes several immunobiological and molecular characteristics, e.g. immunophenotypical characteristics, TCR gene rearrangement status, and also genetic alterations including TCR-associated oncogenic events. These latter events concern translocations involving one of the TCR loci and result from aberrant V(D)J recombination. This type of translocation plays an important role in malignant transformation of especially immature T cells.

In this General Introduction, several aspects of normal human T-cell development are summarized with special attention on the basic aspects of V(D)J recombination in TCR gene complexes. In addition, human T-cell malignancies are described with emphasis on mature T-cell malignancies (i.e. mature T-cell leukemias and cutaneous T-cell lymphomas). Also, several diagnostic and research applications of TCR gene studies in human T-cell malignancies are discussed.

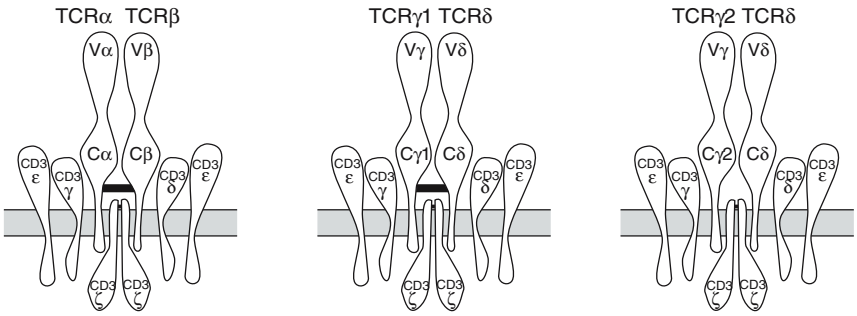

Figure 1. Schematic diagram of human T-cell receptor (TCR)αβ and TCRγδ molecules.
Both types of TCR molecules are associated with CD3 protein chains, which are involved in signal transduction. The proteins of TCRγδ receptor that are derived from Cγ1 sequences are disulfide linked, whereas this interchain disulfide bond is lacking if the TCRγ chain is derived from Cγ2 sequences. Vα, Vβ, Vγ, and Vδ are variable domains of TCR chains; Cα, Cβ, Cγ, and Cδ are constant domains of TCR chains (see reference 130).

HUMAN T-CELL DEVELOPMENT AND V(D)J RECOMBINATION

T-cell development in the thymus

Multipotent hematopoietic progenitors seed the thymus from the fetal liver or bone marrow and start T-cell development.[1-3] The different events of T-cell development take place during a series of discrete phenotypic stages that can be recognized by the expression of CD4 and CD8 coreceptors. In human, thymocytes successively present as $CD4^-/CD8^-$ (double negative, DN), $CD4^+/CD3^-$ (immature single positive, ISP), $CD4^+/CD8^+$ (double positive, DP) and finally $CD4^+/CD3^+$ or $CD8^+/CD3^+$ (single positive, SP) cells. The subset of DN thymocytes can be further subdivided on the basis of CD34 and CD1 expression into the consecutive $CD34^+CD1a^-$, $CD34^+CD1a^+$, and $CD34^-CD1a^+$ stages.[4,5]

The ultimate goal of T-cell development is the expression of the CD3/TCR complex, after which SP T cells exit the thymus to further mature in the periphery.

Peripheral T-cell developmental stages

In healthy individuals, T cells are diverse in structure, immunophenotype, and function. Structural diversity among T cells is the result of TCR recombination in the thymus, leading to expression of different TCR molecules. Naïve (unstimulated) SP T cells that exit the thymus become acitivated and undergo clonal expansion if stimulated with appropriate antigens. These cells acquire effector functions and migratory properties that allow them to clear antigens in both lymphoid and non-lymphoid organs. Effector T cells are short-lived, oligoclonal expansions of antigen-specific T cells. Most of the effector T cells die by apoptosis and only a small fraction survives and differentiates into memory T cells. T cells expressing the same TCR can thus exist in different functional states: naïve, effector, or memory.[6] In addition, it has been shown that memory T cells are heterogeneous in terms of development, effector functions, surface phenotype and trafficking properties.[7-9] Central-memory T cells are long-lived, reside in the lymphoid organs and expand upon re-stimulation by appropriate antigen. In contrast, effector-memory T cells reside in the non-lymphoid organs and exhibit immediate effector function without first undergoing proliferation. Using combinations of different immunophenotypical markers, several functional T-cell subsets can thus be discerned: naïve ($CD45RA^+/CD45RO^-/CD197$ $(CCR7)^+/CD28^+/CD27^+$), central-memory ($CD45RA^-/CD45RO^+/CD197$ $(CCR7)^{+/-}/CD28^+/CD27^+$), effector-memory ($CD45RA^-/CD45RO^+/CD197$ $(CCR7)^-/CD28^-/CD27^-$), and effector ($CD45RA^+/CD45RO^-/CD197$ $(CCR7)^-/CD28^-/CD27^-$) T cells (Table 1).

T-cell receptor molecules and their encoding genes

A TCR molecule consists of two chains, either a TCRα and a TCRβ chain (TCRαβ) or a TCRγ and a TCRδ chain (TCRγδ). The vast majority of mature T lymphocytes (85-95%) expresses TCRαβ; a minority expresses TCRγδ (5-15%).[10] The TCRα, β, γ, and δ proteins all consist of a variable and a constant domain. The variable domain is involved in actual antigen recognition and the constant domain mediates the effector function, resulting in signalling through CD3 (Figure 1).

As the number of possible antigens or antigenic epitopes is innumerable, an enormous diversity of TCR molecules is needed. This is achieved by random recombination of discrete

Table 1. Peripheral TCRαβ$^+$ T-cell subsets.

	CD45 RA	CD45 RO	CD197 (CCR7)	CD28	CD27
Naïve	+	-	+	+	+
Central-memory	-	+	+/-	+	+
Effector-memory	-	+	-	-	-
Effector	+	-	-	-	-

Adapted from Hamann *et al*.[126]

TCR gene segments. Only a limited set of gene segments is able to encode the required diversity of TCR molecules. The variable domain is encoded by a combination of one of the many available V (variable) and J (joining) gene segments (*TCRA* and *TCRG* loci), or by a combination of the available V, D (diversity), and J gene segments (*TCRB* and *TCRD* loci) (Table 2).[10,11] The constant domains of the TCR chains are encoded by C gene segments. One C gene segment is present for the constant domain of the TCRα chain and one for the TCRδ chain. Two C gene segments are available for the constant domains of the TCRβ and TCRγ chains (Figure 2).[12-16]

The generation of a T-cell receptor

During early T-cell differentiation, the germline V, (D), and J gene segments of the TCR gene complexes are coupled and each T cell thereby obtains a specific V-(D)-J exon.[11,17] Figure 3 illustrates an example of a *TCRB* gene rearrangement: one of the Jβ gene segments is coupled to one of the Dβ gene segments, and subsequently a Vβ to Dβ-Jβ joining occurs, resulting in a specific Vβ-Dβ-Jβ exon that can be transcribed into *TCRB* mRNA and translated into TCRβ protein.

Table 2. Estimation of potential primary repertoire of human T-cell receptor (TCR) molecules.

	TCRαβ molecules		TCRγδ molecules	
Repertoire	TCRα	TCRβ	TCRγ	TCRδ
Number of functional gene segments[a]				
V gene segments	45	44-47	6	6
D gene segments	-	2[b]	-	3[b]
J gene segments	50	13	5	4
Combinatorial diversity	>2 x 10^6		>5000	
Junctional diversity	+	++	+	+++
Estimation of total repertoire	>10^{12}		>10^{12}	

+, limited (range 0-20 nt); ++, extensive (range 0-35 nt); +++, very extensive (range 7-55 nt)
[a] Numbers are based on the international ImMunoGeneTics database.[36]
[b] In *TCRD* gene rearrangements, multiple D gene segments might be used; this implies that the number of junctions can vary from one to four. In *TCRB* gene rearrangements, generally only one D gene segment is used

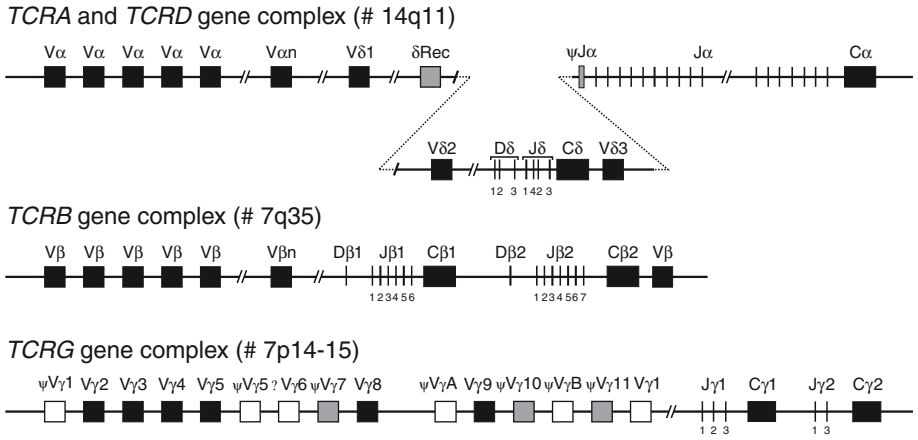

Figure 2. Schematic diagram of the human T-cell receptor gene complexes.
The *TCRA* gene complex consists of approximately 60 V gene segments, a stretch of 61 J gene segments, and one C gene segment. The *TCRB* gene complex contains appromimately 65 V gene segments and two C gene segments, both of which are preceded by a D gene segment and six or seven J gene segments. The *TCRG* gene complex consists of a restricted number of V gene segments (six functional segments and nine pseudosegments) and two C gene segments, each preceded by two or three J gene segments. The *TCRD* gene complex comprises several V (three true Vδ segments and several Vα/Vδ segments), three D, four J, and one C gene segment. The major part of the *TCRD* gene complex is located between the Vα and Jα gene segments and is flanked by the δREC and ψJα gene segments, which are involved in *TCRD* gene deletions that occur before *TCRA* gene rearrangements. Pseudogenes (ψ) are indicated as open symbols (see reference 130).

All V, D, and J gene segments are flanked by specific homologous recombination signal sequences (RSS). These RSS consist of a conserved palindromic heptamer consensus sequence (CACAGTG) adjacent to the coding sequence and a conserved nonamer consensus sequence (ACAAAAACC) that are separated by a less conserved spacer region of either 12 or 23 base pairs.[18,19] In principle, only RSS with different spacer length join efficiently, known as the 12/23 rule (Figure 4).[18] Complete RSS flank the 3' side of V gene segments, both sides of D gene segments and the 5' side of J gene segments.

RSS are recognized by recombination activation gene 1 and 2 proteins (RAG1 and RAG2), which are able to cleave the DNA between the RSS heptamer and the coding sequence of the involved gene segment.[20,21] Timing and efficiency of rearrangement of the various TCR genes is primarily determined by the accessibility of gene segments to the RAG enzymes. Evidence suggests that promoter and enhancer activity is controlled by transcription factors that regulate V(D)J recombination by modulating chromatin structures and rendering gene segments accessible to RAG cleavage.[22-25]

The DNA cleavage results in hairpinned coding ends and blunt 5' phosphorylated signal ends. A so-called coding joint is formed after cleavage and ligation of the hairpinned coding ends. During this ligation process, further (junctional) diversity of the coding joints is obtained by deletion and insertion of nucleotides, resulting in a highly diverse junctional region. The signal ends are also ligated and thereby form the extrachromosomal circular excision

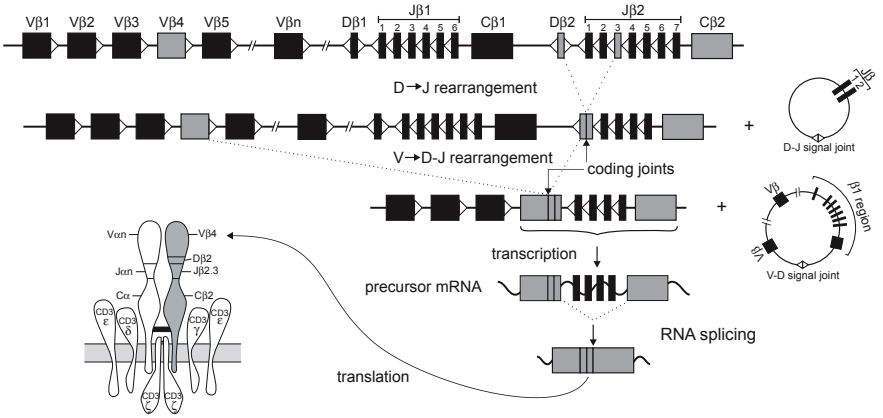

Figure 3. Schematic diagram of sequential rearrangement steps, transcription, and translation of the *TCRB* gene during T-cell differentiation.
In this example, first a Dβ2 to Jβ2.3 rearrangement occurs, followed by Vβ4 to Dβ2-Jβ2.3 rearrangement, resulting in the formation of a Vβ4Dβ2Jβ2.3 coding joint. The rearranged *TCRB* gene is transcribed into precursor messenger RNA (mRNA), spliced into mature mRNA, and finally translated into a TCRβ protein. The two extrachromosomal TCR excision circles (TRECs) that are fomed during this recombination process are indicated as well; they contain the D-J signal joint and the V-D signal joint, respectively (see reference 130).

product containing the two coupled RSS, which is referred to as the signal joint (Figure 3). In case of TCR gene rearrangements, such excision products are called "T-cell receptor excision circles" (TRECs).[19]

TCR gene rearrangements during human T-cell development

V(D)J recombination is a tightly regulated, hierarchical process, which starts in the DN stages of T-cell development (Figure 5).[26-28] Recombinations of TCR genes occur in a sequential manner: *TCRD* > *TCRG* > *TCRB* > *TCRA*.[28,29] During T-cell development in the thymus, the *TCRD* genes start the multistep rearrangement process (Dδ to Dδ, DDδ to Jδ, and Vδ to DDJδ joinings) at the $CD34^{+}CD38^{-}CD1a^{-}$ stage. *TCRD* rearrangement is followed by the single step *TCRG* gene rearrangements with Vγ to Jγ joinings at the $CD34^{+}CD38^{+}CD1a^{-}$ stage. If functional *TCRD* and *TCRG* gene rearrangements are obtained, TCRγδ protein molecules can be produced, allowing T cells to further develop into the γδ T-cell lineage (Figure 5).[10,28] T cells with non-functional *TCRD/TCRG* gene rearrangements continue their TCR gene rearrangement process via the two-step *TCRB* gene rearrangements (Dβ to Jβ followed by Vβ to DJβ joinings). Initiation of TCRβ-selection and *TCRA* rearrangement already occurs at the $CD34^{+}CD38^{+}CD1a^{+}$ stage of human T cell development.[28] *TCRB* rearrangement is followed by complex rearrangement steps in the *TCRD/A* locus, i.e. the *TCRD* gene deletion (particularly by the δRec-ψJα rearrangement), and the subsequent single-step Vα-Jα rearrangement.[30-34] If the *TCRB/TCRA* gene rearrangements are functional, TCRαβ protein molecules can be produced, allowing T cells to further develop into the αβ T-cell lineage (Figure 5).

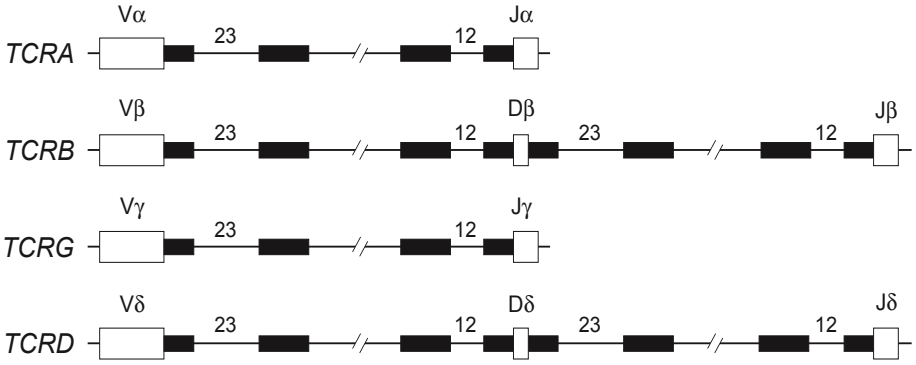

Figure 4. Schematic representation of the V, (D), and J gene segments with the recombination signal sequences (RSS).
The size of the spacer (12 or 23 nucleotides) is also indicated.

TCR repertoire

The many different functional Vβ (n=44-47), Dβ (n=2), and Jβ (n=13) gene segments in the *TCRB* locus and the many different functional Vα (n=45) and Jα (n=53) gene segments in the *TCRA* locus determine the potential V(D)J combinatorial diversity of TCRαβ molecules, which is estimated to be > 2 x 10^6 different combinations in the memory T-cell compartment (Table 2).[35] The combinatorial diversity of TCRγδ molecules is limited, because of the limited number of functional gene segments: 6 Vγ and 5 Jγ gene segments and 6 Vδ, 3 Dδ, and 4 Jδ gene segments (Figure 2 and Table 2).[11,36] However, the extensive junctional diversity (i.e. diversity due to imprecise joining of the V, (D), and J gene segments) of *TCRD* and *TCRG* gene rearrangements compensates for the limited combinatorial diversity. The combinatorial diversity together with the junctional diversity determine the potential primary repertoire of TCR receptors (Table 2).[11,37,38]

Calculations on the combinatorial repertoire are based on the assumption that the available functional V, D, and J gene segments are used randomly. This is not always the case. In healthy individuals, some Vβ families predominate in the PB T-cell repertoire, while others are only rarely used.[39] The representation of J gene segments is also far from random. The Jβ2 family segments are used more frequently than the Jβ1 family segments.[40] Skewing of the TCRβ chain repertoire takes place both in the thymus and the periphery. The thymic repertoire is predominated by only few Vβ gene segments and further modifications in the repertoire are mediated by antigen-induced selection in the periphery.[41] Peripheral TCRγδ$^+$ T cells exhibit preferential usage of particular gene segments. It might well be that particular TCRγδ receptor specificities dominate due to antigenic selection and expansion.[42] In neonatal cord blood and infancy, the Vγ9$^+$/Vδ2$^+$ T-lymphocytes represent only 5 to 15% of all TCRγδ$^+$ T-lymphocytes. However in older children and adults 80 to 95% of TCRγδ$^+$ T-lymphocytes in blood express the Vγ9/Vδ2 receptor. Remarkably, also the junctional regions of the Vδ2-Jδ1 and Vγ9-Jγ1.2 rearrangements show a selection determinant, which implies that the selection and expansion

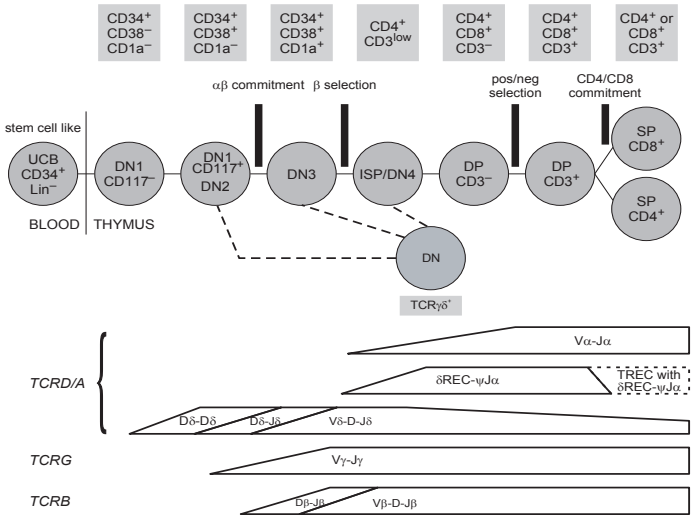

Figure 5. Stages of human T-cell development in the thymus.
The different phenotypic definitions are shown, as well as the main checkpoints during T-cell development. The major stages of T-cell development are depicted with the corresponding start of rearrangements of the TCR loci. DN, double negative; DP, double positive; ISP, immature single positive; SP, single positive. (Adapted from Dik *et al*.[28]).

of the Vγ9^{+}/Vδ2^{+} T-cells after birth is antigen-based.[42,43]

Reduction of the TCR repertoire and expansion of oligoclonal T cells can be seen in physiological and pathophysiological conditions. This phenomenon is commonly observed in healthy elderly individuals and is thought to contribute significantly to the increased morbidity and mortality from infectious disease at old age.[44-48] This especially applies to the CD8^{+}/CD28^{-} effector memory T cells, which have become the majority of circulating CD8^{+} T cells in the elderly.[45] Accumulation of clonally expanded memory CD8^{+} T-cells is thought to be a consequence of prolonged antigenic stimulation throughout life and it has been demonstrated that for example cytomegalovirus (CMV) is a major factor in driving these T-cell expansions.[49-51] However, other viral epitopes can also induce a narrow oligoclonal repertoire and even clonal dominance.[52] Aging not only affects repertoire diversity, but also T-cell homeostasis, which is demonstrated by a substantial decline in the production of naïve lymphocytes as a result of involution of the thymus after puberty.[53,54]

Oligoclonal T-cell expansions in peripheral blood and tissues are also found in various disease entities. Immunodominant T-cell clones are thought to play a causative role in the development of particular autoimmune diseases and lineage restricted cytopenias.[55-57] In addition, the heterogeneity of the TCR repertoire is disrupted in particular T-cell neoplasms. An aberrant immune response resulting in (oligo)clonal T-cell expansions has been hypothesized to be the first step in the development of these malignancies.[58,59]

HUMAN T-CELL MALIGNANCIES

Human T cells can undergo malignant transformation at the various differentiation stages. T-cell malignancies can thus be classified according to their presumed normal T-cell differentiation stage based on immunophenotype and immunogenotype. Clear evidence has demonstrated that natural killer (NK) cells are most probably derived from a lymphoid precursor cell type as well. Since they are closely related and share some immunophenotypic and functional properties,[60] mature T- and NK-cell malignancies are often classified together. The various types of immature T-cell and mature T- and NK-cell neoplasms as well as their presumed normal counterparts are schematically depicted in Figure 6.

Precursor T-ALL

Human precursor T lymphoblastic leukemias (T-ALL) are considered to be the malignant counterparts of thymic differentiation subsets. Approximately 15% to 20% of childhood acute lymphoblastic leukemias and 20% to 25% of adult acute lymphoblastic leukemias belong to the T-cell lineage.[61] Virtually all T-ALL are positive for terminal deoxynucleotidyl transferase (TdT), CD2, CD7, and CyCD3; further discrimination is possible on basis of CD1, CD3, CD4, CD5, and CD8 reactivity. T-ALL are often classified

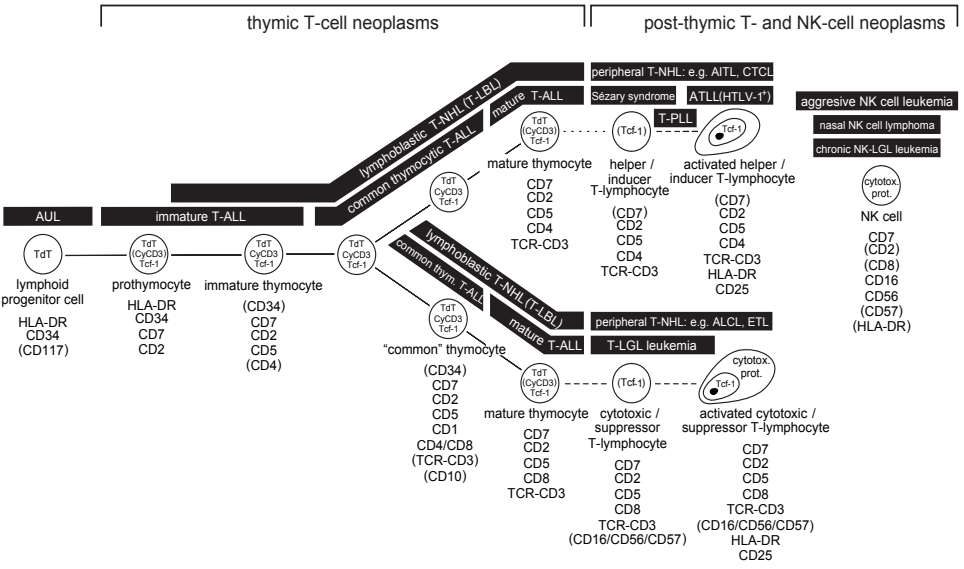

Figure 6. Hypothetical scheme of T/natural killer (NK) lymphoid differentiation.
The expression of relevant immunologic markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias and non-Hodgkin lymphomas (NHL) as presumed malignant counterparts of lymphoid T and NK cells on maturational arrest. AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; ALL, acute lymphoblastic leukemia; ATLL, adult T-cell leukemia lymphoma; AUL, acute undifferentiated leukemia; CTCL, cutaneous T-cell lymphoma; ETL, enteropathy-type T-cell lymphoma; HTLV-I human T-cell leukemia virus type I; LBL, lymphoblastic lymphoma; LGL, large granular lymphocyte; PLL, prolymphocytic leukemia. (Adapted from van Dongen *et al*.[130]).

in three main subtypes using CD1 and CD3: immature T-ALL ($CD1^-/CD3^-$), common (cortical) thymocytic T-ALL ($CD1^+/CD3^{+\text{ or }-}$) and mature T-ALL ($CD1^-/CD3^+$).[62,63] $CD3^+$ T-ALL are further subdivided in TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T-ALL. In another classification, human T-ALL, and especially immature $CD3^-$ T-ALL are further subdivided on basis of their TCR recombination status, thereby reflecting the different stages of early T-cell development.[64] Gene expression profiling studies confirmed that T-ALLs indeed reflect their normal thymic counterparts.[65,66]

Mature T-cell malignancies

Mature T- and NK-cell neoplasms are derived from post-thymic T cells. The current World Health Organization (WHO) classification of hematopoietic tumors recognizes 13 different entities of mature T- and NK-cell malignancies (Table 3).[67] They comprise approximately 5% to 10% of all mature lymphoid neoplasms. Each subtype is characterized by its own disease course, prognosis and treatment possibilities. Hence clinical features are of particular importance in the subclassification of mature T-cell malignancies. However, due to the lack of specific diagnostic parameters, there are overlapping features between the different disease entities.[68] Nevertheless, immunophenotypic analysis can be very helpful in identifying all different entities. Mature T-cell malignancies are by definition CD1 and TdT negative and nearly all express the CD3/TCR complex on the membrane. Analysis of CD3 expression helps to distinguish the $CD3^+$ T-cell malignancies from the $CD3^-$ NK-cell neoplasms. Most post-thymic $CD3^+$ T-cell malignancies express TCR$\alpha\beta$, with only a minority expressing TCR$\gamma\delta$. A major subdivision within the group of TCR$\alpha\beta^+$ T-cell neoplasms concerns CD4 or CD8 positivity. The WHO defined post-thymic T- and NK-cell neoplasms and their major immunophenotypic characteristics are summarized in Table 3. As this thesis mainly focuses on specific entities of mature T-cell neoplasms, i.e. primary cutaneous T-cell lymphoma (CTCL), T-cell prolymphocytic leukemia (T-PLL) and T-cell large granular lymphocyte (T-LGL) leukemia, these malignancies are introduced more extensively in Chapters 3 and 4.

ONCOGENIC RECOMBINATION IN HUMAN T CELLS

As in many other hematologic and mesenchymal malignancies, chromosomal translocations and inversions appear as essential events in T-cell oncogenesis.[69,70] Especially T-lineage acute lymphoid leukemias (ALLs) are characterized by a relatively high proportion of cases with well-defined chromosomal translocations or other genetic alterations. T-ALL are typically characterized by the presence of TCR gene-associated oncogenic events. In contrast, in mature T-cell leukemias the cells most frequently have a seemingly normal karyotype. In fact, specific cytogenetic abnormalities have been defined in only few of the mature T- and NK-cell neoplasms.

Translocations involving TCR loci

TCR gene-associated reciprocal chromosomal translocations constitute a major type of recurrent oncogenic events in T-ALL leukemogenesis.[71-73] Virtually all of these TCR-associated chromosome aberrations involve transcription factor-encoding oncogenes (e.g. *LYL1*, *HOX11*,

Table 3. Major immunophenotypic characteristics of post-thymic T- and NK-cell neoplasms.

Post-thymic T- and NK-cell neoplasms[a]		CD7	CD2	CD5	sCD3	CD4	CD8	TCR	CD25	CD30	CD16	CD56	CD57
T-cell prolymphocytic leukemia	T-PLL	++	+	+	+	+[b]	-	αβ	-	-	-	-	-
T-cell large granular lymphocyte leukemia	T-LGL	+	+	+	+	-	+	αβ> γδ[c]	-	-	+/-	+/-	+
Aggressive NK-cell leukemia	ANKL	+	+	-	-	-	-	-	-	-	+/-	+	-
Adult T-cell lymphoma/leukemia	ATLL	-	+	+	+	+	-	αβ	+	+/-	-	-	-
Extranodal NK/T-cell lymphoma, nasal type	NK/T-cell lymphoma	+/-	+	-	-	-	-	-	-	-	-	+	-
Enteropathy-type T-cell lymphoma	ETCL	+	+	-	+	-	+/-	αβ	-	-	-	+/-	-
Hepatosplenic T-cell lymphoma	HSTCL	+	+	-	+	-	-	γδ>>αβ	-	-	-	+/-	-
Subcutaneous panniculitis-like T-cell lymphoma	SPTCL	+	+	+	+	-	+	αβ>γδ[d]	-	-	-	+/-	-
Mycosis fungoides/Sézary syndrome	MF/SS	+/-	+	+	+	+	-	αβ	+/-	-	-	-	-
Primary cutaneous anaplastic large cell lymphoma	C-ALCL	+	+/-	+/-	+/-	+	-	αβ	-	+	-	-	-
Peripheral T-cell lymphoma, unspecified	PTL, NOS	+/-	+	+/-	+	+	-	αβ	-	+/-	-	-	-
Angioimmunoblastic T-cell lymphoma	AILT	+	+	+	+	+	+/-	αβ	-	-	-	-	-
Anaplastic large cell lymphoma	ALCL	-	+	-	-[e]	+/-	-	αβ	+	+	-	-	-

-, negative; +/-, weakly positive; +, positive; ++, strongly positive

[a] Post-thymic T- and NK-cell neoplasms as defined according to the World Health Organization Classification of hematopoietic and lymphoid tissues.[67]

[b] In 25% of cases there is coexpression of CD4 and CD8; 15% are $CD4^{-}/CD8^{+}$.[127],

[c] ~10% of cases are positive for TCRγδ.[68],

[d] ~25% of cases are positive for TCRγδ[128],

[e] CD3 is negative in more than 75% of cases.[129]

LMO1, *LMO2*, *TAL1, HOXA* genes), which are translocated to TCR loci (Table 4).[61,66,74-77] As a consequence, transcriptional deregulation of the involved oncogene or tumor suppressor gene occurs by regulatory elements of the translocated TCR gene or by loss of negative regulatory elements, eventually resulting in a differentiation block. This block leads to a pre-leukemic cell population. Multiple additional genetic hits can finally result in overt T-ALL. To date, 40-45% of T-ALL cases are characterized by such TCR gene-associated activation of oncogenes. Other recurrent genetic events involved in T-ALL multistep leukemogenesis concern mutations in cell cycle deregulating genes (e.g. *CDKN2A/p16/ARF*), genes involved in stem cell renewal (e.g. *NOTCH1* mutations) or in proliferation and survival (e.g. *ABL1* fusions).[71,78,79]

TCR gene-associated chromosome aberrations are most likely mediated via mechanisms closely resembling physiologic V(D)J recombination.[80] This is supported by the presence of RSS or RSS-like elements in the breakpoint regions of several of the T-ALL oncogenes. Such cryptic RSS can act as incorrect substrates for the V(D)J recombinase (i.e. the RAG proteins).[81] and be involved in TCR-associated translocations.[82,83] Failure in post-synaptic complex repair has been shown as an alternative mechanism of formation of these TCR aberrations.[84]

Presently, in mature T-cell malignancies illegitimate V(D)J recombination involving TCR loci has only been described for T-PLL. Translocations or inversions at 14q32.1 cause rearrangement of the *TCL1* (T-cell leukemia/lymphoma 1) locus involving regulatory elements of the *TCRA/D* locus at 14q11. This results in increased expression of the oncogenes *TCL1*, *TCL1b*, *TNG1* (*TCL1* neighboring gene 1), and *TNG2* (Table 5).[85,86]

TCR related chromosomal aberrations as prognostic markers

Detection of TCR gene-associated translocations is of diagnostic significance and contributes to further disease prognostification in malignant lymphoproliferations. Cytogenetic abnormalities are frequent among T-ALL patients and may identify biologically

Table 4. Non-random TCR-associated chromosome aberrations in T-cell acute lymphobastic leukemia.

Chromosome aberration	Relative frequency of T-ALL (%)	Involved gene	Involved TCR gene
1p32 aberrations	20-25	*TAL1*	*TCRD/TCRB*
t(11;14)(p13;q11)/t(7;11)(q35;p13)	7	*LMO2*	*TCRD/TCRB*
t(11;14)(p15;q11)	1	*LMO1*	*TCRD*
t(10;14)(q24;q11)/t(7;10)(q35;q24)	4	*HOX11*	*TCRD/TCRB*
t(8;14)(q24;q11)	2	*MYC*	*TCRD*
t(7;9)(q34;q32)	2	*TAL2*	*TCRB*
t(7;9)(q34;q34)	2	*TAN1*	*TCRB*
t(1;7)(p32;q34)	1	*LCK*	*TCRB*
t(7;19)(q34;p13)	1	*LYL1*	*TCRB*
t(5;14)(q35;q11)/(t(5;14)(q35;q32))	15-20	*HOX11L2*	*TCRD/(BCL11B)*

distinct prognostic subgroups. Better survival has been associated with normal karyotypes and translocation t(10;14)(q24;q11)[87,88], while for example the t(11;14)(p13;q11) has been identified as a poor prognosis factor.[89] The outcome of patients with poor prognosis may be improved by more intensive or alternative treatment modalities.

Non-TCR related translocations in T-cell malignancies

Less than 10% of T-ALL cases appear to have non-TCR related translocations leading to oncogenic fusion proteins, with the most common corresponding to *MLL*/chromosome 11q23 fusions.[90,91] The t(10;11)(p13-14;q14-21) associated with *CALM-AF10* fusion transcripts, is found in a minority of T-ALL cases, especially of TCRγδ lineage.[92,93]

So far, in mature T-cell malignancies recurrent non-TCR related chromosomal translocations have only been found in anaplastic large cell lymphoma (ALCL), which is associated with the translocation t(2;5) resulting in expression of the oncogenic NPM-ALK fusion protein (Table 5).[94] In other mature T- and NK-cell neoplasms, genetic instability is less apparent and the genetic events involved are probably more subtle.

Table 5. Recurrent genetic aberrations in post-thymic T- and NK-cell malignancies.

Post-thymic T and NK-cell neoplasms	Chromosome aberration	Involved genes	Relative frequency (%)
T-cell prolymphocytic leukemia	inv(14)(q11;q32)/ t(14;14)(q11;q32)	*TCL1, TCL1b, TNG1, TNG2*	90
T-cell large granular lymphocyte leukemia	-	-	-
Aggressive NK-cell leukemia	del 6q	-	-
Adult T-cell lymphoma/leukemia	clonally integrated HTLV-1	various	100
Extranodal NK/T-cell lymphoma, nasal type	-	-	-
Hepatosplenic T-cell lymphoma	isochromosome 7q	unknown	100
Entereopathy-type T-cell lymphoma	-	-	-
Subcutaneous panniculitis-like T-cell lymphoma	-	-	-
Mycosis fungoides/Sézary syndrome	-	-	-
Primary cutaneous anaplastic large cell lymphoma	-	-	-
Peripheral T-cell lymphoma, unspecified	-	-	-
Angioimmunoblastic T-cell lymphoma	-	-	-
Anaplastic large cell lymphoma	t(2;5)(p23;35)	*NPM-ALK*	80

APPLICATIONS OF TCR RECOMBINATION ANALYSIS IN HUMAN T-CELL NEOPLASMS

Since the various types of T-cell lymphomas and leukemias strongly resemble normal lymphoid cells, the vast majority also contains TCR gene rearrangements. Analysis of TCR recombination events has several diagnostic applications. Firstly, because T-cell malignancies are clonal cell proliferations, the TCR gene rearrangements are assumed to be identical in all cells of the malignant clone.[95-97] The diagnosis of malignant T-cell proliferations is therefore supported by the finding of clonally rearranged TCR genes, whereas reactive lymphoproliferations show polyclonally rearranged TCR genes. TCR clonality detection either at the DNA and/or RNA (cDNA) level is a major application of TCR gene recombination studies.

Secondly, the finding of TCR monoclonality not only supports the diagnosis of a T-cell malignancy, but detection of clonal TCR gene rearrangements can also be applied as fingerprints in molecular staging and disease monitoring during therapy (minimal residual disease (MRD) analysis).

Thirdly, since TCR$\alpha\beta^+$/TCR$\gamma\delta^+$ T-ALL and mature T-cell malignancies have membrane expression of the CD3/TCR complex, these malignancies also allow immunophenotypical analysis of the TCR molecules. Well-defined sets of antibodies against the variable parts of the TCR molecules have been developed, which are highly useful in identifying clonal T-cell populations. Additionally, these antibodies can be used for analysis of the TCR repertoire in both physiological and pathological conditions.[46,98-101]

Fourthly, oligoclonal expansions of cytotoxic T cells have been identified in a number of immune-mediated conditions, including infections (e.g. EBV and CMV), graft-versus-host disease (GVHD), and various autoimmune conditions. Furthermore clear monoclonality of cytotoxic T lymphocyte expansions is seen in T-cell large granular lymphocyte (T-LGL) leukemia. Most probably these proliferations represent a dysregulated immune response to an immunodominant viral or self-antigen. Flowcytometric Vβ analysis and *TCRB* gene rearrangement analysis in CD3$^+$/CD8$^+$/TCR$\alpha\beta^+$ T-LGL leukemia clearly demonstrated a skewed TCRβ repertoire with restriction of the antigen-specific part of the TCR molecule, the complementarity-determining region 3 (CDR3).[58,59] Clonotypic analysis of T-cell proliferations can therefore provide in-depth insight into the (molecular) pathogenesis of these proliferations.

Molecular TCR gene rearrangement analysis

The most prominent application of TCR recombination analysis in suspected T-cell proliferations is the detection of clonal TCR gene rearrangements, which is possible via Southern blotting (SB) and polymerase chain reaction (PCR) methods. SB analysis allows detection of deletion and relocation of gene segments based on changes in distances between cleavage sites of restriction enzymes in the DNA. It can easily discriminate between clonal rearrangements and polyclonal TCR gene rearrangements and has long been the "gold-standard" technique for clonality detection.[17,102-104] Clonal cell populations can be detected with a sensitivity of approximately 5%, whereas the detection limit is 10%-15% if a clonal cell population has to be identified within a large background of polyclonal cells. Due to the

greater sensitivity and efficiency, PCR techniques are replacing SB analysis as a diagnostic tool in lymphoproliferative disorders.[105,106] PCR analysis of TCR gene rearrangements is based on the (selective) amplification of junctional regions of rearranged TCR gene segments. Such amplification is only possible when the TCR gene segments are juxtaposed through rearrangement, as the distance between these gene segments in germline configuration is far too large for efficient PCR amplification. Following PCR amplification of TCR gene rearrangements, discrimination between clonal (leukemia/lymphoma-derived) and polyclonal (reactive) PCR products is needed. Several analytical methods exploiting the junctional diversity of rearranged TCR genes have been developed, including heteroduplex analysis and GeneScan/fragment analysis.[107-112]

However, in comparison to SB analysis, PCR based clonality analysis has several limitations and pitfalls.[113-115] These mainly concern a higher rate of false-negative results and difficulties in discriminating monoclonal and polyclonal gene rearrangements. Therefore, a reliable and easy interpretable PCR strategy has recently been developed in the BIOMED-2 Concerted Action BMH4-CT98-3936.[39] This DNA-based multiplex PCR approach combines all V, (D) and J primers in multiplex tubes and allows easy and rapid detection of *TCRD*, *TCRG*, and *TCRB* gene rearrangements. Although this approach has been extensively validated in well-defined samples,[39,116,117] its usage in a routine diagnostic setting is less established.

PCR analysis of *TCRA* rearrangements is also possible, but it requires more primers, especially for the many different V and J gene segments. An alternative would be reverse transcriptase (RT) PCR analysis of *TCRA* VJ-C transcripts, which still requires many different Vα primers, although they can be used in combination with a single Cα primer.[118] Unlike *TCRB* gene rearrangements that have been extensively studied by RT-PCR analysis in normal and malignant T-cell populations,[100,119] little is known about *TCRA* gene rearrangements and Vα/Jα gene segment usage.

MRD analysis and staging

Routine and reliable identification of very low numbers of malignant cells, i.e. MRD detection, is possible via stable leukemia/lymphoma-specific markers, such as clonal TCR gene rearrangements. The junctional regions of the clonally rearranged TCR genes are unique "fingerprint-like" sequences, which can be used as sensitive and specific PCR targets for MRD detection. For this application, the various clonal TCR gene rearrangements have to be identified in each T-cell malignancy at diagnosis by use of selected PCR primer sets. The oligonucleotide sequence of the junctional region is determined and used to develop a patient-specific junctional region oligonucleotide. Many studies have shown that monitoring of MRD in hematopoietic malignant disease, especially ALL, predicts clinical outcome.[120-122] So far, MRD detection has not been broadly applied for mature T-cell malignancies. Identification and monitoring of mature $CD3^+$/$TCR\alpha\beta^+$ T-cell proliferations by flowcytometry using the Vβ antibody kit has been demonstrated to be a suitable method to assess clinical responses to different therapies.[101,123]

TCR-V chain detection via monoclonal antibodies

Another application is the rapid analysis of the V chain repertoire via flowcytometry. Membrane $CD3^+$ T-ALL and mature T-cell malignancies show expression of the CD3/TCR

complex, which can be analysed with well-defined anti-Vα, anti-Vβ, anti-Vγ, and anti-Vδ antibodies. Using normal values in healthy controls of different age groups as reference, such antibodies allow detection of restricted V usage in a quantitative manner.[46,124] It was shown that Vβ flowcytometric results completely correlate with *TCRB* PCR results.[100] Approximately 65% of mature TCRαβ$^+$ T-cell proliferations can now be detected via single Vβ domain expression using the currently available Vβ antibody panel. Quantitative flowcytometric analysis of the Vβ repertoire could therefore (at least partly) replace the more expensive and more cumbersome molecular TCR repertoire studies. Likewise, Vγ and Vδ analysis in the case of TCRγδ$^+$ T-cell proliferations might also (partly) be performed by flowcytometric analysis using Vγ and Vδ antibodies, although interpretation is more complicated than Vβ analysis due to the small Vγ/Vδ repertoire.

Molecular analysis of TCR clonotypes

The antigen-specific portion of the TCR molecule, the variable domain CDR3 region, can serve as a molecular signature (clonotype) of a T-cell clone. Analysis of clonal in-frame *TCRB* gene rearrangements in CD8$^+$ TCRαβ$^+$ T-LGL leukemias demonstrated a common *TCRB* CDR3 signature between different patients.[58,59] This highlights the role of an antigenic stimulus and strengthens the thought that CD8$^+$ TCRαβ$^+$ T-LGL leukemia develops from an oligoclonally dysregulated proliferation. The possible role of antigenic stimulation in the pathogenesis of CD4$^+$ TCRαβ$^+$ T-LGL leukemia and TCRγδ$^+$ T-LGL leukemia still has to be determined. Next to T-LGL leukemia, molecular clonotypic analysis has also identified immunodominant clonotypes associated with other immune mediated disease conditions, such as graft-versus host disease, allogeneic hematopietic stem cell transplantation, aplastic anemia, and paroxysmal nocturnal hemoglobinuria.[57,59,125] Identification of immunodominant clonotypes not only has several pathophysiologic implications, but may also be useful for diagnostic purposes.

OUTLINE OF THE THESIS

The overall objective of the research described in this thesis is to obtain more insight into TCR gene recombinations and their applicability in mature T-cell malignancies. Three main topics are addressed. The first concerns basic and technological aspects of TCR gene recombinations (Chapter 2). Initiation and selection of TCR gene rearrangements during normal thymocyte development are studied in detail. In addition, the clinical applicability of TCR gene rearrangement analysis is studied in lymphoproliferative disorders. The second topic focuses on the applications of molecular analysis of TCR gene rearrangements in cutaneous T-cell lymphomas (Chapter 3). The additional value of molecular clonality analysis in diagnosing and staging of cutaneous lymphoproliferations is described. The studies described also underline the relevance of molecular techniques for determination of extracutaneous dissemination and clonal relationship. The third topic addresses the etiopathogenic aspects of TCR gene rearrangement analysis in mature T-cell leukemias, especially T-cell prolymphocytic leukemia and T-cell large granular lymphocyte leukemia (Chapter 4).

Finally, the results as described in Chapters 2-4 are summarized and discussed in the General Discussion where also some future directions are outlined (Chapter 5).

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Chapter 2

BASIC AND TECHNICAL ASPECTS OF TCR GENE RECOMBINATION STUDIES IN NORMAL AND MALIGNANT T-CELL DIFFERENTIATION

2.1 TCR gene recombination patterns during human T-cell development are determined by selection for functionality and by peripheral antigen-induced selection and expansion of T lymphocytes

2.2 Human T-cell lines with well-defined T-cell receptor gene rearrangements as controls for the BIOMED-2 multiplex PCR tubes
Leukemia 2007; 21: 230-237

2.3 BIOMED-2 multiplex immunoglobulin/T-cell receptor polymerase chain reaction protocols can reliably replace Southern blot analysis in routine clonality diagnostics
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Chapter 2.1

TCR gene recombination patterns during human T-cell development are determined by selection for functionality and by peripheral antigen-induced selection and expansion of T lymphocytes

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ABSTRACT

The different steps of T-cell development all aim at the generation of mature T cells, expressing unique, self MHC restricted and non-autoreactive T-cell receptors. In order to express a functional T-cell receptor (TCR) molecule on their membrane, T cells must undergo TCR gene rearrangements. We selected representative V-J rearrangements for all four TCR loci (*TCRD, TCRG, TCRB, TCRA*) to study the timing and selection of TCR gene rearrangements during human T-cell development. To this end, PCR based GeneScan analysis of TCR gene rearrangements was performed on purified thymic subsets, as well as umbilical cord blood mononuclear cells, peripheral blood mononuclear cells, mature peripheral TCR$\gamma\delta^+$ T cells, and mature peripheral TCR$\alpha\beta^+$ T-cells. For TCR$\alpha\beta^+$ T cells, selection of in-frame *TCRB* and *TCRA* gene rearrangements primarily takes place in the thymus and limited expansion occurs in the periphery. Non-Vγ9/Vδ1$^+$ T cells are selected in the thymus as well, whereas in the periphery TCR$\gamma\delta^+$ T cells are further selected resulting in massive expansion of Vγ9/Vδ2$^+$ T lymphocytes. The timing and extent of selection for in-frame TCR gene rearrangements during thymic development varied between the different TCR loci. Peripheral (super)antigenic selection was more abundant in TCR$\gamma\delta^+$ T cells than in TCR$\alpha\beta^+$ T cells.

INTRODUCTION

Human T cells develop from progenitors that migrate from the fetal liver or bone marrow into the thymus.[1] The main thymocyte subsets are defined according to their CD4/CD8 expression pattern: double negative (DN), double positive (DP), or single positive (SP). Human DN thymocytes mature via an immature SP (CD3$^-$/CD4$^+$) stage, and DP CD3$^-$ and DP CD3$^+$ stages into CD4$^+$ or CD8$^+$ SP thymocytes that exit the thymus to further mature in the periphery.

The ultimate purpose of T-cell differentiation is the generation of T cells expressing a functional T-cell receptor (TCR). Two types of TCR have been recognized: a TCR consisting of a TCRα and a TCRβ chain (TCR$\alpha\beta$) and a TCR consisting of a TCRγ and a TCRδ chain (TCR$\gamma\delta$). The majority of mature T lymphocytes (85% to 98%) in peripheral blood (PB) and most lymphoid tissues express TCR$\alpha\beta$; a minority (2% to 15%) expresses TCR$\gamma\delta$. Each TCR chain consists of a variable domain and a constant domain. The variable domains are highly polymorphic and contain the unique antigen (Ag) binding properties of each individual TCR. During T-cell development, the variable domains of the *TCRA, TCRB, TCRG*, and *TCRD* (located within the *TCRA* locus[2]) genes are assembled through rearrangement of variable (V), diversity (D), and joining (J) gene segments. This process is called V(D)J recombination and involves the lymphoid-specific recombination activating gene (*RAG*) enzymes.

TCR gene recombinations are hierarchically ordered (*TCRD>TCRG>TCRB>TCRA*) during normal human T-cell development.[3] Dik *et al*. unravelled the timing and efficiency of the rearrangements in the various TCR gene complexes.[4]

After complete V(D)J recombination, only T cells with complete in-frame TCR gene rearrangements can express functional proteins and can subsequently be selected. As TCR recombinations are complex processes with imprecise joining of gene segments, approximately

two out of three joinings are out-of-frame; that is, an mRNA is produced without the correct three-nucleotide reading frame preventing translation into a complete protein.[5] Selection for in-frame (functional) TCR gene rearrangements already occurs in an early stage of T-cell development and these thymocytes are rescued from programmed cell death via interaction of their functional TCR with peptide/self-MHC complex. This process is called positive selection and ensures that mature T-cells can recognize foreign antigens preferentially in the context of self-MHC molecules.[6] Thymocytes that recognize autoantigens as well as thymocytes with out-of-frame (non-functional) genes are eliminated via a process called negative selection.[7]

Imprecise joining of gene segments is mediated through exonuclease activity during recombination and through the non-templated nucleotide (N) addition by the enzyme terminal deoxyribonucleotidyl transferase (TdT). As a result, the coupling areas or junctional regions are highly variable in size. This size heterogeneity as well as the three nucleotide ("triplet") reading frame can be accurately assessed by PCR based GeneScan analysis of PCR products from TCR gene rearrangements.[8]

In the current study, we performed GeneScan analysis of multiple TCR gene rearrangements in thymic subsets, umbilical cord blood (UCB) cells and mature peripheral T cells. Our aim was to assess the timing and extent of selection of in-frame TCR gene rearrangements during human T-cell development. Furthermore, we also aimed to identify selection determinants in TCR junctional regions, involved in peripheral expansion of mature T cells. To this end, representative V-J rearrangements were selected for all four TCR loci. For *TCRD* and *TCRG*, Vδ1-Jδ1 and VγI-Jγ1.3/2.3 gene rearrangements were studied; Vδ2-Jδ1 and Vγ9-Jγ1.2 gene rearrangements were analyzed as well since peripheral blood TCRγδ$^+$ T lymphocytes exhibit preferential usage of these gene segments.[9,10] In the *TCRB* locus the Vβ17-Jβ2.7 gene rearrangement was studied since both gene segments are frequently used by thymocytes and peripheral T lymphocytes.[11,12] Finally, as representative *TCRA* recombination Vα4-Cα transcripts were analyzed because of the relative high frequency of Vα4 gene segment usage in TCRαβ expressing T cells.

MATERIALS AND METHODS

Isolation of cell samples from thymus, umbilical cord blood and peripheral blood

Thymi were obtained as surgical tissue discards from children aged 7 weeks to 3 years (median of 6 months) undergoing cardiac surgery at the Erasmus MC Rotterdam, with informed consent from the parents. The children did not present with immunological abnormalities. Thymocytes were isolated by cutting the thymic lobes into small pieces and squeezing them through a metal mesh, and stored at -80ºC until further analyses. Mononuclear cells (MNCs) were isolated by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density centrifugation from human umbilical cord blood (UCB) obtained from full-term normal deliveries and from peripheral blood of healthy volunteers. All samples were obtained according to the guidelines of the Medical Ethical Committee of the Erasmus MC.

Purification of thymocyte subsets and mature peripheral T cells

For the isolation of thymocyte subsets, total thymocytes from 5 donors were pooled to reduce intra-sample variation. After thawing, pooling and Ficoll density separation, thymocytes were labeled with fluorochrome-conjugated monoclonal antibodies. For initial enrichment of the immature single positive (ISP $CD4^+$) population, thymocytes were depleted of CD3-expressing cells using magnetic beads prior to high speed cell sorting. For isolation of $CD3^+/TCR\gamma\delta^+$ thymocytes, magnetic beads were used to enrich for $TCR\gamma\delta^+$ T cells. All magnetic beads were used according to the manufacturer's protocol. A cocktail of monoclonal antibodies was used to deplete NK cells (CD16 and CD56), B lymphocytes (CD19), and myeloid cells (CD13 and CD33). After MACS sorting, the enriched cells were labeled with fluorochrome-conjugated monoclonal antibodies for further purification by high speed cell sorting. In order to isolate mature $CD3^+/TCR\alpha\beta^+$ and $CD3^+/TCR\gamma\delta^+$ T cells, peripheral blood MNCs were labeled with fluorochrome-conjugated monoclonal antibodies for cell sorting. All cell sorting was performed on a FACS DiVa cell sorter (BD Biosciences, Santa Clara, CA, USA). Monoclonal antibodies used, with the clone in brackets, were: CD4-FITC (SK3), TCRαβ-FITC (WT31), CD3-PE (SK7), CD16-PE (B73.1), CD19-PE (4G7), CD56-PE (M431), TCRγδ-PE (11F2), CD3-PerCP (SK7), CD8-PerCP (SK1), CD19-PerCP (SJ25C1), CD3-APC (SK7), CD8-APC (SK1) (all from BD Biosciences), CD13-RDI (MY7) and CD33-RDI (906) (from Beckman Coulter, Fullerton, CA, USA). Purity of the sorted populations was determined on the FACS Calibur flow cytometer (BD Biosciences) and shown to be > 95% for all populations. All populations were sorted twice using different donors for each sort.

DNA and RNA isolation and cDNA synthesis

DNA from thymic subsets, total UCB cells, PB MNCs and peripheral $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells was extracted with the GenElute Mammalian Genomic DNA miniprep kit according to the manufacturer's protocol. RNA was isolated from all samples using RNeasy columns according to the manufacturer's protocol (Qiagen) and reverse transcribed into cDNA as described previously.[13]

PCR GeneScan analysis of TCR gene rearrangements

Amplification reactions were performed in an automated thermocycler (model ABI 2700; Applied Biosystems, Foster City, CA, USA) according to the BIOMED-2 multiplex PCR protocol.[8] The following TCR recombinations were determined in "singleplex" PCR reactions: Vδ1-Jδ1, Vδ2-Jδ1, Vγ-Jγ1.3/2.3, Vγ9-Jγ1.2, Vβ17-Jβ2.7, and Vα4-Cα. Primers that we used were: BIOMED-2 Vδ1, Vδ2, Jδ1, VγIf, Vγ9, Vβ17, Jβ2.7[8], and BIOMED-1 Jγ1.2 specific primers.[14]

Specific primers for Vα4 ($5'$AGAAGTGAACATAACCTGTAGCCA$3'$) and Cα ($5'$ GGTACACGGCAGGGTCAG $3'$) were newly developed. Complete TCRD, TCRG, TCRB V-J rearrangements and Vα-Cα transcripts were amplified with FAM-labeled Jδ1, Jγ1.2, Jβ2.7, and Cα primers, respectively.

In order to analyze V(D)J rearrangements and selection of the TCR chain (i.e. in-frame *TCRD, TCRG, TCRB*, and *TCRA* gene rearrangements) in the isolated populations, the obtained PCR products were subsequently subjected to GeneScan analysis as described before.[8]

RESULTS AND DISCUSSION

Vδ1-Jδ1 GeneScan patterns

Complete Vδ1-Jδ1 gene rearrangements were detected from the $CD4^+$ ISP stage onwards and were present in $TCR\alpha\beta^+$ thymocytes as well (Figure 1). The GeneScan patterns of these fractions showed a merely random pattern. In contrast, the GeneScan pattern of $TCR\gamma\delta^+$ thymocytes showed a symmetrical unimodal pattern with peaks at every third nucleotide (triplet peaks) and deep indentation between the peaks, illustrating selection on the basis of in-frame gene rearrangements. This is consistent with Vδ1-Jδ1-Cδ chain expression by the majority of post-natal $TCR\gamma\delta^+$ thymocytes. The selection for in-frame Vδ1-Jδ1 rearrangements is not detectable in adult PB MNCs and mature $TCR\gamma\delta^+$ T cells, due to preferential Vδ2 usage in peripheral $TCR\gamma\delta^+$ T cells.[15] Also in UCB cells no straightforward in-frame Vδ1-Jδ1 pattern is detected, probably explained by the presence of many $TCR\alpha\beta^+$ cells part of which contain monoallelic out-of-frame *TCRD* gene rearrangements.

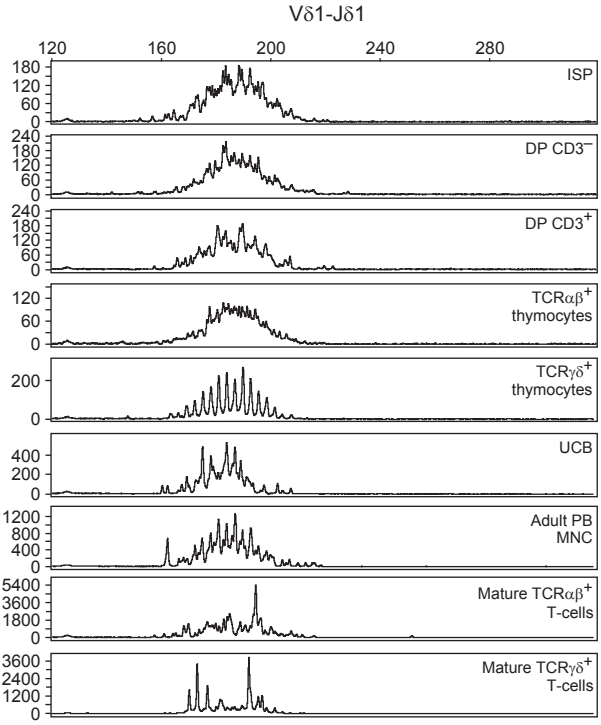

Figure 1. GeneScan analysis of Vδ1-Jδ1 gene rearrangements in thymic subsets, umbilical cord blood cells, and mature T lymphocytes.
Clearly visible triplet peaks are detectable in $TCR\gamma\delta^+$ thymocytes.

Vδ2-Jδ1 GeneScan patterns

Complete Vδ2-Jδ1 gene rearrangements could easily be detected in $CD4^+$ ISP cells (Figure 2). These rearrangements remain present in both the DP $CD3^-$ and $CD3^+$ stages as well as $TCR\alpha\beta^+$ thymocytes, which is consistent with previous findings.[16] The size heterogeneity of the junctional regions in these thymocyte fractions as well as in UCB cells and mature $TCR\alpha\beta^+$ T-cells demonstrated a random pattern. In contrast, the GeneScan patterns of PB MNCs and purified peripheral $TCR\gamma\delta^+$ T-cells showed clear selection on the basis of in-frame gene rearrangements as illustrated by the symmetrical pattern with triplet peaks. The triplet peaks of Vδ2-Jδ1 gene rearrangements were less clear in total PB MNCs as compared to the purified $TCR\gamma\delta^+$ T cells, most likely because the PB MNC population consists of a mixture of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells.

The strong selection for in-frame Vδ2-Jδ1 gene rearrangements in adult peripheral $TCR\gamma\delta^+$ T cells is in line with the well-described preferential Vδ2 usage by these cells. This selection is not detectable in post-natal $TCR\gamma\delta^+$ thymocytes and UCB MNCs, in which the Vδ1 gene segment is preferentially used. During early childhood the distribution of Vδ gene usage

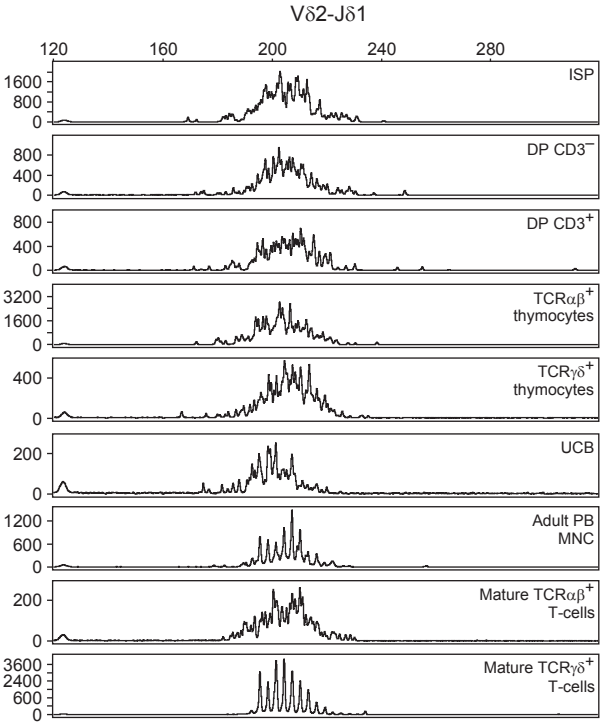

Figure 2. GeneScan analysis of Vδ2-Jδ1 gene rearrangements in thymic subsets, umbilical cord blood cells, and mature T lymphocytes.

Clearly visible triplet peaks are detectable in adult PB MNCs and particularly in mature $TCR\gamma\delta^+$ T lymphocytes.

changes from 60-65% Vδ1, and 15-20% Vδ2 in neonates to 10-15% Vδ1, and 80-85% Vδ2 in older children and adults.[17, 18] The deep indentation between the triplet peaks fits with the high frequency of in-frame Vδ2-Jδ1 rearrangements and the virtual absence of out-of-frame Vδ2-Jδ1 rearrangements in adult TCRγδ$^+$ T lymphocytes (Figure 2).

VγI-Jγ1.3/2.3 GeneScan patterns

Typical polyclonal Gaussian curves can be detected in all immature thymic subsets as well as TCRαβ$^+$ thymocytes (Figure 3). Selection on in-frame VγI-Jγ1.3/2.3 gene rearrangments can be detected in TCRγδ$^+$ thymocytes, whereas it is virtually absent in UCB cells and peripheral T cells. The in-frame VγI-Jγ1.3/2.3 gene rearrangements of TCRγδ$^+$ T lymphocytes are not detectable in UCB cells because of the high frequency of out-of-frame VγI-Jγ1.3/2.3 gene rearrangements in TCRαβ$^+$ T lymphocytes. No selection on in-frame VγI-Jγ1.3/2.3 gene rearrangements can be detected in mature TCRγδ$^+$ T cells, which is explained by the preferential usage of the Jγ1.2 gene segment in peripheral TCRγδ$^+$ T cells.

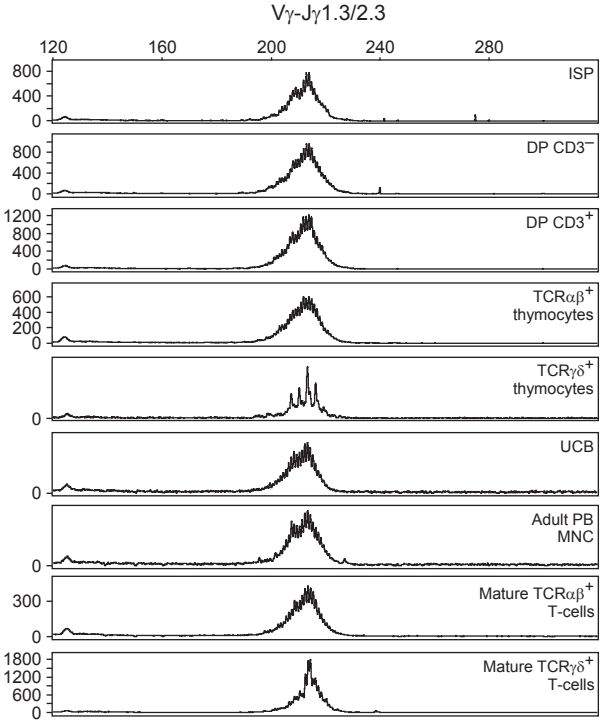

Figure 3. GeneScan analysis of Vγ-Jγ1.3/2.3 gene rearrangements in thymic subsets, umbilical cord blood cells and mature T lymphocytes.

Vγ9-Jγ1.2 GeneScan patterns

Vγ9-Jγ1.2 gene rearrangements were observed at low frequency in all immature thymic subsets from ISP onwards, as well as in mature TCRαβ$^+$ thymocytes, and UCB cells. As shown in Figure 4, the size distribution of the Vγ9-Jγ1.2 junctional regions in these fractions was random; without clear triplet peaks. Vγ9-Jγ1.2 rearrangements are rare in TCRγδ$^+$ thymocytes, but massive positive selection occurred in PB MNCs and mature TCRγδ$^+$ T cells. In adult mature peripheral T cells, the GeneScan patterns of Vγ9-Jγ1.2 gene rearrangements showed a much smaller size distribution of the junctional region than in thymic subsets. Such reduction of the antigen receptor repertoire in the periphery can be the result of antigen-induced proliferation of a specific T-cell subset. It is well-established that mature TCRγδ$^+$ lymphocytes are selected for reactivity with a (super)antigen:[15,19,20] the single peak in the GeneScan patterns of adult PB MNCs and especially mature TCRγδ$^+$ T cells (Figure 4) represents the Vγ9-Jγ1.2 canonical rearrangement. The majority of expanded Vγ9-Jγ1.2$^+$ T-lymphocytes have canonical junctions, lacking random N-nucleotide insertion and joining the Vγ9 to the Jγ1.2 gene segment in an identical manner.[15]

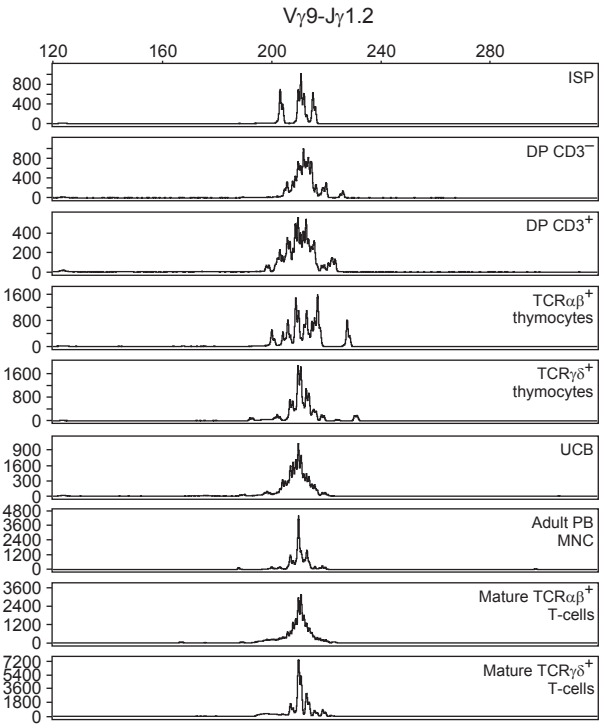

Figure 4. GeneScan analysis of Vγ9-Jγ1.2 gene rearrangements in thymic subsets, umbilical cord blood cells and mature T lymphocytes.
The single peak that can be detected in adult PB MNCs and especially mature TCRγδ$^+$ T-cells represents the Vγ9-Jγ1.2 canonical rearrangement.

Vβ17-Jβ2.7 GeneScan patterns

From the ISP subset onwards, Vβ17-Jβ2.7 rearrangements were in-frame as shown by the triplet peaks and were retained throughout all stages of development except for the TCRγδ$^+$ T cells (Figure 5). In contrast to the Vδ2-Jδ1 rearrangements in TCRγδ$^+$ cells, no clear difference between the GeneScan patterns of immature and mature TCRαβ$^+$ cells could be observed. This suggests that *TCRB* gene rearrangements in TCRαβ$^+$ T cells are primarily selected in the thymus and that there is no further (super)antigenic selection of *TCRB* gene rearrangements in the periphery. TCRγδ$^+$ thymocytes contained non-selected (out-of-frame) Vβ17-Jβ2.7 gene rearrangements which is in agreement with previous findings.[4,16] Peripheral TCRγδ$^+$ T cells also showed a random pattern representing non-selected gene rearrangements.

Vα4-Cα GeneScan patterns

Based on a *TCRA* multiplex RT-PCR approach, we selected Vα4-Cα as a representative rearranged transcript of the *TCRA* locus, because of its frequent usage in PB MNCs.

Non-selected Vα4-Cα transcripts (lacking the three-nucleotide spacing) were clearly

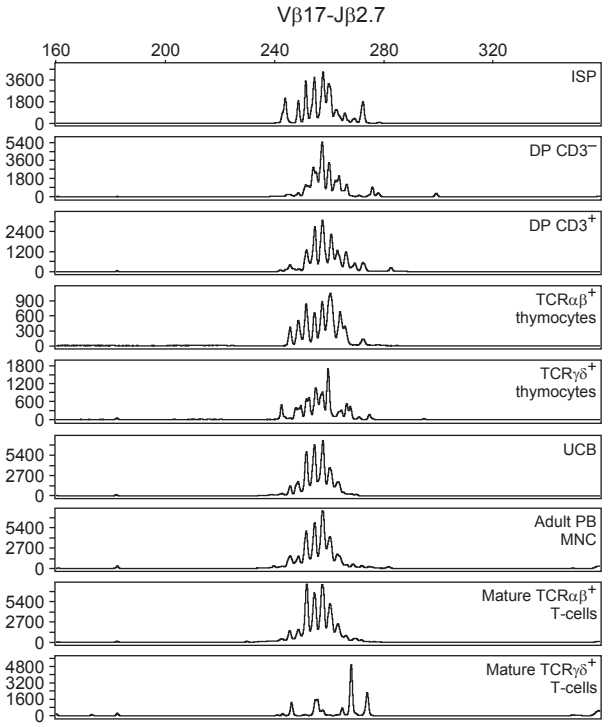

Figure 5. GeneScan analysis of Vβ17-Jβ2.7 gene rearrangements in thymic. subsets, umbilical cord blood cells, and mature T lymphocytes.

present in ISP cells and DP CD3$^-$ cells (Figure 6). The GeneScan pattern of DP CD3$^+$ cells showed clear triplet peaks (Figure 6), indicating that they have undergone in-frame selection of Vα4-Cα rearranged transcripts. The triplet peaks can also be easily identified in TCRαβ$^+$ thymocytes, UCB cells, PB MNCs, and mature TCRαβ$^+$ T cells. This confirms the data from Vβ17-Jβ2.7 GeneScan patterns suggesting that peripheral (antigenic) selection occurs at a lower level in TCRαβ$^+$ T cells than in TCRγδ$^+$ cells. TCRγδ$^+$ thymocytes and TCRγδ$^+$ lymphocytes hardly contained Vα4-Cα transcripts and the three-nucleotide spacing reflecting selection is clearly absent (Figure 6). The very low frequency of *TCRA* gene rearrangements in TCRγδ$^+$ T cells fits with the observation that TCRγδ$^+$ T cells rarely underwent *TCRD* gene deletion with subsequent *TCRA* rearrangement.

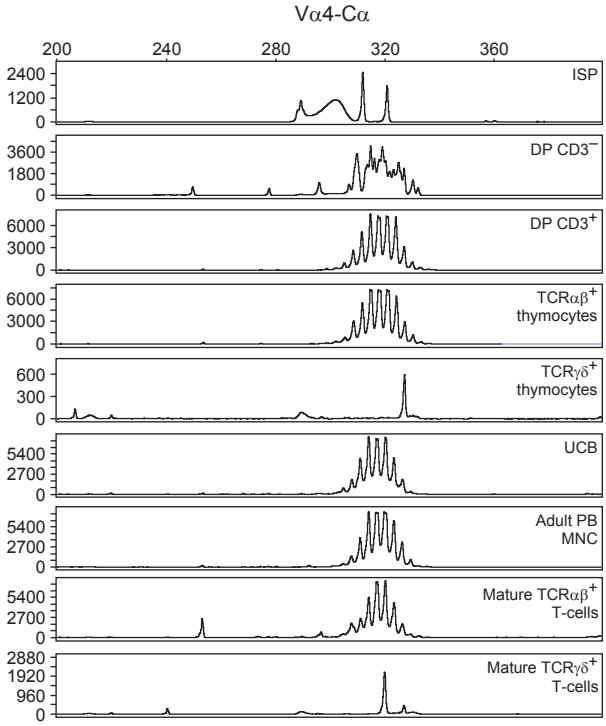

Figure 6. GeneScan analysis of Vα4-Cα rearranged transcripts in thymic subsets, umbilical cord blood cells, and mature T lymphocytes.

CONCLUSION

Selection for in-frame TCR gene rearrangements ensures that mature T-cells express functional TCR molecules on their membrane. For T cells of the TCRαβ lineage, selection of in-frame *TCRB* gene rearrangements already takes place at the ISP CD4$^+$ stage, while in-frame rearrangements of the *TCRA* locus were present from the DP CD3$^+$ thymic subset onwards. However, selection on functional TCR gene rearrangements not only occurs in the thymus but also in the periphery. This peripheral selection and expansion holds especially true for TCRγδ$^+$ T cells. In adults, the majority of TCRγδ$^+$ T cells present in the PB express a TCR consisting of a Vδ2-Jδ1-Cδ chain and a Vγ9-Jγ1.2-Cγ1 chain.[21] The massive peripheral expansion of Vγ9/Vδ2 expressing T cells is most probably antigen driven.[15,20] This selection is proven by the finding of an invariant T nucleotide in ~90% of the in-frame Vδ2-Jδ1 junctional regions in peripheral T lymphocytes. The invariant T nucleotide is a peripheral antigen selection marker that is absent in DNA isolated from thymocytes and UCB cells, but is present in DNA from normal adult PB MNC.[15,18]

In summary, this study shows the timing and extent of selection for in-frame TCR gene rearrangements during T-cell development (Table 1). PCR based GeneScan analysis of TCR gene rearrangements is demonstrated to be a very useful method to detect selected and unselected TCR gene patterns during normal T-cell differentiation. Since reduction of TCR repertoire and expansion of oligoclonal T cells is associated with various physiological and non-physiological conditions (e.g. aging, autoimmune diseases, Omenn syndrome),[22-24] the here described technology for TCR repertoire studies is broadly applicable.

Table 1. Selection on TCR gene rearrangements during human T-cell development.

	Thymocytes					Peripheral			
	ISP	DP CD3$^-$	DP CD3$^+$	TCRαβ$^+$	TCRγδ$^+$	UCB	MNC	TCRαβ$^+$	TCRγδ$^+$
Vδ1-Jδ1	-	-	-	-	++	+/-	-	-	-
Vδ2-Jδ1	-	-	-	-	-	-	+	-	++
VγI-Jγ1.3/2.3	-	-	-	-	+	-	-	-	-
Vγ9-Jγ1.2	-	-	-	-	-	-	+	-	++
Vβ17-Jβ2.7	+/-	+	+	+	-	++	++	++	-
Vα4-Cα	-	+/-	+	+	-	++	++	++	-

(-) No selection
(+/-) Limited selection
(+) Selection
(++) Strong selection
DP, double positive; ISP, immature single positive; MNC, mononuclear cell; UCB, umbilical cord blood

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Chapter 2.2

Human T-cell lines with well-defined T-cell receptor gene rearrangements as controls for the BIOMED-2 multiplex polymerase chain reaction tubes

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ABSTRACT

The BIOMED-2 multiplex polymerase chain reaction (PCR) tubes for analysis of immunoglobulin and T-cell receptor (TCR) gene rearrangements have recently been introduced as a reliable and easy tool for clonality diagnostics in suspected lymphoproliferations. Quality and performance assessment of PCR-based clonality diagnostics is generally performed using human leukemia/lymphoma cell lines as controls. We evaluated the utility of 30 well-defined human T-cell lines for quality performance testing of the BIOMED-2 PCR primers and protocols. The PCR analyses of the TCR loci were backed up by Southern blot analysis. The clonal *TCRB*, *TCRG*, and *TCRD* gene rearrangements were analyzed for gene segment usage and for the size and composition of their junctional regions. In 29 out of 30 cell lines, unique clonal TCR gene rearrangements could be easily detected. Besides their usefulness in molecular clonality diagnostics, these cell lines can now be authenticated based on their TCR gene rearrangement profile. This enables their correct use in molecular clonality diagnostics and in other cancer research studies.

INTRODUCTION

Diagnostics of suspected lymphoproliferations is mainly based on histomorphology or cytomorphology, supplemented with immunohistology and flowcytometric immunophenotyping. However, difficulties in making the final diagnosis occur in ~5-15% of cases. Especially in suspected T-cell proliferations discrimination between a malignant and a reactive T-cell population can be complicated, despite extensive immunophenotyping. Therefore, molecular analysis of T-cell receptor (TCR) genes is widely used to support the final diagnosis in such complicated cases. Based on the fact that T-cell malignancies are derived from a single malignantly transformed cell, all cells in principle have a common clonal origin and show clonally (identically) rearranged TCR genes. The finding of clonal TCR gene rearrangements therefore supports the diagnosis of a malignant T-cell proliferation, whereas in reactive lymphoproliferations the TCR genes are polyclonally rearranged. In addition to clonality assessment, TCR gene rearrangement studies are also used to evaluate the clonal relationship between two lymphoid malignancies in one patient and for staging of the disease.

The well-defined and fully standardized set of oligonucleotide primers and polymerase chain reaction (PCR) protocols of the BIOMED-2 Concerted Action BMH4-CT98-3936 has recently been introduced as a reliable strategy for clonality diagnostics.[1] The BIOMED-2 primers and protocols for multiplex PCR analysis of immunoglobulin (Ig) and TCR gene rearrangements, have now been further validated in large series of well-defined clinical samples, underlining the diagnostic specificity and applicability.[2-6] In addition, we recently demonstrated that the BIOMED-2 multiplex PCR approach can reliably replace "gold standard" Southern blot (SB) analysis in routine clonality diagnostics of lymphoproliferative disorders.[7] Because of the world-wide introduction of the BIOMED-2 primers and PCR protocols in routine clonality diagnostics, easily accessible positive controls for quality performance testing are required.

Quality and performance of molecular Ig/TCR clonality assays are assessed with control samples that are analyzed in parallel with the suspected patient samples. Both validated human leukemia/lymphoma cell lines and patient specimens can be used as positive controls. Cell lines are preferred, because they allow the usage of the same control material in unlimited amounts in all diagnostic laboratories.[8] To facilitate this process, we studied the TCR beta (*TCRB*), TCR gamma (*TCRG*) and TCR delta (*TCRD*) gene rearrangement pattern of a large series of 30 well-defined human T-cell lines to be used as controls for the BIOMED-2 multiplex PCR tubes.

MATERIALS AND METHODS

T-cell lines

Thirty human T-cell lines were cultured in Rosewell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum and antibiotics. All cell lines were free of mycoplasma contamination, as tested by indirect Hoechst 33258 DNA staining.[9] Immunophenotypical data of these cell lines were collected from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; www.dsmz.de) GmbH (Braunschweig, Germany) supplemented with our own results (Table 1). Table 1 also shows the disease-origin of the 30 evaluated T-cell lines. Virtually all cell lines are available at the DSMZ (www.dsmz.de), except for HUT 78/H9, HUT 102 and TALL-104, which were obtained from the American Type Culture Collection (ATCC; www.lgcpromochem-atcc.com Manassas, VA, USA). In addition, cell lines ARR,[10] DU.528,[11] JB6,[12] K-T1[13] and SUP-T3[13] were obtained via the original authors. For obtaining viable cells or DNA of the presented T-cell lines, the above-mentioned sources should be contacted.

DNA isolation

High molecular weight DNA was isolated from the cell lines, using a phenol-chloroform extraction-based protocol, followed by ethanol precipitation and resolution in Tris ethy lenediaminetetraacetic acid buffer. Alternatively, DNA was isolated using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol.

Southern blot analysis

DNA (20 μg) was digested with the appropriate restriction enzymes, size fractionated in 0.7% agarose gels and transferred to nylon filters as described before.[14] The TCR gene rearrangements were detected using ^{32}P random oligonucleotide labeled probes. *TCRB* rearrangements were analyzed by use of the Dβ1 upstream (TCRBD1U), Dβ1 (TCRBD1), Jβ1 (TCRBJ1), Dβ2 upstream (TCRBD2U), Dβ2 (TCRBD2), Jβ2 (TCRBJ2) and Cβ (TCRBC) probes in *Bgl*II, *Eco*RI and *Hin*dIII digests.[15] *TCRG* rearrangements were analyzed by use of the Jγ (TCRGJ1.3) (cross-hybridization with Jγ2.3), TCRGJ2.1 (cross-hybridization with Jγ1 region) and Cγ probes in *Bgl*II and *Eco*RI digests.[16] *TCRD* rearrangements were analyzed by use of the Jδ1 (TCRDJ1), Jδ2 (TCRDJ2), Jδ3 (TCRDJ3) and Cδ (TCRDC4) probes in *Bgl*II, *Eco*RI and *Hin*dIII digests. *TCRD* deletions were analyzed using the δREC (TCRDRE) and pseudo Jα (TCRAPJ) probes in *Bgl*II, *Eco*RI and *Hin*dIII digests.[17] All probes were obtained from Dako A/S (Carpinteria, CA, USA).

Table 1. Immunophenotype of 30 human T-cell lines.

Cell line	T-cell malignancy of origin[a]	CD7	CD5	CD1[a]	CyCD3	CD3	TCR	CD4[b]	CD8	TdT	CD2
ARR	CD3- T-ALL	+	-	-	ND	-	-	-	-	ND	-
DU.528	CD3- T-ALL	+	ND	-	ND	-	-	-	-	-	-
H-SB2	CD3- T-ALL	+	+	-	+	-	-	-	-	-	-
RPMI 8402	CD3- T-ALL	+	+	-	+	-	-	-	-	+	+
CML-T1	CD3- T-ALL	+	+	-	ND	-	-	+	+	-	-
Karpas 45	CD3- T-ALL	+	+	-	+	-	-	+	+	+	+
KE-37/SKW-3	CD3- T-ALL	+	+	+	+	-	-	+	-	ND	+
SUP-T1	CD3- T-ALL	+	+	+	ND	-	-	+	+	+	-
SUP-T3	CD3- T-ALL	+	+	+	ND	-	-	+	+	+	+
MOLT 3/4	CD3- T-ALL	+	+	+	+	-	-	+	+	+	+
P12-Ichikawa	CD3- T-ALL	+	+	+	+	-	-	+	-	+	+
PF-382	CD3- T-ALL	+	+	+	ND	-	-	+	+	-	+
CCRF-CEM	TCRαβ$^+$ T-ALL	+	+	+	+	+	αβ	+	-	+	-
HPB-ALL	TCRαβ$^+$ T-ALL	+	+	+	+	+	αβ	+	+	+	+
JURKAT	TCRαβ$^+$ T-ALL	+	+	+	+	+	αβ	+	-	+	+
K-T1	TCRαβ$^+$ T-ALL	+	+	+	ND	+	αβ	-	-	+	+
TALL-1	TCRαβ$^+$ T-ALL	+	+	+	+	+	αβ	+	+	+	+
MOLT 16/17	TCRαβ$^+$ T-ALL	+	+	-	+	+	αβ	-	-	+	+
TALL-104	TCRαβ$^+$ T-ALL	+	ND	-	ND	+	αβ	-	+	ND	+
DND-41	TCRβδ$^+$ T-ALL	+	+	+	+	+	βδ	+	-	+	+
Loucy	TCRγδ$^+$ T-ALL	+	+	-	ND	+	γδ	-	-	-	-
MOLT 13	TCRγδ$^+$ T-ALL	+	+	-	+	+	γδ	-	-	+	-
Peer/Be13	TCRγδ$^+$ T-ALL	+	+	-	+	+	γδ	+	+	+	-
HUT 78/H9	CTCL (SS)	+	+	-	+	+	αβ	+	-	-	-
HUT 102	CTCL (MF)	-	+	-	-	-	-	+	-	-	+
MT-1	ATLL	-	+	-	ND	-	-	-	-	-	-
DEL[c]	ALCL	-	-	-	ND	-	ND	-	-	ND	-
JB6[c]	ALCL	+	-	ND	ND	-	-	ND	ND	ND	+
Karpas 299[c]	ALCL	-	+	ND	ND	-	-	+	-	ND	-
SU-DHL1[c]	ALCL	-	+	-	ND	-	-	-	-	-	-

Abbreviations: ALCL, anaplastic large cell lymphoma; ATLL, adult T-cell lymphoma leukemia; CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; SS, Sézary syndrome; T-ALL, T-cell acute lymphoblastic leukemia; ND, not done. Tint is used to emphasize the expression pattern of the tested leukocyte markers.

[a] Clone T6.

[b] Clone Leu 3a.

[c] Cell lines express CD30.

PCR amplification

Amplification reactions were performed in an automated thermocycler (model ABI 9600/2700; Applied Biosystems, Foster City, CA, USA) according to the BIOMED-2 multiplex PCR protocol.[1] All BIOMED-2 multiplex PCR kits were obtained from InVivoScribe Technologies (San Diego, CA, USA) (www.invivoscribe.com). After PCR amplification of *TCRB*, *TCRG* and *TCRD* gene rearrangements, products were subjected to heteroduplex analysis and/or GeneScan analysis for confirmation of the monoclonal character of the TCR gene rearrangements.[1,18]

Sequence analysis of TCR gene rearrangements

PCR products were in principle directly sequenced except for cell lines with more than one clonal PCR product. In such cases homoduplexes were excised from the polyacrylamide gel and eluted as described before.[19] The eluted PCR products were directly sequenced. Sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) either on the ABI377 fluorescent sequencer (Applied Biosystems), as previously described[20] or on an ABI 3100 Genetic Analyzer. Sequence reactions were carried out in both directions using the same mixture of multiple V, D, or J primers as used in the multiplex PCR reaction for which the sample was positive. Vβ, Dβ, Jβ, Vγ, Jγ, Vδ, Dδ, and Jδ gene segments as well as reading frames of the involved *TCRB*, *TCRG* and *TCRD* gene rearrangements were identified by comparison to published sequences, using the ImMunoGeneTics database.[21] In case of *TCRB* rearrangements Vβ gene designation was carried out according to Rowen *et al*.[22]

RESULTS

Human T-cell lines and their TCR gene rearrangement patterns

Table 2 summarizes the rearrangement patterns of the *TCRB*, *TCRG* and *TCRD* genes, as assessed by the BIOMED-2 multiplex PCR primers and protocols. For all multiplex combinations at least one positive cell line control was identified. In 29 cell lines clonal *TCRB* and *TCRG* gene rearrangements could be detected, which is in line with their T-cell origin. In seven out of these cell lines clonal *TCRD* gene rearrangements were found as well. No clonally rearranged TCR genes could be detected in cell line DEL.

The *TCRB* gene rearrangements found in the cell lines covered 18 out of 23 Vβ primers (not Vβ4, Vβ11, Vβ19, Vβ21, and Vβ22) both Dβ primers and ten out of thirteen Jβ primers (not Jβ1.4, Jβ2.4, and Jβ2.6). In case of *TCRG* gene rearrangements for each individual Vγ and Jγ primer a positive cell line control is available. Clonally rearranged *TCRD* genes of the total cell line panel covered two out of six Vδ primers, one out of two Dδ primers and two out of four Jδ primers.

The following cell lines, which cover the most frequently used gene segments can be used as positive controls for the multiplex combinations: RPMI 8402, JURKAT (*TCRB* tube A), MOLT 3/4, Peer/Be13 (*TCRB* tube B), HUT 78/H9, JURKAT (*TCRB* tube C), MOLT 13, RPMI 8402 (*TCRG* tube A), P12-Ichikawa, JURKAT (*TCRG* tube B), Loucy, and MOLT 13 (*TCRD*).

Table 2. Results of BIOMED-2 TCR multiplex PCR tubes in 30 human T-cell lines.

Cell lines	*TCRB*			*TCRG*		*TCRD*
	Tube A (Vβ-Jβ)	Tube B (Vβ-Jβ)	Tube C (Dβ-Jβ)	Tube A	Tube B	
ARR	+ (267)[a]	-	+ (304) / +(308)	+ (183)	+ (212)	+ (201)
DU.528	+ (272)	-	-	+ (223) / + (202)	-	-
H-SB2	+ (259)	-	-	+ (162)	+ (177)	-
RPMI 8402	+ (263) / +(261)	-	-	+ (224) / + (157)	-	-
CML-T1	+ (248) / +(241)	+ (271)	-	+ (197) / + (222)	-	-
Karpas 45	-	+ (275)	-	+ (203) / + (158)	-	-
KE-37/SKW-3[b]	+ (271)	-	-	+ (226)	+ (181)	-
SUP-T1	-	+ (257)	-	+ (215) / + (220)	-	-
SUP-T3	-	+ (267)	-	+ (214) / + (203)	-	+ (170)
MOLT 3/4	-	+ (274) /+ (264)	-	+ (223) / + (242)	-	-
P12-Ichikawa	-	+ (269)	+ (301)	+ (223)	+ (175)	+ (185)
PF-382	+ (261)	+ (272)	-	+ (183) / + (158)	-	-
CCRF-CEM	-	+ (254)	-	+ (212) / + (203)	-	-
HPB-ALL	-	+ (251) / +(263)	-	+ (219) / + (231)	-	-
JURKAT	+ (265)	-	+ (311)	+ (212)	+ (116)	-
K-T1	+ (257)	+ (266)	-	+ (203)	+ (167)	+
TALL-1	-	+ (260) /+ (263)	-	+ (212)	+ (97)	-
MOLT 16/17	+ (262)	+ (272)	-	+ (218) / + (229)	-	-
TALL-104	-	+ (272)	-	+ (214) / + (157)	-	-
DND-41	+ (259) / (269)	-	-	+ (213) / + (193)	-	+ (186)
Loucy	+ (270)	+ (269)	-	+ (209)	+ (187)	+ (151) / +(211)
MOLT 13	+ (267)	-	+ (301) / (192)	+ (215) / + (236)	-	+ (174)
Peer/Be13[b]	+ (260)	+ (269)	-	+ (212)	+ (167)	+ (183)
HUT 78/H9	+ (264)	-	+ (299) / + 192)	+ (221) / + (239)	-	-
HUT 102	+ (256)	-	+ (199)	+ (212) /+ (240)	-	-
MT-1	-	+ (266)	-	+ (212) / + (156)	-	-
DEL	-	-	-	-	-	-
JB6	-	+ (268)	+ (303)	+ (224) / + (157)	-	-
Karpas 299	+ (271)	-	+ (306)	+ (251) / + (213)	-	-
SU-DHL-1	-	+ (260)	-	+ (202) / + (223)	-	-

Abbreviations: PCR, polymerase chain reaction; TCR, T-cell receptor.

[a] Numbers in brackets indicate exact sizes of PCR products in nucleotides as calculated based on sequence results.

[b] Identical TCR gene rearrangements were detected in these cell lines which is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that SKW-3 is a derivative of KE-37 and Be13 is derived from cell line Peer; see discussion.

Detailed configuration of *TCRB* genes

The *TCRB* gene configuration of all cell lines is summarized in Table 3. Known translocations involving the *TCRB* locus are indicated in the footnotes of the table. PCR analysis was performed on all cell lines, whereas SB data were obtained in 28 cell lines. Biallelic complete (Vβ-Jβ) and/or incomplete (Dβ-Jβ) gene rearrangements were found by multiplex PCR and SB analysis in 20 cell lines. In addition, complete Vβ-Jβ gene rearrangements were found in cell line KE-37, in which no SB analysis was performed.

In seven cell lines the *TCRB* gene rearrangements on the second allele as detected by SB analysis, could not be identified by multiplex *TCRB* PCR analysis. In cell lines H-SB2, SUP-T1, and SUP-T3 this fits with the presence of a *TCRB* gene translocation involving the genes *LCK*, *NOTCH1*, and *TAL2*, respectively.[13,23,24] Split signal FISH using *TCRB* probes was performed in cell lines Karpas 45 and TALL-104 and did not demonstrate chromosomal aberrations (Cauwelier *et al*.[25] and MacLeod, personal communication). The *TCRB* gene rearrangement on the second allele was identified by reverse transcriptase PCR analysis in these cell lines. Thereafter, these gene rearrangements were confirmed by singleplex PCR analysis using BIOMED-2 primers, suggesting competition for the second allele in the multiplex PCR. In cell line DU.528 a second rearrangement between Dβ1 and Dβ2 was identified by ligation-mediated (LM) PCR using protocols as described.[26] Logically, this specific *TCRB* recombination will not be detected by the BIOMED-2 primers. Previously, MT-1 was demonstrated to contain a non-functional recombination between Jβ2.3 and a suggested Vβ like sequence,[27] which can not be detected by the Vβ primers used in the current study.

PCR and SB analysis of TCR genes demonstrated a biallelic germline configuration of the *TCRB* locus in cell line DEL, whereas cell line SU-DHL-1 contained a monoallelic clonal *TCRB* gene rearrangement with a germline *TCRB* configuration on the second allele.

Detailed configuration of *TCRG* genes

Table 4 summarizes the *TCRG* gene configuration of all cell lines. Of the 30 cell lines, 28 were analyzed by multiplex PCR and SB analysis, giving completely concordant results. Biallelic clonally rearranged *TCRG* genes could be detected in all cell lines, except for cell line DEL.

Detailed configuration of *TCRD* genes

The *TCRD* gene configuration of all cell lines is summarized in Table 5. Known translocations involving the *TCRD* locus are indicated in the footnotes of Table 5. All 30 cell lines were studied by multiplex PCR analysis, whereas SB analysis was performed in 28 cell lines; cell lines DEL and KE-37 were studied by multiplex PCR analysis only and no clonal *TCRD* gene rearrangements were detected. Biallelic deletions of the *TCRD* gene were found in 19 T-cell lines by SB analysis; no monoclonal *TCRD* gene rearrangements were found by BIOMED-2 multiplex PCR analysis in 18 out of these cell lines. However, in KT-1 sequence analysis of the clonal band revealed a Vδ4(Vα14)-Jα45 gene rearrangement, which is due to cross annealing of the Dδ2 primer in a reverse manner with the Jα45 gene segment.

Table 3. *TCRB* gene configuration in 30 human T-cell lines.

Cell lines	SB analysis	Heteroduplex PCR analysis and sequencing											
		Jβ gene segments											
		Jβ1.1	Jβ1.2	Jβ1.3	Jβ1.4	Jβ1.5	Jβ1.6	Jβ2.1	Jβ2.2	Jβ2.3	Jβ2.4	Jβ2.5	Jβ2.6	Jβ2.7
ARR	R/R/R	Dβ1 (0)					Dβ1 (0)							Vβ16 (+)
DU.528	R/R[a]								Vβ3 (+)					
H-SB2	R/R[b]	Vβ5 (+)												
RPMI 8402	R/R					Vβ24 (+)								Vβ17 (-)
CML-T1	R/R/R											Vβ17 (+)		Vβ5 (+) / Vβ12 (+)
Karpas 45	R/R[c]											Vβ2 (+)		
KE-37/SKW-3[d]	ND					Vβ9 (-)								
SUP-T1	R/R[e]							Vβ1 (+)						
SUP-T3	R/R[f]									Vβ3 (+)				
MOLT 3/4	R/R							Vβ2 (+)				Vβ12 (-)		
P12-Ichikawa	R/R					Dβ1 (0)		Vβ13 (+)						
PF-382	R/R							Vβ2 (+)						Vβ6 (-)
CCRF-CEM	R/R[g]									Vβ9 (+)				
HPB-ALL	R/R											Vβ5 (+) / Vβ6 (-)		
JURKAT	R/R		Vβ8 (+)	Dβ1 (0)										
K-T1	R/R		Vβ15 (+)							Vβ18 (+)				
TALL-1	R/R									Vβ5 (-)		Vβ2 (+)		
MOLT 16/17	R/R	Vβ14 (-)								Vβ2 (+)				
TALL-104	R/R[h]									Vβ2 (+)				
DND-41	R/R		Vβ18 (+)											Vβ13 (-)
Loucy	R/R							Vβ5 (+)	Vβ2 (-)					
MOLT 13	R/R/R	Vβ12 (+)				Dβ1 (0)				Dβ2 (0)				
Peer/Be13[d]	R/R									Vβ7 (+)				Vβ10 (-)
HUT 78/H9	R/R/R		Dβ1 (0) / Vβ23 (+)							Dβ2 (0)				
HUT 102	R/R		Vβ20 (+)					Dβ2 (0)						
MT-1	R/R[i]									Vβ2 (+)				
DEL	G/G[j]													
JB6	R/R								Dβ1 (0)	Vβ8 (+)				
Karpas 299	R/R						Dβ1 (0)							Vβ2 (+)
SU-DHL-1	R/G									Vβ14 (+)				

Abbreviations: G, germline configuration; ND, not done; PCR, polymerase chain reaction; R, rearranged allele; SB, Southern blot.
(+) in-frame gene rearrangement , (-) out-of-frame gene rearrangement, (0) reading frame not applicable.
[a]A second rearrangement between Dβ1 and Dβ2 was identified by ligation mediated (LM) PCR, [b] t(1;7)(p34;q34)[23], [c] A Vβ5-Jβ2.1 (-) gene rearrangement was detected by reverse transcriptase (RT) PCR analysis, [d] Identical *TCRB* gene rearrangements were detected in these cell lines which is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that SKW-3 is a derivative of KE-37 and Be13 is derived from cell line Peer, [e] t(7;9)(q34;q34.3)[13], [f] t(7;9)(q34;q32)[24], [g] In addition clonal Vβ1-Dβ2 and Jβ1.5-Dβ-Jβ2.1 gene rearrangements were detected as previously described[36], [h] A Vβ15-Jβ2.2 (-) gene rearrangement was detected by RT PCR analysis, [i] MT-1 was demonstrated to contain a non-functional recombination between Jβ2.3 and a suggested Vβ like sequence[27], [j] Germline configuration of the *TCRB* locus has been reported.[37]

Table 4. *TCRG* gene configuration in 30 human T-cell lines.

Cell lines	SB analysis	Heteroduplex PCR analysis and sequencing								
		Vγ gene segments								
		Vγ2	Vγ3	Vγ4	Vγ5	ψVγ7	Vγ8	Vγ9	ψVγ10	ψVγ11
ARR	R/R			Jγ1.3/2.3 (-)				Jγ1.1 (+)		
DU.528	R/R	Jγ1.3/2.3 (-)					Jγ1.3/2.3 (-)			
H-SB2	R/R							Jγ1.3/2.3 (-)	Jγ1.3/2.3 (-)	
RPMI 8402	R/R			Jγ1.3/2.3 (+)					Jγ1.3/2.3 (-)	
CML-T1	R/R	Jγ1.3/2.3 (+)		Jγ1.3/2.3 (-)						
Karpas 45	R/R						Jγ1.3/2.3 (+)		Jγ1.3/2.3 (+)	
KE-37/SKW-3[a]	ND						Jγ1.3/2.3 (-)	Jγ1.3/2.3 (+)		
SUP-T1	R/R		Jγ1.3/2.3 (+)	Jγ1.3/2.3 (-)						
SUP-T3	R/R				Jγ1.3/2.3 (-)		Jγ1.3/2.3 (+)			
MOLT 3/4	R/R	Jγ1.1 (-) Jγ2.1 (-)								
P12-Ichikawa	R/R			Jγ1.3/2.3 (-)				Jγ1.3/2.3 (+)		
PF-382	R/R			Jγ1.3/2.3 (-)					Jγ1.3/2.3 (+)	
CCRF-CEM	R/R		Jγ1.3/2.3 (+)	Jγ1.3/2.3 (+)						
HPB-ALL	R/R		Jγ1.3/2.3 (-) / Jγ1.3/2.3 (-)							
JURKAT	R/R						Jγ1.3/2.3 (+)			Jγ1.3/2.3 (-)
K-T1	R/R						Jγ1.3/2.3 (+)	Jγ1.3/2.3 (-)		
TALL-1	R/R				Jγ1.3/2.3 (+)					Jγ1.3/2.3 (-)
MOLT 16/17	R/R						Jγ1.3/2.3 (+) / Jγ2.1 (+)			
TALL-104	R/R						Jγ1.3/2.3 (-)		Jγ1.3/2.3 (-)	
DND-41	R/R				Jγ1.3/2.3 (-)		Jγ1.3/2.3 (-)			
Loucy	R/R	Jγ1.3/2.3 (+)						Jγ1.3/2.3 (+)		
MOLT 13	R/R		Jγ1.3/2.3 (+)				Jγ1.1 (-)			
Peer/Be13[a]	R/R						Jγ1.3/2.3 (+)	Jγ1.3/2.3 (-)		
HUT 78/H9	R/R				Jγ1.3/2.3 (+)		Jγ1.1 (-)			
HUT 102	R/R					Jγ1.3/2.3 (+)	Jγ1.1 (+)			
MT-1	R/R	Jγ1.3/2.3 (+)							Jγ1.3/2.3 (-)	
DEL	ND									
JB6	R/R	Jγ1.3/2.3 (+)							Jγ1.3/2.3 (-)	
Karpas 299	R/R	Jγ2.1 (-)					Jγ1.3/2.3 (-)			
SU-DHL-1	R/R	Jγ1.3/2.3 (-)		Jγ1.3/2.3 (-)						

Abbreviations: ND, not done; PCR, polymerase chain reaction; R, rearrangement of the involved gene segment; SB, Southern blot.
(+) in-frame gene rearrangement , (-) out-of-frame gene rearrangement, (0) reading frame not applicable.
R, rearrangement of the involved gene segment; ND, not done.
[a] identical *TCRG* gene rearrangements were detected in these cell lines which is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that SKW-3 is a derivative of KE-37 and Be13 is derived from cell line Peer.

In five cell lines monoallelic clonal *TCRD* gene rearrangements were found by both SB and PCR analysis (Table 5). SB analysis demonstrated biallelic clonally rearranged *TCRD* genes in cell lines Loucy and MOLT 13, while this could be confirmed by PCR analysis only in cell line Loucy (Table 5). In cell line MOLT 13, a monoallelic *TCRD* gene rearrangement was detected by PCR analysis. A second rearrangement between Jδ1 and a sequence located ~11kb downstream of the *TCRD* deleting element δREC and ~25 kb upstream of Vδ2 was identified by LM PCR analysis. Of note, 20 nucleotides downstream of the junction we observed a recombination signal sequence (RSS) resembling element (CACCATTATGCATG CTGGATATCACACTGAACAAACACT). Using the RSS Project Data Base Search Program (http://host10.bioinfo3.servers.ifom-ieo-campus.it/rss/) that is based on the 'recombination information content' (RIC) algorithm as described by Cowell *et al.*[28] this element was predicted to be a functional 23 base-pair spacer RSS.

In addition, SB analysis of cell lines DU.528, RPMI 8402, and TALL-104 detected monoallelic *TCRD* gene rearrangements, which have been shown earlier to represent translocations involving the genes *TAL1*, *LMO1* and *LMO2*, respectively;[11,29,30] logically these *TCRD* gene translocations were not detected by the applied BIOMED-2 *TCRD* PCR analysis.

Identification of gene segments and junctional regions

Gene segment usage and junctional region composition of *TCRB*, *TCRG*, and *TCRD* gene rearrangements are summarized in Supplementary Tables 1-3 (designated for website publication only).

DISCUSSION

We studied a large group of 30 human well-defined T-cell lines in order to characterize the *TCRB*, *TCRG*, and *TCRD* gene configuration by SB analysis and BIOMED-2 multiplex PCR analysis. This immunogenotypic characterization was in accordance with their T-cell origin in 29 cell lines, as clonal TCR gene rearrangements could be easily demonstrated. Only cell line DEL appeared to have germline TCR genes, fitting with its origin of anaplastic large cell lymphoma (null-cell type).[31]

As the BIOMED-2 primers and PCR protocols are now widely used in routine clonality diagnostics, appropriate controls are required for quality control. We defined the configuration of the *TCRB*, *TCRG*, and *TCRD* loci of the 29 positive cell lines, making them suitable as positive and negative controls in both multiplex and singleplex PCR assays. The exact sizes of the BIOMED-2 PCR products as presented in Table 2 are especially helpful in GeneScan experiments, in which PCR products are easily discriminated on basis of their size.

The cell lines are derived from different types of lymphoid malignancies, which represent the malignant counterparts of the different stages of lymphoid development.[32] Our study included 23 cell lines from immature (IM) T-cell malignancies and seven cell lines from mature (post-thymic) T-cell malignancies. Recently, Asnafi *et al.*[33] demonstrated that IM T-cell malignancies can be classified on basis of their TCR gene status, reflecting the different stages of early T-cell development. On basis of immunophenotypic and molecular data, we

Table 5. *TCRD* gene configuration in 30 human T-cell lines.

Cell lines	SB analysis	Heteroduplex PCR analysis and sequencing
ARR	R/D	Vδ1-Jδ1 (+)
DU.528	D/R[a]	
H-SB2	D/D[b]	
RPMI 8402	D/R[c]	
CML-T1	D/D	
Karpas 45	D/D	
KE-37/SKW-3	ND	
SUP-T1	D/D	
SUP-T3	D/R	Vδ1-Jδ1 (-)
MOLT 3/4	D/D	
P12-Ichikawa	D/R	Vδ1-Jδ1 (-)
PF-382	D/D	
CCRF-CEM	D/D	
HPB-ALL	D/D	
JURKAT	D/D	
K-T1	D/D	Vδ4(Vα14)-Jα45[d]
TALL-1	D/D	
MOLT 16/17	D/D	
TALL-104	D/R[e]	
DND-41	D/R	Vδ1-Jδ1 (+)
Loucy	R/R	Dδ2-Jδ1 (0) / Vδ6-Jδ2 (+)
MOLT 13	R/R[f]	Vδ1-Jδ1 (+)
Peer/Be13[g]	D/R	Vδ1-Jδ1 (+)
HUT 78/H9	D/D	
HUT 102	D/D	
MT-1	D/D	
DEL	ND	
JB6	D/D	
Karpas 299	D/D	
SU-DHL-1	D/D	

Abbreviations: D, deleted; ND, not done; PCR, polymerase chain reaction; R, rearrangement of the involved gene segment; SB, Southern blot.
(+) in-frame gene rearrangement , (-) out-of-frame gene rearrangement, (0) reading frame not applicable.
[a] t(1;14)(p33;q11)[11].
[b] Clonal δREC-ψJα and δREC-Jα gene rearrangements could be found.
[c] t(11;14)(p15;q11)[29].
[d] Gene rearrangement was detected due to cross reactivity of Dδ2 primer in a reverse manner with the Jα45 gene segment.
[e] t(11;14)(p13;q11)[30].
[f] A second rearrangement between Jδ1 and a sequence located ~11kb downstream of the *TCRD* deleting element δREC and ~25 kb upstream of Vδ2 was identified by ligation mediated (LM) PCR.
[g] Identical *TCRD* gene rearrangements were detected in these cell lines which is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that Be13 is derived from cell line Peer.

tried to classify the more IM T-cell lines according to the scheme proposed by Asnafi *et al*.:[33] IM β/pre αβ (n=12), sCD3$^+$/TCRαβ$^+$ (n=7), and sCD3$^+$/TCRγδ$^+$ (n=3) (Table 1). The curious cell line DND-41 is TCRβδ$^+$ and can therefore not be classified according to this scheme. Remarkably, no IMδ or IMγ cell lines were found. Previously, it has been suggested that IM T-cell lines might be used as model system for early T-cell differentiation.[34] However, 18 of the 23 T-ALL/T-LBL derived T-cell lines had a variable immunophenotype but a comparable TCR gene configuration, suggesting that these cell lines might not necessarily reflect T-cell development in all aspects. Nevertheless, the T-cell lines that carry known translocations might be used as model systems for further oncogenetic studies.

Cross-contamination of cell lines during long-term culture is a well-recognized problem and might have major impact on cancer research in general.[35] It is crucial in all cell-line experiments that the used cell lines faithfully correspond to their original source. As human T-cell lines are established from T-cell malignancies or normal T-cells,[32] each cell line contains unique monoclonal TCR gene rearrangements. Molecular gene rearrangement analysis and additional sequencing is therefore an excellent tool for cell line authentication. The 29 positive cell lines analyzed in our study can now be easily identified on basis of their unique TCR gene rearrangement profile as provided in detail in Supplementary Tables 1-3. We detected an identical TCR gene rearrangement profile in cell lines KE-37 and SKW-3. Cell lines Peer and Be13 showed identical *TCRB*, *TCRG*, and *TCRD* gene rearrangements as well. This is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that SKW-3 is a derivative of KE-37 and Be13 is derived from cell line Peer (DSMZ; www.dsmz.de).

The aim of this study was to evaluate the TCR gene rearrangement pattern of a large group of human T-cell lines in order to define appropriate positive controls for application of the BIOMED-2 multiplex PCR tubes in routine clonality diagnostics of T-cell proliferations. We calculated the exact oligonucleotide sizes of all clonal *TCRB*, *TCRG*, and *TCRD* gene rearrangements and analyzed their junctional regions. Except for cell line DEL, unique clonal TCR gene rearrangements could be easily identified. Besides their usefulness in a diagnostic setting, authentication of these cell lines based on their TCR profile, enables their correct use in cancer research studies.

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Supplementary Table 1. *TCRB* gene configuration.

T-cell line	Vβ		Junction		Jβ	In-frame	
ARR		Dβ1	GGGACAGGAGGAAAGG	ACTGAAGCTTTC	Jβ1.1	n.a.	-4 (8) -5
ARR		Dβ1	GGGACAGGGAAGG	CTATAATTC	Jβ1.6	n.a.	-3 (4) -3
ARR	Vβ16S1A1N2	TGTGCCAGCAGC	CTTCTAGCGGGAGTCC	CCTACGAGCAGTACTTC	Jβ2.7	Y	0 (0) -4 (10) -2 (3) -2
DU.528[a]	Vβ3S1	TGTGCCAGCAGTT	CACGACAGGGGGCGCTTTTTAA	GAACACCGGGGAGCTGTTTTTT	Jβ2.2	Y	-4 (3) -2 (10) 0 (9) -1
H-SB2	Vβ5S1A1	TGCGCCAGCAGCT	GGACAGGGTATGG	GAACACTGAAGCTTTC	Jβ1.1	Y	-3 (0) -1 (8) -3 (5) -1
RPMI 8402	Vβ17S1A3T	TGTGCC	TATCAGGGGAGAGTGGGCT	CTCCTACGAGCAGTAC	Jβ2.7	N	-6 (3) -4 (6) -2 (10) 0
RPMI 8402	Vβ24S1A3T	TGTGCCACCAGCAGAGA	TGGAAAGGGC	AGCAATCAGCCCCAGCAT	Jβ1.5	Y	0 (5) -5 (4) -3 (1) -1
CML-T1	Vβ5S4A1T	TGTGCCAGCAG	-	CTCCTACGAGCAGTAC	Jβ2.7	Y	-1 (0) 0
CML-T1	Vβ12S1A1N2	TGTGCCATCAGT	-	TACGAGCAGTAC	Jβ2.7	Y	-5 (0) -4
CML-T1	Vβ17S1A3T	TGTGCC	TCCACTACCCGGACTAGCGGGGGG	CAAGAGACCCAGTAC	Jβ2.5	Y	-6 (10) -1 (14) -1 (0) -2
Karpas 45	Vβ2S1A1	TGCAGTGCTAG	TGGTTCTAGCGGAGCCAGCA	AAGAGACCCAGTACTTC	Jβ2.5	Y	-3 (5) -4 (7) -5 (8) -3
Karpas 45	Vβ5S1A1	TGCGCCAG	TATCTCAGCAGGTAACTAGGAC	CAATGAGCAGTTC	Jβ2.1	N	-8 (14) -3 (5) -8 (3) -6
KE-37/SKW-3[d]	Vβ9S2A2PT	TGTGCCAGCAGCCAAGA	CTCCGGGACAGGGGCGGGTA	GCAATCAGCCCCAGCATTTT	Jβ1.5	N	0 (4) 0 (10) -2 (6) -2
SUP-T1	Vβ1S1A1N1	TGTGCCAGCAGCGTAG	GTGGTAGCTTGAAA	CAGTTCTTC	Jβ2.1	Y	0 (14) -13
SUP-T3	Vβ3S1	TGTGCCAGCAGTTTA	GGACAGGCCCTC	AGCACAGATACGCAGTAT	Jβ2.3	Y	-2 (0) -1 (7) -4 (5) 0
MOLT 3/4	Vβ2S1A1	TGCAGTGCTAGAG	GTCGACTAGCGATCCAAAA	AATGAGCAGTTC	Jβ2.1	Y	-1 (3) -2 (8) -6 (8) -7
MOLT 3/4	Vβ12S1A1N2	TGTGCCATCAGTGAG	CCGACAGGGATCAG	AAGAGACCCAGTAC	Jβ2.5	N	-2 (2) -2 (7) -3 (5) -3
P12-Ichikawa	Vβ13S3	TGTGCCAGCAGT	ATCCGGACGCCTCACCCTCA	CTACAATGAGCAGTTC	Jβ2.1	Y	-5 (4) -1 (4) -7 (12) -3
P12-Ichikawa		Dβ1	GGGACAGGTATT	TCAGCCCCAGCATTTT	Jβ1.5	n.a.	-4 (4) -6
PF-382	Vβ2S1A2	TGCAGTGC	CACGTCGGGACTAGCGGGCTTA	AATGAGCAGTTC	Jβ2.1	Y	-1 (6) 0 (12) -4 (4) -7
PF-382	Vβ6S9P	TGTGCCAGAAGCT	CAGAGGCTTTCAGGGCGAG	CGAGCAGTACTTCG	Jβ2.7	N	-3 (10) -4 (5) -3 (4) -6
CCRF-CEM[b]	Vβ9S1A1T	TGTGCCAGCAGCC	TTGGG	ACAGATACGCAGTAT	Jβ2.3	Y	-4 (5) -3
HPB-ALL	Vβ5S3A2T	TGTGCCAGCAGCT	CGCGGAAA	ACCCAGTAC	Jβ2.5	Y	-3 (8) -8
HPB-ALL	Vβ6S1A3T	TGTGCCAGCAGC	CCAAGGGAGG	GAGACCCAGTAC	Jβ2.5	N	0 (4) -9 (6) -1 (0) -5
JURKAT	Vβ8S2A1T	TGTGCCAGCAGTTT	CTCGACCTGTTCGG	CTAACTATGGCTACACC	Jβ1.2	Y	-3 (14) 0
JURKAT		Dβ1	GGGACAGGGTTAGAA	CTCTGGAAACACC	Jβ1.3	n.a.	-3 (6) 0

T-cell line	Vβ		Junction		Jβ	In-frame	
K-T1	Vβ15S1	TGTGCCAC	GCCCCCCCGACAGTTTG	TAACTATGGCTACACC	Jβ1.2	Y	-10 (17) -1
K-T1	Vβ18S1	TGTGCCAGCTCACC	GAGACAGGCGACGG	CAGATACGCAGTATT	Jβ2.3	Y	-3 (14) -4
TALL-1	Vβ2S1A1	TGCAGTG	ACCCCAGCAG	AGAGACCCAGTAC	Jβ2.5	Y	-7 (10) -4
TALL-1	Vβ5S2	TGTGCCAGCAGC	GGGGACTAGCGGGTGAGG	GATACGCAGTATT	Jβ2.3	N	-4 (1) 0 (12) -4 (5) -6
MOLT 16/17	Vβ2S1A1	TGCAGTGC	AGGGGAGTCGGGGGGCGTA	ACAGATACGCAGTAT	Jβ2.3	Y	-6(10) -6 (6) 0 (3) -3
MOLT 16/17	Vβ14S1	TGTGCCAGCA	CCGACCCGGACAGGGGAATGG	ACTGAAGCTTTCTTT	Jβ1.1	N	-7 (7) -1 (9) -2 (5) -5
TALL-104	Vβ2S1A1	TGCAGTGCTA	AGCTTAGGGGGGGGCGCC	CAGATACGCAGTAT	Jβ2.3	Y	-4 (9)-6(6) 0 (3) -4
TALL-104	Vβ15S1	TGTGCCACCAGTGAT	CTGGGACAGGGA	ACCGGGGAGCTGT	Jβ2.2	N	-3 (2) 0 (9) -3 (1) -5
DND-41	Vβ13S2A1T	TGTGCCAGCAGTT	ACCCCCGACAGGGAGGAGAGGGG	CTCCTACGAGCAG	Jβ2.7	N	-4 (6) -2 (7) -3 (10) 0
DND-41	Vβ18S1	TGTGCCAGCTCACC	CGGGACAGGGAGA	TATGGCTACACC	Jβ1.2	Y	-3 (1) 0 (9) -3 (3) -5
Loucy	Vβ2S1A1	TGCAGTGCTAGAG	CAGAGGCGCAGG	GAACACCGGGGAGCTGTTT	Jβ2.2	N	-1 (8) -4 (4) -4 (0) -1
Loucy	Vβ5S2	TGTGCCAGCAGC	CGAGGGGAACCCCCCTGGA	CCTACAATGAGCAGTTC	Jβ2.1	Y	-4 (2) -5 (5) -2 (12) -2
MOLT 13	VB12S2A3T	TGCGCCAGCAG	GAGAGTTAGACGGGACAG	GAACACTGAAG	Jβ1.1	Y	-1 (11) 0 (7) -5 (0) -1
MOLT 13		Dβ1	GGGGGCTCAA	CCCCAGCATTTT	Jβ1.5	n.a.	0 (4) -10
MOLT 13		Dβ2	CGGGAGGGCCCG	ACAGATACG	Jβ2.3	n.a.	0 (4) -3
Peer/Be13[d]	Vβ7S3A2T	TGTGCCAGCAGCCAAGA	GACTCTACCACTCCGCCTGGG	TACGCAGTAT	Jβ2.3	Y	0 (21) -8
Peer/Be13[d]	Vβ10S1P	TGTGCCAGCA	CGGTGGAGCAGTACCC	CCTACGAGCAGTAC	Jβ2.7	N	-7 (16) -2
HUT 78/H9	Vβ23S1A1T	TGTGCCAGCAGC	ACCAGCCCAGGGGGCCGG	GGCTACACC	Jβ1.2	Y	0 (7) -4 (8) 0 (3) -8
HUT 78/H9		Dβ1	GGGACAGGGAGA	ATGGCTACACC	Jβ1.2	n.a.	-3 (3) -6
HUT 78/H9		Dβ2	GGGACTAGCGGGGGGA	ACAGATACGCAGTAT	Jβ2.3	n.a.	-1 (1) -3
HUT 102	Vβ20S1A3T	TGTGCCTGGAGT	AGGCAGGAGGCGGTTGGC	GGCTACACCTTC	Jβ1.2	Y	0 (5) -10 (5) -1 (8) -8
HUT 102		Dβ2	GGGACTAGCGGGGGGGTTG	CTCCTACAATGAGCAGTTC	Jβ2.1	n.a.	-4 (7) 0
MT-1[c]	Vβ2S1A1	TGCAGTGCTA	AACTAGTAGGT	ACAGATACGCAGTAT	Jβ2.3	Y	-4 (1) -3 (5) -8 (5) -3
DEL							
JB6	Vβ8S2A2N2T	TGTGCCAGCAG	ACTAGCGGGGGGAC	CAGATACGCAGTATT	Jβ2.3	Y	-1 (0) -3 (12) -1 (2) -4
JB6		Dβ1	TGTAACATTGTGGGGACGGAGAT	CACCGGGGAGCTGT	Jβ2.2	n.a.	-7 (6) -4
Karpas 299	Vβ2S1A1	TGCAGTGCTAGAG	CCCAGATAGGGTCTAGCCCCCT	CGAGCAGTACTTC	Jβ2.7	Y	-1 (12) -4 (5) -7 (5) -6

T-cell line	Vβ		Junction		Jβ	In-frame	
Karpas 299	Vβ2S1A1	TGCAGTGCTAGAG	CCCAGATAGGGTCTAGCCCCCT	CGAGCAGTACTTC	Jβ2.7	Y	-1 (12) -4 (5) -7 (5) -6
Karpas 299		Dβ1	GGGACAGGGGGCCT	TAATTCACCCCTCCACTTT	Jβ1.6	n.a.	0 (2) -6
SU-DHL-1	Vβ14S1	TGTGCCAGCAGTTT	CAGTAGGGGA	ACAGATACGCAGTATTTT	Jβ2.3	Y	-3 (4) -5 (5) -2 (1) -3
DU.528		Dβ1	GGGACAGGGGACTAGCGGGAGGGC	Dβ2			
CCRF-CEM	Jβ1.5	AATGCTGGGGCTGAT	AGCCCGATGTCC	GTTCTTCGGGCCAGG	Jβ2.1	n.a.	
CCRF-CEM	Vβ1S1A1N2T	TGTGCCAGCAGC	CCTGGACCCGGTACCAGGG	GTTACACCATGATACAAAAA	5' Dβ2RSS	n.a.	
MT-1		CAGACCTCTCTGTACATTTG	CACCAGCAGTCCATGGACTAGCG GGATT	ACAGATACGCAGTATTTT	Jβ2.3	n.a.	

[a] A second rearrangement between Dβ1 and Dβ2 was identified by ligation mediated (LM) PCR analysis
[b] In addition clonal Jβ1.5-Dβ-Jβ2.1 and Vβ1-Dβ2 gene rearrangements were detected as previously described (Duby et al, 1991)
[c] MT-1 was demonstrated to contain a non-functional recombination between Jβ2.3 and a suggested Vβ like sequence (Ikuta et al, 1985)
[d] Identical *TCRB* gene rearrangements were detected in these cell lines, which is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that SKW-3 is a derivative of KE-37 and Be13 is derived from cell line Peer.

Germline Dβ1: GGGACAGGGGGC Polymorphism in Dβ2 gene segment.
Germline Dβ2 according to X02987 database: GGGACTAGCGGGGGGG
Germline Dβ2 according to U66061 database: GGGACTAGCGGGAGGG
n.a., not applicable

N nucleotides P nucleotides

Gene segment usage and junctional region composition of *TCRB* gene rearrangements in 30 human T-cell lines

Supplementary Table 2. *TCRG* gene configuration.

T-cell line	Vγ		Junction		Jγ	In-frame	
ARR	Vγ4	TGTGCCACCTGGGA	-	GAACAACACTT	Jγ1.3/2.3	N	-4 (0) -29
ARR	Vγ9	TGTGCCTTGTGGGA	CTTTAAATTGGGTTCA	ACTGGTTGGTTCAAGATATTT	Jγ1.1	Y	-4 (16) -5
DU.528	Vγ8	TGTGCCA	AA	ATTATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N	-11 (2) -5
DU.528	Vγ2	TGTGCCACCTGGGACGG	CCCCTCTC	GAATTATTATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N	-1 (8) 0
H-SB2	Vγ9	TGTGCCTTGTGGGAGGTG	-	ATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	0 (0) -2
H-SB2	Vγ10	TGTGCTGCGTGG	GATTATCAG	TTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	-8 (9) -3
RPMI 8402	Vγ4	TGTGCCACCTGGGATGG	AGCGTCTAC	GAATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-1 (9) 0
RPMI 8402	Vγ10	TGTGCTGCGTGG	GGT	ATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	-8 (3) -2
CML-T1	Vγ2	TGTGCCACCTGGGA	GGGCCCCA	GCAGTGGAACAACACTG	Jγ1.3/2.3	Y	-4 (8) -23
CML-T1	Vγ4	TGTGCCACCTGGGATGGG	ACCAGGAG	ATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	0 (8) -2
Karpas 45	Vγ10	TGTGCTGCGTGG	GTA	AATTATTATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	Y	-8 (3) -1
Karpas 45	Vγ8	TGTGCCACCTGG	TCTA	AGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	Y	-6 (4) -11
KE-37/SKW-3[a]	Vγ8	TGTGCCACCTGGGA	CCGTCCGCCATCCCGT	ATTATTATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N	-4 (16) -2
KE-37/SKW-3[a]	Vγ9	TGTGCCTTGTGGGAGGTG	CC	GAATTATTATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	Y	0 (2) 0
SUP-T1	Vγ3	TGTGCCACCTGG	CGGAC	GAATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-6 (5) 0
SUP-T1	Vγ4	TGTGCCACCTGGGATGGG	CAATA	AATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	0 (5) -1
SUP-T3	Vγ5	TGTGCCACCTGGGACAGG	CCGGTGCAGG	GAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	0 (10) -12
SUP-T3	Vγ8	TGTGCCACCTGGG	TC	AAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-5 (2) -10
MOLT 3/4	Vγ2	TGTGCCACCT	TCG	GATATTT	Jγ1.1	N	-8 (3) -19
MOLT 3/4	Vγ2	TGTGCCACCTGGGA	GGGGGGTGTCT	TGATTGGATCAAGACGTTT	Jγ2.1	N	-4 (11) -7
P12-Ichikawa	Vγ4	TGTGCCACCTGGG	TTAAAATTCCCCT	AATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	-5 (13) -1
P12-Ichikawa	Vγ9	TGTGCCTTGTGGGAGGT	CTCCGCTT	AGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-1 (8) -11
PF-382	Vγ4	TGTGCCACCTGGGA	-	GAACAACACTT	Jγ1.3/2.3	N	-4 (0) -29
PF-382	Vγ10	TGTGCTGCGTGG	GATGAGGGC	TATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-8 (9) -7
CCRF-CEM	Vγ3	TGTGCCACCTGGGACAG	TTCCC	ATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-1 (5) -8
CCRF-CEM	Vγ4	TGTGCCACCTGGGAT	TAAGGGGGGTTG	GGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-3 (12) -22

T-cell line	Vγ	Junction		Jγ	In-frame	
HPB-ALL	Vγ3	TGTGCCACCTGGGACAGG	CAAA	AATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N 0 (4) -1
HPB-ALL	Vγ3	TGTGCCACCTGGGA	TAAACGCTGCCTTTCTGACGGGGGT	TTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N -4 (25) -6
JURKAT	Vγ8	TGTGCCACCTGG	AAATT	TTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y -6 (5) -3
JURKAT	Vγ11	TGTGCCTG	TCAGATCCTCACAGGGCGGGTT	TAAGAAACTCTTTGGCAGTGGAACAACACTG	Jγ1.3/2.3	N -13 (22) -9
K-T1	Vγ9	TGTGCCTT	CCGGCCCG	AAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N -10 (8) -10
K-T1	Vγ8	TGTGCCACCTGG	TC	TAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	Y -6 (2) -9
TALL-1	Vγ5	TGTGCCACCTGG	TC	GAATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTG	Jγ1.3/2.3	Y -6 (2) 0
TALL-1	Vγ11	TGTGCCTGCTGGATTAGGCAC	CC	AATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N 0 (2) -1
MOLT 16/17	Vγ8	TGTGCC	CCCCTGGAGG	TGGATCAAGACGTTT	Jγ2.1	Y -12 (10) -11
MOLT 16/17	Vγ8	TGTGCCACCTGG	ACAGATAGGATC	TATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y -6 (12) -4
TALL-104	Vγ8	TGTGCCACCTGGGAT	CTGG	TTATTATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N -3 (4) -3
TALL-104	Vγ10	TGTGCTGCGTGG	GACCGCAC	TATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N -8 (8) -7
DND-41	Vγ5	TGTGCCACC	GGCCACCTT	TTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N -9 (9) -3
DND-41	Vγ8	TGTGCCACCTGGGA	CACATAAGGGGG	ACAACACTT	Jγ1.3/2.3	N -4 (12) -31
Loucy	Vγ2	TGTGCCACCTGGG	GTTCCCAGGATTTCAC	CTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y -5 (16) -18
Loucy	Vγ9	TGTGCCTTGTGGGAG	CCCCCCCCCTC	GAATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y -3 (11) 0
MOLT 13	Vγ3	TGTGCCACCTGGGACAGG	CCCCGCCT	TAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y 0 (8) -9
MOLT 13	Vγ8	TGTGCCACCTGGGAT	-	CCACTGGTTGGTTCAAGATATTT	Jγ1.1	N -3 (0) -3
Peer/Be13[a]	Vγ8	TGTGCCACCTGG	AAGG	ATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y -6 (4) -2
Peer/Be13[a]	Vγ9	TGTGCCTT	CCGGCCCG	AAGAAACTCT	Jγ1.3/2.3	N -10 (8) -10
HUT 78/H9	Vγ5	TGTGCCACCTGGGACAGG	CTTACCT	ATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y 0 (7) -2
HUT 78/H9	Vγ8	TGTGCCACCTGG	ACACCTT	CACTGGTTGGTTCAAGATATTT	Jγ1.1	N -6 (7) -4
HUT 102	Vγ7	TGTGCCACCTGGGAC	GCGGCTG	ATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	Y -3 (7) -8
HUT 102	Vγ8	TGTGCCACCTGGGAT	GAGC	CCACTGGTTGGTTCAAGATATTT	Jγ1.1	Y -3 (4) -3
MT-1	Vγ2	TGTGCCACCTGGGACGGG	CC	TTATAAGAAACTCTTTGGCAGTGGAACAACACTG	Jγ1.3/2.3	Y 0 (2) -6
MT-1	Vγ10	TGTGCTGCGTGG	GAT	TTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N -8 (3) -3
DEL						

T-cell line	Vγ		Junction		Jγ	In-frame	
JB6	Vγ2	TGTGCCACCTGGG	GATCCATATAGGT	GAATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	Y	-5 (13) 0
JB6	Vγ10	TGTGCTGCGTGGT	CTTGAGGCTGGC	GAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	-7 (12) -12
Karpas 299	Vγ2	TGTGCCACCTGGGACGGG	GGTTAAGTTCCTT	TAGTGATTGGATCAAGACGTTT	Jγ2.1	N	0 (13) -4
Karpas 299	Vγ8	TGTGCCACCTGGGATAGG	CC	ATTATAAGAAACTCTTTGGCAGTGGAACAACACTT	Jγ1.3/2.3	N	0 (2) -5
SU-DHL-1	Vγ4	TGTGCCACCT	CTCCCAAAACCCCCAAG	ATTATTATAAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N	-8 (17) -2
SU-DHL-1	Vγ2	TGTGCCACCTGGG	C	AAGAAACTCTTTGGCAGTGGAACAACA	Jγ1.3/2.3	N	-5 (1) -10

[a] Identical *TCRG* gene rearrangements were detected in these cell lines, which is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that SKW-3 is a derivative of KE-37 and Be13 is derived from cell line Peer.

n.a., not applicable

N nucleotides P nucleotides

Gene segment usage and junctional region composition of *TCRG* gene rearrangements in 30 human T-cell lines

Supplementary Table 3. *TCRD* gene configuration.

T-cell line	Vδ		Junction		Jδ	In-frame	
ARR	Vδ1	TGTGCTCTT	CGGGAACGGGGGGATTTCTACCTGTTTGGGTCGGGGGAAGCGTACACGGAC	AAACTCATCTTT	Jδ1	Y	-8 (8) -3 (7) -3 (36) -8
DU.528							
H-SB2							
RPMI 8402							
CML-T1							
Karpas 45							
KE-37/SKW-3							
SUP-T1							
SUP-T3	Vδ1	TGTGCTCTTGGGGA	GGGGGCCTCC	CCGATAAACTCATCTTT	Jδ1	N	-3 (0) -3 (5) -5 (5) -3
MOLT 3/4							
P12-Ichikawa	Vδ1	TGTGCTCTTGGGGAACT	ACGACCTCTACTGGTCGAAG	CACCGATAAACTCATCTTT	Jδ1	N	0 (9) 0 (5) ~8 (6) ~1
PF-382							
CCRF-CEM							
HPB-ALL							
JURKAT							
K-T1							
TALL-1							
MOLT 16/17							
TALL-104							
DND-41	Vδ1	TGTGCTCTTGGGGAA	GCCTTCCGACCATCACGCACCTT	CACCGATAAACTCATCTTT	Jδ1	Y	-2 (1) 0 (6) -3 (16) -1
Loucy	Vδ6	TGTGCAGCAAG	CAAAGCTGCCGG	TTTGACAGCACAACTCTTCTTT	Jδ2	Y	-1 (12) -1
Loucy		Dδ2	TGTTTCATTGGCCCGGGGGGGGGGTTTACCGATAT	ACACCGATAAACTCATCTTT	Jδ1	n.a.	-12 (4) -3 (5) -5 (16) 0
MOLT 13[a]	Vδ1	TGTGCTCTTGGGGAAC	CTGGGGGGT	ACACCGATAAACTCATCTTT	Jδ1	Y	-1 (0) -1 (7) -5 (2) 0
Peer/Be13[b]	Vδ1	TGTGCTCTTGGG	ACGGGGGTGAGGGGACTCCAGG	ACACCGATAAACTCATCTTT	Jδ1	Y	-5 (10) -4 (5) -4 (7) 0
HUT 78/H9							
HUT 102							

T-cell line	Vδ	Junction	Jδ	In-frame
MT-1				
DEL				
JB6				
Karpas 299				
SU-DHL-1				
MOLT 13	ATGTGCAGCCAAGAGA CTGAGCCATT	ACTGCTGGCACTACTGGGGGA	Jδ1	n.a.

[a] A second rearrangement between Jδ1 and a sequence located ~11kb downstream of the *TCRD* deleting element δREC and ~25 kb upstream of Vδ2 was identified by ligation mediated (LM) PCR analysis
[b] Identical *TCRD* gene rearrangements were detected in these cell lines, which is in line with the findings of DSMZ investigators, who demonstrated by DNA fingerprinting that Be13 is derived from cell line Peer.

Dδ2 germline CCTTCCTAC
Dδ3 germline ACTGGGGGATACG

n.a., not applicable

N nucleotides P nucleotides

Gene segment usage and junctional region composition of *TCRD* gene rearrangements in 30 human T-cell lines

Chapter 2.3

BIOMED-2 multiplex immunoglobulin/T-cell receptor polymerase chain reaction protocols can reliably replace Southern blot analysis in routine clonality diagnostics

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ABSTRACT

To establish the most sensitive and efficient strategy of clonality diagnostics via immunoglobulin and T-cell receptor gene rearrangement studies in suspected lymphoproliferative disorders, we evaluated 300 samples (from 218 patients) submitted consecutively for routine diagnostics. All samples were studied using the BIOMED-2 multiplex polymerase chain reaction (PCR) protocol. In 176 samples Southern blot (SB) data were also available, and the two types of molecular results were compared. Results of PCR and SB analysis of both T-cell receptor and immunoglobulin loci were concordant in 85% of samples. For discordant results, PCR results were more consistent with the final diagnosis in 73% of samples. No false-negative results were obtained by PCR analysis. In contrast, SB analysis failed to detect clonality in a relatively high number of samples, mainly in cases of low tumor burden. We conclude that the novel BIOMED-2 multiplex PCR strategy is of great value in diagnosing patients with suspected B- and T-cell proliferations. Because of its higher speed, efficiency, and sensitivity, it can reliably replace SB analysis in clonality diagnostics in a routine laboratory setting. Just as with SB results, PCR results should always be interpreted in the context of clinical, immunophenotypical, and histopathological data.

INTRODUCTION

In most patients with suspected lymphoproliferative disorders, discrimination between reactive and malignant cell populations can be assessed by histomorphology or cytomorphology supplemented with immunohistochemistry or flowcytometric immunophenotyping. However, in 5 to 10% of patients, diagnosis is more complicated and less straightforward. In such cases, molecular gene rearrangement studies have proved useful as an additional diagnostic tool. Molecular clonality analysis is based on the fact that, in principle, all cells of a malignancy have a common clonal origin and show clonally (identically) rearranged immunoglobulin (Ig) or T-cell receptor (TCR) genes. The diagnosis of malignant B- and T-cell proliferations is therefore supported by the finding of Ig/TCR gene clonality, whereas reactive lymphoproliferations show polyclonally rearranged Ig/TCR genes.[1]

Gene rearrangement analysis can be performed by Southern blot (SB)- and polymerase chain reaction (PCR)-based techniques. Despite the high reliability of SB analysis, it is increasingly replaced by PCR techniques because of the greater efficiency and sensitivity of PCR. Moreover, PCR is relatively easy, less labor intensive and requires much less high-molecular-weight DNA. Also, SB analysis cannot be performed on paraffin-embedded tissue because the isolated DNA is often degraded. Therefore, there is a strong need to replace SB analysis with reliable PCR techniques. However, PCR studies have often suffered from false-negative results due to improper annealing of primers and/or the presence of somatic hypermutation.[2]

Both SB and PCR analysis of the immunoglobulin heavy chain (*IGH*) locus have been demonstrated to be very useful and reliable techniques in clonality assessment of suspected B-cell malignancies. However, the most useful gene target for identifying T-cell clonality is less well established. In virtually all PCR studies, only the T-cell receptor-γ (*TCRG*)

locus was analyzed at the DNA level because of the relative structural simplicity of the gene.[3,4] PCR analysis of the T-cell receptor-β (*TCRB*) locus as a diagnostic test has been performed mostly on cDNA using Vβ and Cβ primers. Recently, the BIOMED-2 based *TCRB* gene rearrangement analysis was evaluated in a large series of well-defined samples from immature and mature T-cell malignancies and was demonstrated to be a very reliable assay.[5] However, these novel BIOMED-2 multiplex PCR methods for detecting B- and T-cell clonality have yet to be validated in routine diagnostic laboratory settings.

The vast majority of lymphoid malignancies encountered in the West belong to the B-cell lineage (90 to 95%). Even though B-cell clonality can be assessed by flowcytometric immunophenotyping, Ig gene rearrangement analysis is the only reliable assay for paraffin-embedded and frozen tissue biopsies. Consequently, in many routine diagnostic laboratories, TCR gene rearrangement analysis is applied to a smaller number of cases per year than Ig analysis, resulting in a lower level of experience. Because our institute is a reference center for clonality analysis in suspected T-cell proliferations, we routinely obtain many T-cell proliferation samples each year.

In our study, we performed a comparative prospective study of SB-PCR Ig/TCR gene rearrangements on a series of 300 specimens consecutively obtained for routine diagnostics. In general, SB analysis was performed with optimized DNA probes for the *IGH* locus and the *TCRB* locus.[6,7] To determine B- or T-cell clonality by PCR analysis, we analyzed the *IGH*, *TCRB*, and *TCRG* genes. To this end we used the well-defined and fully standardized set of oligonucleotide primers and PCR protocols of the BIOMED-2 Concerted Action BMH4-CT98-3936.[8,9] Resulting PCR products were analyzed by both heteroduplex (HD) and GeneScan (GS) analysis to evaluate the diagnostic value of these methods.

Our results show that the BIOMED-2 PCR-based TCR gene rearrangement analysis is more sensitive in detecting T-cell clonality than SB analysis. In instances of discordance, PCR results demonstrated agreement with histopathological diagnosis more often than SB analysis. The higher sensitivity of PCR analysis over SB analysis also holds for *IGH* gene rearrangements. Based on these results, we discuss the most sensitive and efficient strategy of molecular clonality analysis.

MATERIALS AND METHODS

Patients

From June 2001 until February 2004, 300 DNA samples from fresh or frozen tissue samples (peripheral blood, PB, n=110; lymph node, LN, n=68; bone marrow, BM, n=28; skin, n=59; bowel, n=7; liver, n=3; spleen, n=1; thyroid, n=1; vitreous fluid, n=15; CSF, n=3; pleural fluid, n=2; adenoid, n=1; brain, n=1; maxillary sinus tissue, n=1) were prospectively collected from a total of 218 patients with suspected malignant lymphoproliferation. Most patients were seen and followed by physicians at the Erasmus MC, University Medical Center (Rotterdam), especially at the Departments of Hematology and Dermatology. Diagnoses were based on a combination of clinical, histological, immunophenotypical and cytomorphological data. Patients diagnosed with a malignancy were classified according to the World Health Organization classification of lymphoid neoplasms.[10]

DNA isolation

High molecular weight DNA from fresh or frozen tissue samples was obtained by one of two methods. In the first, DNA was extracted using a phenol-chloroform extraction-based protocol, followed by ethanol precipitation and re-solubilization in TE buffer.[1] Alternatively, DNA was isolated using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

Southern blot analysis

DNA (20 μg) was digested with appropriate restriction enzymes, size fractionated in 0.7% agarose gels and transferred to nylon filters as described elsewhere.[1] The Ig and TCR gene rearrangements were detected by use of ^{32}P random oligonucleotide-labeled probes. The IGHJ6 probe (DakoCytomation California, Inc., Carpinteria, CA, USA) was used for analyzing *IGH* genes in combination with *Bgl*II or *Bam*HI/*Hin*dIII digests[6], whereas the TCRBJ1 and TCRBJ2 probes (DakoCytomation) were used for analyzing *TCRB* genes in combination with *Eco*RI, *Bgl*II and *Bam*HI/*Hin*dIII digests.[7] In a selected number of cases (n=11), the *TCRG* and TCR delta (*TCRD*) gene rearrangements were studied as well. For analysis of *TCRG* gene rearrangements, the TCRGJ13 probe was used in combination with *Eco*RI and *Pst*I digests,[11] whereas the TCRDJ1 probe was used in combination with *Eco*RI digests for analysis of *TCRD* gene rearrangements.[12] In case of clinical suspicion of NK-cell lymphoma and EBV infection, the presence of (clonal) EBV genome was assessed using the *Xho*I probe in *Bam*HI/*Hin*dIII-digested DNA.[13]

PCR amplification

All amplification reactions were performed in an automated thermocycler (model ABI 9600/9700; Applied Biosystem, Foster City, CA, USA) according to the BIOMED-2 multiplex PCR protocol.[8] Each 50-μl PCR reaction included 100 ng DNA, 10 pmol of 5' and 3' oligonucleotide primers, 0.2 mmol/L dNTP, 5 μl 10x buffer II (*TCRB*, *TCRG*) or 5 μl 10x Gold buffer (*IGH*, *IGK*), and 1 to 2 U Ampli-*Taq* Gold polymerase (Applied Biosystems). The concentration of $MgCl_2$ ranged from 1.5 mmol/L (*TCRB* tube C, *TCRG*, *IGH*, *IGK*) to 3 mmol/L (*TCRB* tubes A and B). The cycling parameters were: pre-activation for 7 minutes at 95°C, followed by 35 cycles of 30 seconds denaturation at 95°C, >30 seconds annealing at 60°C, and >30 seconds extension at 72°C. After the last cycle a final extension step of at least 10 minutes at 72°C was performed.

For amplification of *IGH* rearrangements, we employed six framework FR1-V_H primers, seven FR2-V_H primers, seven FR3-V_H primers, and one FAM-labeled J_H consensus primer in three multiplex combinations (*IGH* multiplex tubes A, B and C). DNA from the precursor B-cell line NALM-6 was employed as a positive control. In case of inconclusive *IGH* gene results, the samples were further analyzed using multiplex PCR reactions for the Igκ (*IGK*) genes. For amplification of the *IGK* locus, we employed seven Vκ primers, two FAM-labeled Jκ primers, one intron RSS primer, and one FAM-labeled Kde primer in two combinations (*IGK* multiplex tubes A and B). For amplification of *TCRB* rearrangements, Vβ family and FAM-labeled Jβ primers were used in two different combinations (*TCRB* multiplex tubes A and B) and in one combination containing Dβ and Jβ primers (*TCRB* multiplex tube C). DNA obtained from the following immature T-cell lines was used as a positive control: RPMI-8402 (tube A), CML-T1 (tube B), and Jurkat (tube C). For amplification of *TCRG*

genes, we used Vγ and FAM-labeled Jγ primers in two multiplex combinations (tubes A and B).[8] Positive controls consisted of DNA from immature T-cell lines MOLT 3, RPMI-8402 (tube A) and Jurkat (tube B). In a few cases *TCRD* rearrangements were analyzed by using a single multiplex tube containing Vδ, Jδ and Dδ primers. All BIOMED-2 multiplex PCR tubes were obtained from InVivoScribe Technologies (Carlsbad, CA, USA; www.invivoscribe.com). All cell lines are available at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Features of these cell lines have been summarized in detail elsewhere.[14]

After Ig/TCR amplification, 10 μl of PCR products was loaded on 1% agarose gels to check whether PCR products had been formed. Subsequently, PCR products were further analyzed by heteroduplex and GeneScan analysis (see below) to assess whether the obtained PCR products were derived from monoclonal or polyclonal cell populations.

Heteroduplex and GeneScan Analyses

The PCR products for heteroduplex (HD) analysis were denatured at 94°C for 5 minutes and subsequently renatured at 4°C for 60 minutes to induce duplex formation.[15] Afterwards the duplexes were immediately loaded on 6% nondenaturing polyacrylamide gels in 0.5X Tris-Boric acid-EDTA buffer, run at ambient temperature, and visualized by ethidium bromide staining. A 100-bp DNA ladder (Promega Corporation, Madison, WI, USA) was used as size marker.

GeneScan analysis was performed using an automated ABI PRISM 377 fluorescent sequencer (Applied Biosystems) for the majority of PCR products, whereas the remaining samples were analyzed using an ABI 3100 Genetic Analyzer (Applied Biosystems). When using the first detection method (ABI 377), 2 μl of 10x diluted PCR products were mixed with 2.0 μl of formamide, 0.5 μl of 6-carboxytetramethylrhodamine-labeled internal standard (Genescan 500-TAMRA; Applied Biosystems), and 0.5 μl of loading buffer (blue dextran). After denaturation at 95°C for 2 min and cooling, 3 μl of the mixture was size-separated on a high-resolution polyacrylamide gel and analyzed. The size and profile of the PCR products was determined using GeneScan Analysis software v. 2.1 (Applied Biosystems).[16,17]

For the second method (ABI 3100), 1 μl of a 5x dilution of PCR products was added to 10 μl of a MilliQ: rhodamine-labeled internal standard (GeneScan-500 ROX; Applied Biosystems) mixture (40:1). After denaturation at 95°C for 2 min and cooling, the samples were size-separated and detected. The size and profile of the PCR products were determined using GeneScan Analysis software v. 3.7.1. (Applied Biosystems). GeneScan analysis results in a Gaussian distribution of multiple peaks, representing many different PCR products in case of reactive lymphoproliferations, but gives a single peak in case of monoclonal lymphoproliferation. Oligoclonality is defined as multiple peaks in a polyclonal background.

RESULTS

We consecutively investigated 300 samples (from 218 patients) that were submitted for routine diagnostics. In all patients a clinical diagnosis of malignant lymphoproliferative disease was initially considered. All samples were studied by multiplex PCR-based HD and

GS analysis of Ig/TCR gene rearrangements, whereas SB analysis could be performed on only 176 out of the 300 samples (Table 1). Some samples were analyzed for both Ig and TCR clonality. Overall, 258 samples were analyzed by PCR for T-cell clonality; of these samples, 150 were analyzed by *TCRB* SB. PCR *IGH* gene rearrangement analysis was performed in 87 samples, and SB analysis was performed in 48 of these samples.

Because of our position as a reference center and our extensive expertise on TCR gene rearrangement analysis, the focus of our study lies on T-cell clonality analysis. SB and PCR analysis were, in principle, performed once. In practice this means that Ig clonality was evaluated with the IGHJ6 probe and in three different FR *IGH* multiplex PCR reactions. In limited cases additional SB and PCR analysis of the *IGK* gene was performed. TCR clonality was analyzed with two *TCRB* SB probes as well as with *TCRB* and *TCRG* multiplex PCR protocols. Few samples were studied by SB analysis for *TCRG/TCRD* gene rearrangements or for *EBV* genome and PCR analysis of *TCRD* gene rearrangements. In case of doubtful or discrepant results, assays were repeated.

Table 1. Summary of performed SB- and PCR-based Ig/TCR analysis.

	Samples (patients)	
Clonality analysis	Multiplex PCR	SB
TCR only	214 (155)	28 (24)
Ig only	39 (30)	3 (3)
Ig and TCR	47 (33)	145 (119)
Total	300 (218)	176 (146)

High level of concordance between SB and PCR Ig/TCR gene rearrangement studies

Of 300 samples 176 were analyzed simultaneously by multiplex PCR and SB analysis (Table 1). Molecular data were concordant for TCR analysis in 127 of 150 samples (85%), whereas Ig gene rearrangement analysis was concordant in 41 of 48 samples (85%). Table 2 shows the proportion of concordant SB-PCR results in polyclonal and clonal cases. The finding of a monoclonal T- or B-cell population both by SB and PCR clonality analysis strongly suggests a clonal (probably malignant) lymphoproliferation. Polyclonal gene rearrangements in both assays are indicative of a reactive (benign) lymphoproliferation.

Table 2. Concordance between SB- and PCR-based Ig/TCR analysis in polyclonal and clonal cases.

	Gene rearrangement target	
SB-PCR concordance	Ig	TCR
Total concordance	41/48 (85%)	127/150 (85%)
Polyclonal cases	25/41 (61%)	83/127 (65%)
Clonal cases	16/41 (39%)	44/127 (35%)

Nevertheless, it should be emphasized that the results of molecular clonality studies should always be interpreted in the context of clinical, histologic and immunophenotypic data.

In 30 samples derived from 27 patients, discordances were found between multiplex PCR and SB analysis. In all these samples, monoclonality was demonstrated by PCR analysis, whereas SB did not show evidence for clonal Ig/TCR gene rearrangements.

Evaluation of TCR clonality as demonstrated by PCR analysis with non-clonal results observed by SB analysis

In 23 samples derived from 21 patients, multiplex PCR analysis demonstrated clonal *TCRB/TCRG* gene rearrangements, which were not detected by SB analysis. In 17 samples the molecular findings correlated with the histopathological diagnosis of malignant T-cell proliferations (74%). (For an example of histologically proven ALCL, see Figure 1.)

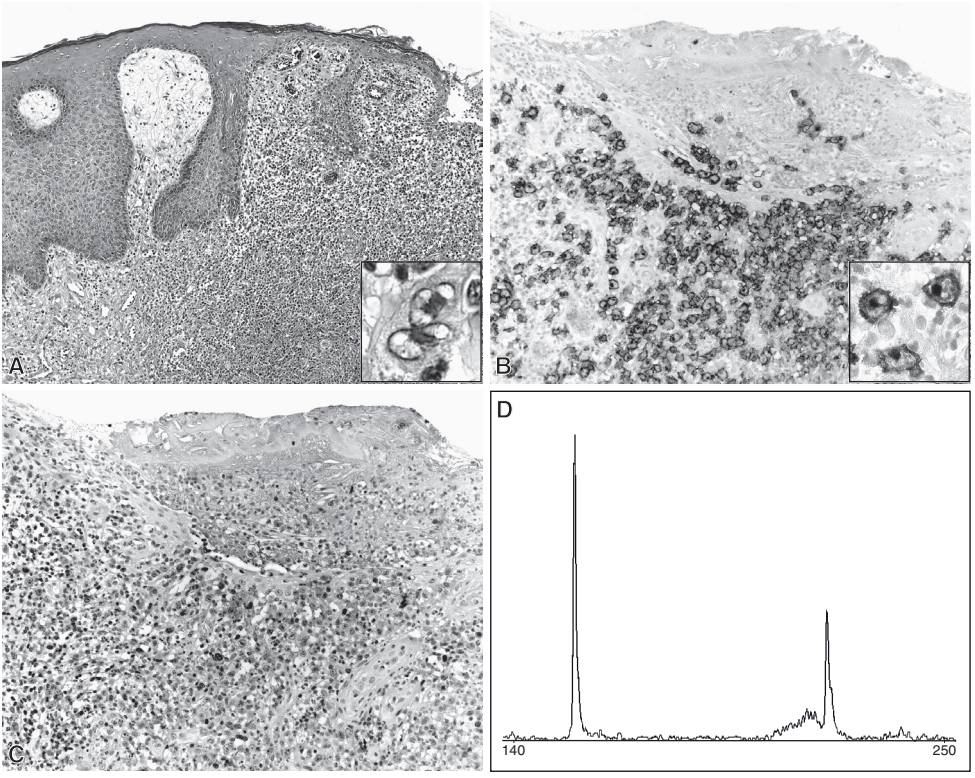

Figure 1. Skin localization of ALCL in case no. 4.
A. Histology of ulcerating skin tumor. Skin biopsy specimen demonstrates localization of dermal infiltrate of large cells with irregular nuclei (HE; magnification x 100). Note the large hallmark cells (inset) (HE; magnification x 600). **B**. The large neoplastic cells are strongly positive for CD30 (magnification x 200). Note the strongly stained membrane and the dot-like staining in the Golgi complex area (inset) (magnification x 600). **C**. The neoplastic cells also stain for CD4 (magnification x 200). **D**. PCR-based GeneScan analysis. Bi-allelic monoclonal *TCRG* gene rearrangements could be identified in skin DNA.

In the remaining samples there was no clear indication for a T-cell malignancy at the time of diagnosis (Table 3).

PCR analysis of the TCR locus identified clonal gene rearrangements in 6 samples derived from 5 patients in whom no diagnosis of T-cell leukemia or lymphoma could be made. Case no. 11 was diagnosed as myeloproliferative disease, unclassifiable (MPD-U). Case no.14, diagnosed as idiopathic hypereosinophilic syndrome (HES), showed identical clonal TCR rearrangements in the BM and PB samples by PCR analysis. Immunophenotyping of the PB sample demonstrated a small T-cell population (0.3% of leukocytes) with an aberrant phenotype ($CD3^-/CD4^+/CD5^+$). It has been shown that in some HES patients abnormal monoclonal T-cells can be found in PB.[18-21] However, in the literature so far, no clonal T-

Table 3. Discordances between SB and PCR results in suspect T-cell proliferations.

Case no.	Sample no.	Final clinico-histological diagnosis[a]	SB			PCR procedure		
			TCRB	*TCRG*	*TCRD*	*TCRB*	*TCRG*	*TCRD*
1	2001-146 (skin)	LyP	G	ND	ND	C	C	ND
2	2001-148 (LN)	AILT	G	ND	ND	C	C	ND
3	2001-152 (PB)	T-LGL	G	G	ND	C	C	ND
4	2001-162 (skin)	ALCL	G	G	ND	C	C	ND
5	2002-003 (LN)	Peripheral T-NHL, NOS	G	ND	ND	C	P	P
6	2002-022 (PB)	MF	G	ND	ND	C	C	ND
7	2002-033 (LN)	$CD30^+$ CTCL	G	ND	ND	C	C	ND
8	2002-047 (PB)	T-LGL	G	ND	ND	C	C	ND
9	2002-050 (LN)	ALCL	G	ND	G	C	P	C
10	2002-113 (BM)	T-ALL	G	ND	G	C	P	C
11	2002-144 (BM)	MPD-U	G	G	G	C	C	P
12	2003-022 (LN)	AILT	G	G	G	C	C	ND
13	2003-172 (BM)	T-LBL	G	G	G	C	C	C
14	2003-236 (BM)	HES	G	ND	ND	C	P	ND
	2003-296 (PB)	HES	G	ND	ND	C	C	ND
15	2003-245 (skin)	MF	G	G	G	C	P	ND
16	2003-270 (PB)	SS	G	ND	ND	C	P	ND
	2004-007 (LN)	SS	G	ND	ND	C	P	ND
17	2004-001 (skin)	SS	G	ND	ND	C	P	ND
18[b]	2004-004 (PB)	Reactive	G	ND	ND	C	C	ND
19	2004-005 (skin)	$CD30^+$ CTCL	G	ND	ND	C	P	ND
20	2004-011 (BM)	Reactive	G	ND	ND	C	P	ND
21	2004-020 (LN)	Reactive	G	ND	ND	C	C	ND

R, rearranged; G, germline; C, clonal; P, polyclonal; ND, not done

[a] *Diagnosis:* AILT, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; CTCL, cutaneous T-cell lymphoma; HES, hypereosinophilic syndrome; LyP, lymphomatoid papulosis; MF, mycosis fungoides; MPD-U, myeloproliferative disease, unclassifiable; PTLD, post-transplant lymphoproliferative disorder; SS, Sézary syndrome; T-ALL, T-cell acute lymphoblastic leukemia; T-LBL, T-cell lymphoblastic lymphoma; T-LGL, T-cell large granular lymphocyte leukemia; T-NHL NOS, T-cell non-Hodgkin lymphoma, not otherwise specified

[b] Case no. 18 was diagnosed with a $CD30^+$ CTCL 10 years before.

cell populations identical to the one in PB have been detected in BM. Further research should reveal the meaning of the clonal T-cell population in BM. Since patients diagnosed with idiopathic HES and associated clonal T-cell populations are at risk of developing T-cell lymphoma,[19,22,23] careful follow-up is required in this case. Case no. 18 was admitted to our hospital in 1994 and was diagnosed with $CD30^+$ CTCL. Although the patient was clinically healthy without any signs of relapse, the PB sample demonstrated monoclonal *TCRB* rearrangements in 2004. The PCR analysis on the paraffin-embedded skin tissue sample from 1994 demonstrated weak clonal *TCRB* rearrangements, identical to the ones found in the PB sample 10 years after presentation. We strongly recommend follow-up of this patient as well. A skin relapse of an ALCL was diagnosed in case no. 20. Staging resulted in the detection of weak clonal *TCRB* gene rearrangements in the BM sample, not identical to the ones found in the skin. Flow cytometry and histopathology did not demonstrate localization of malignant lymphoma. Finally, case no. 21 was diagnosed as reactive lymphadenopathy after extensive immunohistochemical and flowcytometric analysis of the LN sample.

Evaluation of Ig clonality as demonstrated by PCR analysis with non-clonal results observed by SB analysis

In 7 samples derived from 6 patients, multiplex PCR analysis demonstrated clonal B-cell populations that were not detected by SB analysis (Table 4). *IGH* monoclonality was detected in the LN sample of case no. 27 who suffered from HIV and EBV infection. The histopathological diagnosis of B-PTLD-associated disease was made from the LN sample (Figure 2). This polymorphic B-cell lymphoproliferative disorder also occurs in immunodeficient states outside the post-transplant setting and comprises 5% of HIV-associated lymphomas. Most cases have clonally rearranged *IGH* and *IGK* genes with the *TCRB* gene in the germline configuration as found by SB analysis.[24] In addition, TCR oligoclonality was detected in the sample by PCR analysis (Figure 2). Although SB analysis of the *IGH* locus did not provide evidence for malignancy, the presence of a clonal EBV genome in the LN sample was identified by SB analysis (data not shown). In 4 of the other 6 samples, the *IGH* PCR results agreed with biopsy histology and immunophenotypical analysis. All together 5 of 7 (71%) B-cell proliferations showed concordant PCR and histology results.

Table 4. Discordances between SB and PCR results in suspect B-cell proliferations.

Case no.	Sample no.	Final clinico-histological diagnosis[a]	SB			PCR procedure			
			IGH	*IGK*	EBV	FR1	FR2	FR3	*IGK*
22	2001-125 (skin)	Dermatitis	G	ND	ND	C	C	C	ND
23	2001-170 (PB)	MM	G	ND	ND	C	C	C	ND
24	2002-011 (BM)	Reactive	G	ND	ND	C	C	P	ND
25	2002-096A (LN)	DLBCL	G	ND	ND	C	C	C	ND
	2002-096B (BM)	DLBCL	G	ND	ND	C	C	C	ND
26	2002-122 (skin)	DLBCL	G	G	ND	C	C	P	C
27[b]	2003-242 (LN)	B-PTLD	G	G	R	C	C	C	ND

G, germline; R, rearranged; C, clonal; P, polyclonal; ND, not done

[a] *Diagnosis:* DLBCL, diffuse large B-cell lymphoma; MM, multiple myeloma

[b] Case no.27 was diagnosed with a PTLD-associated disorder (HIV and EBV positive) and demonstrated oligoclonal TCR rearrangements in the LN sample by PCR analysis (Figure 2).

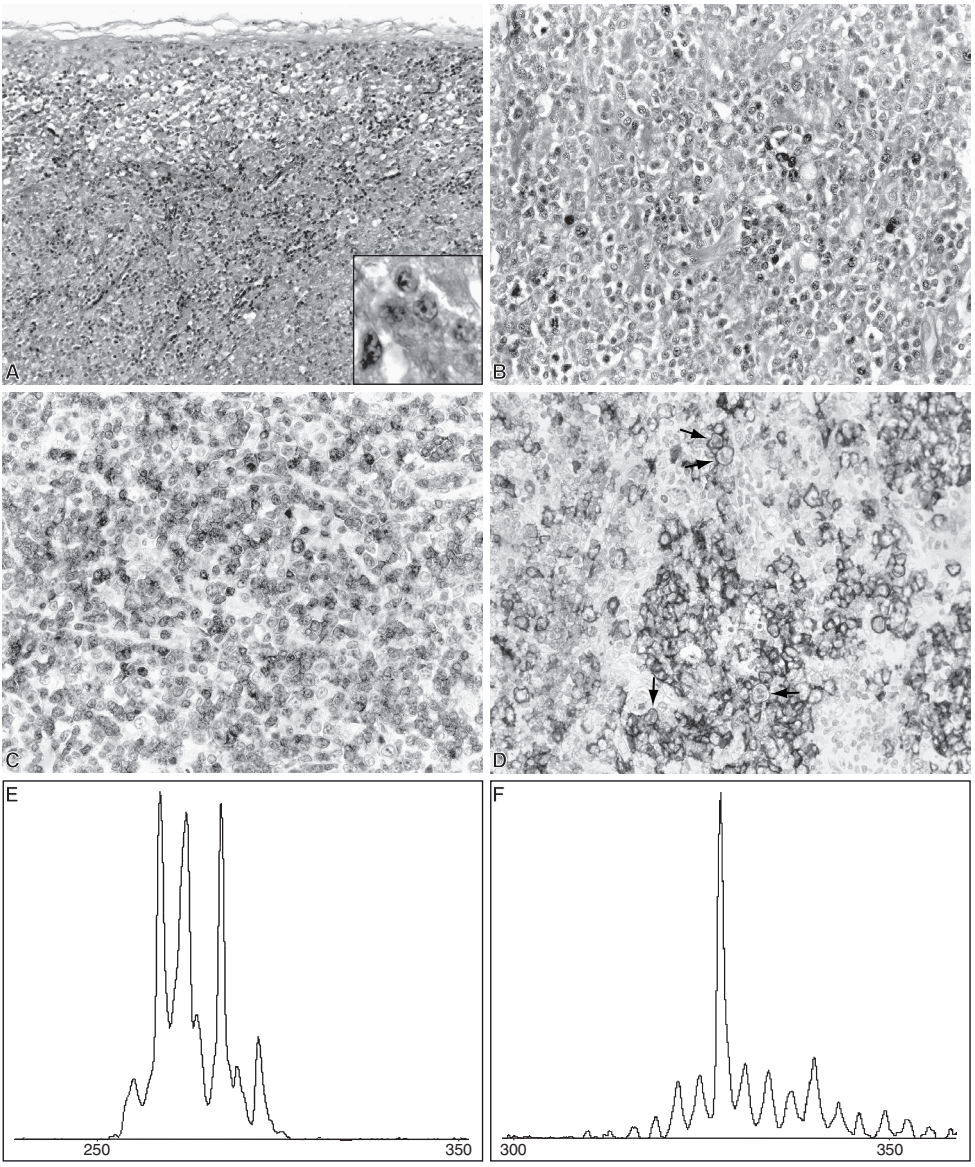

Figure 2. Polymorphic B-cell PTLD-associated disease in case no. 27.
A. Histology of LN. There was no recognizable lymph node architecture (HE; magnification x 200). There were numerous blasts (inset) (HE; magnification x 600) intermingled with an infiltrate of smaller lymphocytes. **B**. EBER-ISH showed scattered positively stained nuclei indicating presence of EBV RNA (magnification x 400). **C**. CD3 staining of the predominantly small T-cells. Occasionally, larger cells stained positively (magnification x 400). **D**. CD20 stained the majority of the large blasts. Note the numerous mitotic figures (arrows) (magnification x 400). **E**, **F**. PCR-based GeneScan analysis of LN DNA. Oligoclonal *TCRB* gene rearrangements could be identified (E), whereas a monoclonal *IGH* gene rearrangement was detected (F).

The two remaining samples were diagnosed as reactive lesions after extensive histopathological evaluation. In the skin sample of case no. 22, a weak monoclonal gene rearrangement was detected by PCR analysis. On histopathological examination the lymphocytic infiltrate almost exclusively contained T-cells. This case was discussed in the Dutch Cutaneous Lymphoma Working Group, and a diagnosis of dermatitis was made. The small amount of polyclonal B-cells in the cutaneous infiltrate might explain the finding of (oligo)clonal *IGH* gene rearrangements.[25,26] Though a weak clonal *IGH* gene rearrangement was detected in the BM sample of case no. 24 by PCR analysis, immunophenotyping only detected a reactive plasma cell population, and no abnormal B-cells were seen by cytomorphological evaluation.

DISCUSSION

We evaluated a group of 218 patients with an initial suspicion of lymphoproliferative disorders to estimate whether the newly designed primers and PCR protocols of the BIOMED-2 Concerted Action method could reliably replace SB analysis in a routine diagnostic setting. A total of 300 DNA samples was analyzed by two PCR-based strategies (HD and GS analysis), whereas a series of 176 DNA samples were analyzed by both SB and PCR analyses. The fact that SB analysis could not be performed in 124 samples further stresses the need for reliable PCR assays. When the SB and PCR approaches were compared and related to the final diagnosis, PCR and SB results of both T- and B-cell clonality analyses were concordant in 85% of samples.

In those samples in which the SB-PCR results were discordant, the PCR results appeared to correlate with the histopathological diagnosis in 22/30 cases (73%). Although PCR analyses showed unconfirmed clonal results in 27% of cases in comparison to the SB analysis, the number of false-negative SB results in cases of true malignancy was unexpectedly high. Further research and follow-up should estimate the meaning of small clonal T- or B-cell populations in non-malignant cases as determined by PCR-based techniques. Most recently the BIOMED-2-based primers and protocols were studied in a series of ~100 well-defined samples of reactive lymphoproliferations, and (oligo)clonality was detected in 10% of cases (A.W. Langerak, unpublished data).

Based on our research and published reports, we strongly recommend careful follow-up of non-malignant cases with clonal lymphoproliferations since Ig/TCR clonality might be an early sign of an underlying hematological malignancy. Frequent clinical and histologic follow-up on PB, BM or LN samples is required. This might result in diagnosing patients in early stage disease, thus favoring prognosis and survival. The detection of identical T- or B-cell clones in a tissue sample and in PB is of great importance in staging lymphoid malignancies and is of prognostic relevance.[27-30] It should, however, be noted that the finding of (oligo)clonality might not always be clinically significant. (Oligo)clonal T-cell populations can be detected in PB of the elderly,[31,32] in patients diagnosed with autoimmune diseases, and in patients with viral infection.[33] In cutaneous T-cell lymphoma (CTCL), the detection of an identical monoclonal TCR gene rearrangement in skin and extracutaneous tissues has been proven to be an independent prognostic marker.[34,35] In addition, detection of the same clonal

gene rearrangements in multiple skin biopsy specimens at the time of diagnosis may provide prognostic information related to disease progression.[36] Therefore, if possible, we recommend studying multiple (parallel) samples from multiple suspect localizations at initial disease presentation. In addition, follow-up samples should be evaluated when disease progression is suspected.

The detection of a clonal B- or T-cell population can facilitate diagnosis in patients in whom clinical, histopathological, and immunophenotypical findings are consistent but not entirely typical of a malignant lymphoproliferation. The importance of accurate molecular clonality diagnostics is supported by the fact that the current diagnostic criteria for patients with SS and T-LGL leukemia include the presence of a clonal TCR gene rearrangement in PB.[37,38]

The BIOMED-2 multiplex PCR approach is a rapid and reliable procedure that is far more sensitive than SB analysis in detecting clonality in suspect lymphoproliferations. The final clinico-histopathological diagnosis correlates well with PCR results in a higher number of patients in comparison to SB results. Therefore, it seems that the "gold standard" of SB analysis can now be reliably replaced in a routine laboratory setting. Essentially, this not only holds true for Ig clonality analysis but also for TCR clonality assessment, as demonstrated in this study. There was no difference in clonality detection rate between *TCRB* and *TCRG* gene rearrangement analysis, whereas the combined analysis clearly showed additional value. We detected the highest number of clonal *IGH* gene rearrangements when using multiplex combination A and B (FR1 + FR2 primers) (Table 5), but the combined information from all three FR PCR reactions resulted in a higher number of clonal cases. Although SB *IGH* gene rearrangement analysis remains a reliable and helpful additional test, the PCR approach is strongly preferred. The finding of polyclonal *IGH* gene rearrangements by PCR analysis in case of strong suspicion of a B-cell malignancy would indicate performing additional PCR-based *IGK* analysis. In two cases of our series, analysis of the *IGK* locus was of additional value. We detected clonal *IGK* gene rearrangements in a case of follicular lymphoma and a case of cutaneous B-cell lymphoma (CBCL), whereas polyclonal gene rearrangements were found upon *IGH* analysis. In case of T-cell clonality diagnostics, our *TCRB/TCRG* protocols are superior to SB analysis of the *TCRB* locus. The latter, however, may still be a valuable test under certain conditions.

In conclusion, both HD and GS are reliable techniques with completely concordant results. Because of its enhanced speed, accuracy, and interpretation, GS analysis is slightly favored over HD analysis. Based on our current results and extensive experience with TCR gene studies in general, we now propose a flowchart that demonstrates the most efficient and sensitive strategy in detecting T-cell clonality (Figure 3). This strategy holds true for fresh or frozen tissue samples, and may be applicable to paraffin-embedded tissue samples, provided that DNA quality is such that PCR products of ~300 base pairs can be amplified.

Table 5. Clonality detection rate of different targets in PCR-based Ig/TCR clonality assessment.

	IGH				TCR		
	FR1	FR2	FR3	FR1+2+3	*TCRB*	*TCRG*	*TCRB+TCRG*
Clonality %	94	88	55	100	87	86	100
Per target	(31/33)	(29/33)	(18/33)	(33/33)	(103/119)	(102/119)	(119/119)

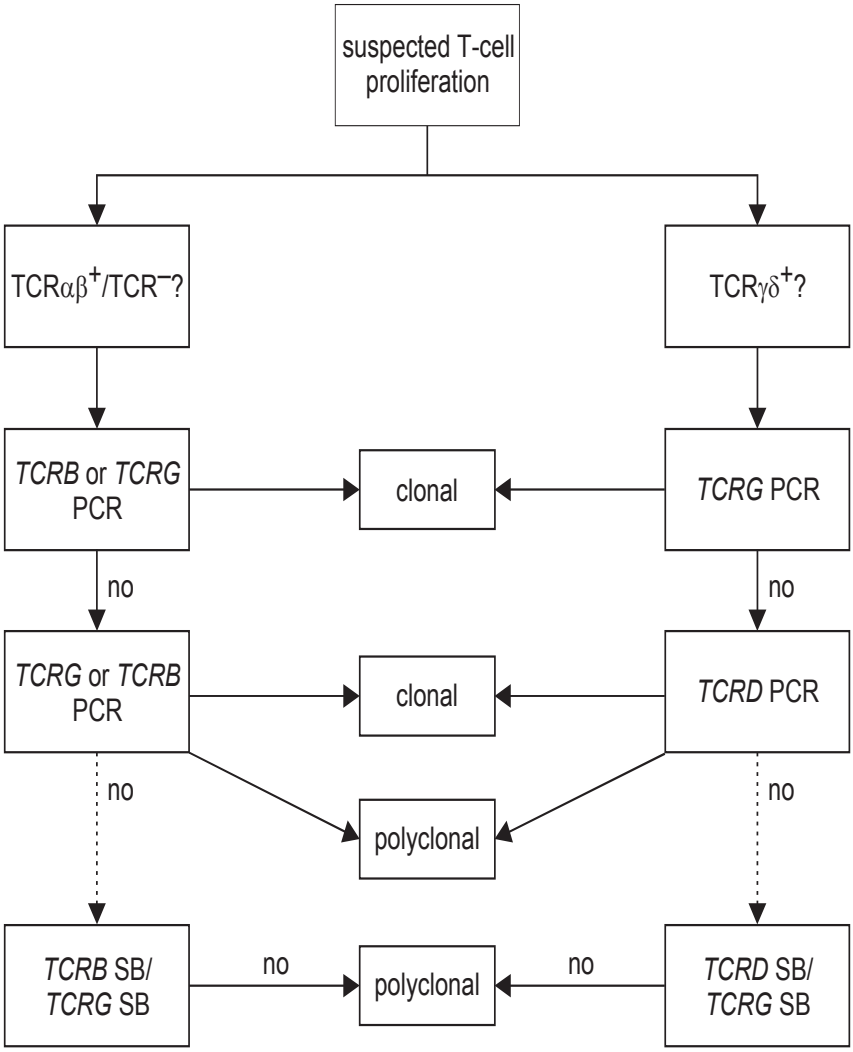

Figure 3. Strategy for BIOMED-2 multiplex PCR TCR clonality analysis in suspect T-cell proliferations.
In TCRαβ+ or TCR negative T-cell proliferations, there is no clear evidence-based preference for either the *TCRB* or *TCRG* locus as the initial target for clonality diagnostics. Thus, either *TCRB* or *TCRG* can be used as the first line target, followed by the other locus as the second target. For TCRγδ+ T-cell proliferations, the *TCRG* locus is the clear first line target for clonality assessment, followed by *TCRD* as the second target. In the absence of clonal TCR gene rearrangements in PCR, SB analysis might still be considered for all suspect T-cell proliferations, provided that enough high quality DNA is available. Although the PCR strategy as described here is suitable for fresh or frozen tissue samples, it could possibly be applied to DNA isolated from paraffin-embedded tissue samples, provided that DNA quality is such that PCR products of ~300 base pairs can be amplified.

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Chapter 3

CUTANEOUS LYMPHOMA

3.1 Primary cutaneous T-cell lymphoma.

3.2 Molecular immunoglobulin/T-cell receptor clonality analysis in cutaneous lymphoproliferations. Experience with the BIOMED-2 standardized polymerase chain reaction protocol
Haematologica 2003; 88: 659-670

3.3 Clonal identity between skin and synovial tissue in a case of mycosis fungoides with polyarthritis
J Am Acad Dermatol 2004; 51: 111-117

3.4 Late relapse of primary cutaneous $CD30^+$ anaplastic large cell lymphoma confirmed by T-cell receptor (TCR) PCR analysis
in: Cutaneous lymphomas: Unusual cases 2, G. Burg, W. Kempf (eds.), Steinkopff Verlag Darmstadt, 2006; 2: 26-27

Chapter 3.1

Primary cutaneous T-cell lymphoma

Primary cutaneous lymphomas are a group of malignant lymphoproliferative disorders that present in the skin without evidence of extracutaneous disease at the time of diagnosis. Following the gastrointestinal tract, the skin is the second most common site of extranodal non-Hodgkin lymphomas, with an estimated annual incidence of 1:100.000. Based on the immunophenotypical characteristics of the malignant lymphocytes, two major subgroups of primary cutaneous lymphomas are distinguished: primary cutaneous T-cell lymphomas (CTCL) and primary cutaneous B-cell lymphomas (CBCL). Approximately 75% of cutaneous lymphoma cases are of T-cell origin and the remaining 25% belong to the B-cell lineage.

Classification of primary cutaneous lymphomas

In 2005, the World Health Organization (WHO) and the European Organization for Research and Treatment of Cancer (EORTC) collectively presented a new classification system for cutaneous lymphomas: the WHO-EORTC classification (Table 1).[1] Following this classification, ~90% of CTCL belong to four major categories: mycosis fungoides, Sézary syndrome, primary cutaneous $CD30^+$ anaplastic large cell lymphoma, and lymphomatoid papulosis. As these entities have been the main subject of study in Chapter 3 of this thesis, they are described in more detail below. Their main clinical, histopathological, immunophenotypical, and molecular characteristics are summarized in Table 2.

Mycosis fungoides and Sézary syndrome

Mycosis fungoides (MF) is the most common subtype of CTCL, accounting for approximately 45% of all primary cutaneous lymphomas. Clinically, MF is characterized by a slow and indolent course with subsequent evolution from patches to more infiltrated plaques and eventually tumors. Extracutaneous manifestations may occur in advanced stages, mainly to lymph nodes, peripheral blood and internal organs. Histologically, the skin lesions show epidermotropic infiltrates of small to medium-sized mononuclear cells with hyperchromatic, indented (cerebriform) nuclei. Pautrier's microabcesses consisting of aggregates of cerebriform cells in the epidermis are highly characteristic, but seen only in a minority of cases. The typical immunophenotype of tumor cells is $CD2^+$, $CD3^+$, $CD4^+$, $CD5^+$, $CD8^-$, $TCR\alpha\beta^+$, and $CD30^-$. Malignant cells often show loss of CD7. *TCRB* and *TCRG* genes are clonally rearranged in most cases. The detectability of molecular clonality analysis is particularly low in patch/plaque stage disease, though advanced PCR based techniques can detect clonality in up to 70% of cases. The disease-specific 5-year survival correlates with the stage of disease and is estimated to be 100% in limited patch/plaque-stage disease, but the prognosis is poor in the more advanced stages.[1-3]

Sézary syndrome is generally regarded as the leukemic variant of MF and is characterized by the presence of erythroderma, lymphadenopathy, and neoplastic T-lymphocytes in the peripheral blood. It is virtually impossible to distinguish MF from Sézary syndrome by histomorphological analysis. In the new WHO-EORTC classification, the term Sézary syndrome refers to cases without a history of MF. Circulating Sézary cells most commonly show similar immunophenotypical characteristics as the malignant T cells in MF. Demonstration of identical clonal TCR gene rearrangements in both skin and peripheral blood is considered as an important diagnostic criterion allowing differentiation between SS and benign forms of

Table 1. WHO-EORTC classification of cutaneous lymphomas with primary cutaneous manifestations.

Cutaneous T-cell and NK-cell lymphomas
Mycosis fungoides (MF)
MF variants and subtypes
• Folliculotropic MF
• Pagetoid reticulosis
• Granulomatous slack skin
Sézary syndrome
Adult T-cell leukemia/lymphoma
Primary cutaneous $CD30^+$ lymphoproliferative disorders
• Primary cutaneous anaplastic large cell lymphoma
• Lymphomatoid papulosis
Subcutaneous panniculitis like T-cell lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Primary cutaneous peripheral T-cell lymphoma, unspecified
• Primary cutaneous epidermotropic aggressive $CD8^+$ T-cell lymphoma (provisional)
• Cutaneous γδ T-cell lymphoma (provisional)
• Primary cutaneous $CD4^+$ small/medium-sized pleomorphic T-cell lymphoma (provisional)
Precursor hematologic neoplasm
$CD4^+/CD56^+$ hematodermic neoplasm (blastic NK-cell lymphoma)
Cutaneous B-cell lymphomas
Primary cutaneous marginal zone B-cell lymphoma
Primary cutaneous follicle center lymphoma
Primary cutaneous diffuse large B-cell lymphoma, leg type
Primary cutaneous diffuse large B-cell lymphoma, other
Intravascular B-cell lymphoma

Adapted from Willemze *et al.*, 2005.[1]

erythroderma.[4] Most patients follow an aggressive clinical course with an overall survival rate at 5 years of 10-20%.[2,3]

Complex karyotypes are present in many cases of MF and Sézary Syndrome and genetic alterations have been reported frequently.[5,6] As yet, clear association with specific recurrent mutations and chromosomal abnormalities resulting in expression of oncogenes or inactivation of tumor suppressor genes have not been identified.[7,8] However, disease progression in MF/SS has been associated with several genetic abnormalities such as p53 expression defects.[9] In addition, gene expression profiling revealed many differentially expressed genes in Sézary Syndrome, as compared to normal controls.[10] Highly overexpressed genes include $CD4^+$ T cell related transcription factors such as *STAT4*, *GATA-3* and *JUN B*.[11]

Table 2. Major characteristics of the most frequently diagnosed primary cutaneous T-cell lymphomas (CTCL).

	Mycosis fungoides	Sézary syndrome	Primary cutaneous $CD30^+$ ALCL	Lymphomatoid papulosis
Clinical features	Patches, plaques, tumors	Erythroderma, generalized lymphadenopathy, neoplastic T-cells in peripheral blood	Solitary or localized nodules or tumors	Papules, nodules Spontaneous regression
Histopathology of skin	Epidermotropism, Pautrier's microabscesses	Epidermotropism, Pautrier's microabscesses	Non-epidermotropic, large $CD30^+$ T-cells	Non-epidermotropic, large $CD30^+$ T-cells
Immunophenotype	$CD2^+/CD3^+/CD4^+/CD5^+/CD7^{+/}$ $CD45RO^+/TCR\alpha\beta^+$	$CD2^+/CD3^+/CD4^+/CD5^+/CD7^{+/}$ $CD26^{+/-}/TCR\alpha\beta^+$	$CD2^{+/-}/CD3^{+/-}/CD4^+/$ $CD5^{+/-}/CD30^+$	$CD2^{+/-}/CD3^{+/-}/CD4^+/$ $CD5^{+/-}/CD30^+$
TCR gene rearrangement	Clonal (>70%)	Clonal (100%)	Clonal (>90%)	Clonal (~50%)
Disease-specific 5-year survival (%)	88	24	95	100
Relative frequency within CTCL (%)	54	3.5	10	16

Spectrum of primary cutaneous CD30$^+$ lymphoproliferative disorders

Primary cutaneous CD30$^+$ lymphoproliferative disorders represent the second most common group of CTCL, accounting for approximately 30% of all cases. This group includes primary cutaneous CD30$^+$ anaplastic large cell lymphoma (C-ALCL), lymphomatoid papulosis (LyP) and borderline cases. It is now generally accepted that C-ALCL and LyP form a spectrum of disease, and that histologic criteria alone are often insufficient to differentiate between these two ends of the spectrum. The clinical appearance and course are used as decisive criteria for the definite diagnosis and choice of therapy.[12]

Primary cutaneous CD30$^+$ anaplastic large cell lymphoma

Primary cutaneous CD30$^+$ anaplastic large cell lymphoma (C-ALCL) is an indolent lymphoma that generally presents with one solitary or a few clustered skin tumors, and often shows ulceration. These lymphomas frequently relapse in the skin, whereas extracutaneous dissemination is uncommon. There is a diffuse, non-epidermotropic infiltrate with cohesive sheets of large CD30$^+$ tumor cells. In most cases the tumor cells have the characteristic morphology of anaplastic cells, showing round, oval, or irregularly shaped nuclei, prominent eosinophilic nucleoli, and abundant cytoplasm. The neoplastic cells often express a CD4$^+$ T-cell phenotype with variable loss of pan T-cell antigens (CD2, CD3, CD5).

Most cases show clonally rearranged TCR genes. The translocation t(2;5)(p23;q35), which is frequently present in nodal ALCL, is not or extremely rarely found in C-ALCL and rather points to a secondary skin localization of a nodular type ALCL. The prognosis is usually favourable with a 5-year survival of more than 90%.

Lymphomatoid papulosis

Lymphomatoid papulosis (LyP) is characterized by the presence of papular, papulonecrotic, and/or nodular skin lesions at different stages of development, predominantly on the trunk and limbs. Individual skin lesions spontaneously regress within 3 to 12 weeks. The histologic picture of LyP is extremely variable. Characteristically, the dermal infiltrates contain scattered or small clusters of large atypical CD30$^+$ cells, interspersed in an extensive inflammatory infiltrate. Clonal TCR gene rearrangements have been detected in approximately 60%-70% of LyP lesions. Patients with LyP generally have an excellent prognosis, although a minority of patients develops systemic lymphoma.[12]

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Chapter 3.2

Molecular immunoglobulin/T-cell receptor clonality analysis in cutaneous lymphoproliferations

Experience with the BIOMED-2 standardized polymerase chain reaction protocol

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ABSTRACT

Molecular clonality analysis of immunoglobulin (Ig) and T-cell receptor (TCR) genes is a widely used diagnostic tool for discrimination between polyclonal, oligoclonal, and monoclonal lymphoproliferative skin lesions. We studied Ig/TCR clonality in a series of 60 patients with an initial suspicion of (primary) cutaneous B- or T-cell lymphoma (CBCL/CTCL). Clonality of Ig/TCR gene rearrangements was assessed by Southern blot (SB) and polymerase chain reaction (PCR) analysis using standardized PCR primers and protocols of the BIOMED-2 Concerted Action BMH4-CT98-3936. The obtained PCR products were subjected to heteroduplex (HD) and GeneScan (GS) analysis. We compared the data of 154 samples with the histopathologic diagnosis, based on the EORTC classification of skin lymphomas.

Molecular results were largely concordant with histopathology. In 12 CBCL patients PCR analysis of Ig gene rearrangements detected clonality in 83% of cases whereas SB did so in 92%. Clonal TCR gene rearrangements were detected by SB in 68% of CTCL patients, whereas *TCRG* and *TCRB* PCR analysis detected clonality in 76% and 66% of cases respectively. PCR GS analysis of TCR rearrangements appeared to be slightly more informative than HD analysis. Clonality assessment was particularly informative for studying involvement of extracutaneous sites, such as regional lymph nodes, peripheral blood, and bone marrow.

Our study shows that the BIOMED-2 multiple PCR analysis strategy is a reliable and useful technique in the diagnostic process of patients with an initial suspicion of (primary) CBCL/CTCL and for assessment of extracutaneous dissemination, provided that the results are interpreted in the context of clinical, histologic, and immunophenotypic data.

INTRODUCTION

Cutaneous lymphoproliferations represent a heterogeneous group of benign and malignant disease entities. In cutaneous lymphoproliferations it is of great prognostic importance, though still not easy, to differentiate between a clinically malignant aggressive primary cutaneous lymphoma, such as mycosis fungoides (MF) and a more benign disease entity, such as dermatitis. Molecular diagnostic tools with a high specificity and sensitivity might contribute to an early and correct diagnosis and thereby better treatment, improving the patient's prognosis.

The first step in diagnosing patients with a clinical suspicion of primary cutaneous B- or T-cell lymphoma (CBCL/CTCL) consists of histomorphology, immunophenotyping, and cytological analysis on various tissue samples such as (involved) skin, (enlarged) lymph nodes (LN), bone marrow (BM), and peripheral blood (PB). Molecular clonality studies employing Southern blot (SB) analysis and more recently also polymerase chain reaction (PCR) analysis have been introduced as an additional diagnostic step.[1-13]

Since cutaneous lymphomas are clonal diseases that are derived from a single malignantly transformed lymphoid cell, all malignant cells contain clonal (identical) rearrangements of immunoglobulin (Ig) or T-cell receptor (TCR) genes.[14,15] Clonal or polyclonal B- or T-cell populations can be discriminated based on the presence or absence of clonally rearranged Ig

or TCR genes.[16] Provided that optimal probe/restriction enzyme combinations are used, SB analysis can be considered the gold-standard molecular technique for clonality studies, because the risk of false-negative and/or false-positive results is very low.[17] Detectability of (clonal) Ig and TCR rearrangements by PCR analysis is limited by the choice of oligonucleotide primers. PCR analysis can yield more false-negative results than does SB analysis in the cases when the applied PCR primer sets are inappropriate for recognizing each rearranged Ig or TCR gene segment. False-negative results can also be caused by the occurrence of somatic mutations in Ig genes of (post-) follicular B-cell lymphomas. However, the higher efficiency and sensitivity of PCR techniques compensate for these disadvantages and may be particularly important for detecting small numbers of malignant cells, for example in early stage CBCL/CTCL. Also, SB analysis requires larger amounts of high quality DNA and cannot be performed on paraffin-embedded tissues. This implies a strong need to replace SB analysis by reliable PCR techniques.

In the present study we evaluated the contribution of both SB and PCR analyses of rearranged Ig and TCR genes to the diagnostic process of patients with an initial suspicion of (primary) CBCL/CTCL. SB analysis was performed with optimized DNA probes for the immunoglobulin heavy chain (*IGH*) locus and T-cell receptor β (*TCRB*) locus.[18,19]

For PCR based analysis of the *IGH*, *TCRB*, and T-cell receptor γ (*TCRG*), loci we used the well-defined and fully standardized set of oligonucleotide primers and PCR protocols of the BIOMED-2 Concerted Action BMH4-CT98-3936, entitled *PCR-based clonality studies for early diagnosis of lymphoproliferative disorders*.[20] To determine their homogeneous or heterogeneous character, the PCR products were analysed by heteroduplex (HD) as well as GeneScan (GS) analysis. GS analysis has been described to be fast, accurate, sensitive, and easy to interpret.[21-23] HD analysis can be a cheap and reliable alternative in a diagnostic setting, not requiring expensive automated sequencing equipment or fluorochrome-labeled oligonucleotides.

A total of 154 different samples (including skin, LN, BM, PB, ascites, and synovium) from 60 patients with an initial suspicion of (primary) CBCL or CTCL were subjected to clonality assessment by SB and PCR. The results were compared with the clinical, histologic, cytological, and immunophenotypic data.

Our results show that the BIOMED-2 PCR-based GS analysis of *TCRB* and *TCRG* rearrangements is a very reliable and sensitive method for detecting clonal T-cells in CTCL. This technique seems to be as informative as SB analysis of the *TCRB* locus. Although SB analysis should still be considered as the most informative method in CBCL, our BIOMED-2 multiplex PCR approach of Ig clonality detection is only slightly less informative. The implications of our results for diagnosing suspected cutaneous lymphoproliferations are discussed.

DESIGN AND METHODS

Patients

From February 1990 until November 2001 154 DNA samples (skin, n=75; LN, n=18; BM, n=12; PB, n=47; ascites, n=1; synovium, n=1) from a total of 60 patients with

an initial suspicion of (primary) CBCL or CTCL were obtained. Routine biopsies were 4 mm punch biopsies, whereas occasionally *in situ* or excision biopsies were taken in case of deeply infiltrated or tumorous lesions. All patients were seen and followed at the Department of Dermatology of the Erasmus MC, University Medical Center Rotterdam. The patients with a strong suspicion of malignant disease (n=48) were discussed in the Dutch Cutaneous Lymphoma Working Group (DCLWG), where a consensus about the definite diagnosis was reached. Twelve patients were not discussed in the DCLWG, because in an early stage of the diagnostic process a diagnosis of non-malignancy was made. The DCLWG uses the European Organization for Research and Treatment of Cancer (EORTC), classification for primary cutaneous lymphomas, which is based on a combination of clinical, histologic and immunophenotypic criteria.[24]

Twelve patients were diagnosed as having (primary or secondary) CBCL. Patients with confirmed primary CBCL (n=7) could be divided according to the EORTC classification into having the following disease categories: primary cutaneous follicular center cell lymphoma (PCFCCL; n= 3), large B-cell lymphoma of the leg (n=3) and immunocytoma (n=1). In addition, five patients were eventually diagnosed as having primary nodal B-NHL with secondary skin involvement. These patients were classified according to the WHO classification of lymphoid neoplasms as having:[25] follicular lymphoma (n=2), diffuse large B-cell lymphoma (n=1) and lymphoplasmacytic lymphoma (n=2).

Thirty-one patients were diagosed as having (primary or secondary) CTCL. The confirmed primary CTCL (n=28) were categorized according to the EORTC classification into: mycosis fungoides (MF; n=16), Sézary's syndrome (SS; n=3), $CD30^+$ anaplastic/pleomorphic large cell CTCL (n=5), pleomorphic small sized CTCL (n=1), and lymphomatoid papulosis (LyP; n=3). There was an extra group of three patients with a proven T cell lymphoma which could not be placed in one of the groups of the EORTC classification. These diagnoses were T-NHL with skin involvement (n=1), $CD8^+$ CTCL (n=1), and adult T-cell leukemia lymphoma (ATLL; n=1). One additional patient was diagnosed as having a natural killer (NK)-cell lymphoma.

Patients with a confirmed diagnosis of non-CTCL, non-CBCL (n=16) lymphoproliferations were divided into the following groups: pseudo B-cell lymphoma (n=4) and benign dermatoses (n=12). The latter could be subdivided in dermatitis (n=8), histiocytosis (n=2), Jessner-Kanoff lymphocytic infiltation of the skin (n=1), and mucinosis follicularis (n=1).

Diagnosis and staging

The 48 patients with a strongly suspected malignant disease underwent extensive examination to assess the diagnosis and the stage of disease. The DCLWG used the staging system according to Fuks.[26] This examination consisted of imaging techniques (chest X-ray, computed tomography (CT) of thorax and abdomen), histologic and immunophenotypic analysis of skin tissue, and cytological analysis of PB. LN excision and examination were performed in patients with palpable lymph nodes. If the PB cytomorphology was suspicious, BM aspiration and biopsy were performed as well. Immunophenotyping on cell suspensions and/or cryostat sections of skin, LN, BM, and PB samples was performed with several distinct markers to determine the differentiation lineage of the suspicious lymphocytes (e.g. CD1, CD2, CD3, CD4, CD5, CD8, CD10, CD19, CD20, CD22 and CD30) and the presence of clonality in case of suspect B-lymphocytes via single Ig light chain expression (Igκ and Igλ).

DNA isolation

High molecular weight DNA was isolated from skin, LN, PB, BM, and ascites specimens that were collected during routine diagnostic procedures, using a phenol-chloroform extraction-based protocol,[14] followed by ethanol precipitation and resolution in TE buffer.

Southern blot analysis

SB analysis was performed as described elsewhere.[14] In short, 10-15 µg of high molecular weight DNA were digested with *Bgl*II, *Bam*HI/*Hin*dIII, *Eco*RI, or *Hin*dIII, electrophoresed in 0.7% (w/v) agarose gels, and transferred to nylon filters, which were hybridized with several well-defined ^{32}P-labeled probes. The IGHJ6 probe (DAKO Corporation, Carpinteria, CA, USA) was used for the analysis of the *IGH* genes in combination with *Bgl*II or *Bam*HI/*Hin*dIII digests,[18] whereas the TCRBJ1, TCRBJ2, and TCRBC probes (DAKO Corporation) were used for analysis of the *TCRB* genes in combination with *Eco*RI and *Hin*dIII digests.[19] Incidentally, in case of inconclusive SB results the samples were further analyzed using specific probes for the Igκ (*IGK*) and *TCRG* genes. For the *IGK* genes this involved the IGKJ5, IGKC, and IGKDE probes (DAKO Corporation) on *Bgl*II or *Bam*HI/*Hin*dIII digests.[27] For the *TCRG* genes, specific probes were used for the Jγ1.1/2.1 and Jγ1.3/2.3 gene segments (in *Eco*RI digests) and for the Jγ1.2 gene segment (in *Bgl*II digests).[14] In one patient, diagnosed as having NK-cell lymphoma, assessment of the presence of (clonal) EBV genome was also performed, using the *Xho*I probe in *Bam*HI/*Hin*dIII digested DNA.[28]

PCR amplification

In each 50 µl PCR reaction, 200 ng DNA, 10 pmol of 5' and 3' oligonucleotide primers, 0.2 mmol/L dNTP, 5 µL 10xGold buffer (*IGH*, *IGK*, *IGL*) or 5 µL 10xbuffer II (*TCRB*, *TCRG*, t(14;18)), and 1-2 U Ampli*Taq* Gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. The concentration of $MgCl_2$ ranged from 1.5 mmol/L (*IGH*, *IGK*, *TCRB* tube C, *TCRG*, t(14;18)) and 2.5 mmol/L (*IGL*) to 3 mmol/L (*TCRB* tubes A and B). All amplification reactions were performed in an automated thermocycler (model ABI 9600; Applied Biosystems) using multiplex PCR according to BIOMED-2 protocol.[20] Cycling conditions were those described in the BIOMED-2 PCR protocol and consisted of the following steps: pre-activation (7 min, 95ºC), followed by 35 cycles of denaturation at 95ºC (45 sec for classical thermocylers; >30 sec for new generation thermocyclers), annealing at 60ºC (>45 sec for classical thermocyclers; >30 sec for new generation thermocyclers), and extension at 72ºC (1.5 min for classical thermocyclers; >30 sec for new generation thermocyclers), and a final extension step of at least 10 min at 72ºC.

For amplification of *IGH* rearrangements six FR (framework)1-V_H family primers, seven FR2-V_H family primers, seven FR3-V_H family primers and one FAM-labeled J_H consensus primer were used in three multiplex combinations (*IGH* multiplex tubes A, B, and C). NALM-6 DNA was employed as a positive control. In case of inconclusive *IGH* gene results, we further analyzed the samples using multiplex PCR reactions for the *IGK* and *IGL* genes. Amplification of the *IGK* locus was performed with six Vκ family primers and two FAM-labeled Jκ primers (*IGK* multiplex tube A) or the same six Vκ family primers, one intron RSS primer, and one FAM-labeled Kde primer (*IGK* multiplex tube B). For amplification of the *IGL* locus two Vλ primers and one FAM-labeled Jλ primer were used in a single

multiplex tube (*IGL* tube A). For amplification of *TCRG* genes we used VγIf and Vγ10 primers (*TCRG* multiplex tube A) or Vγ9 and Vγ11 primers (*TCRG* multiplex tube B) in combination with two FAM-labeled Jγ primers. Positive controls consisted of Molt 3 and ALL 1 (Tube A) and Jurkat, and patient's DNA samples (tube B). For amplification of *TCRB* rearrangements 23 Vβ family primers were used with 9 FAM-labeled Jβ primers (*TCRB* multiplex tube A), the same 23 Vβ family primers with the remaining 4 FAM-labeled Jβ primers (*TCRB* multiplex tube B), and 2 Dβ primers with all 13 FAM-labeled Jβ primers (*TCRB* multiplex tube C).[20] Positive controls consisted of ALL 1 (tube A), CML-T1 (tube B), and Jurkat (tube C).

Following Ig/TCR amplifications, 10 μL of PCR product were loaded on 1% agarose gels to check whether any product had been formed. Subsequently the rearranged Ig/TCR products were further analyzed by heteroduplex analysis (*see below*) and GeneScan analysis (*see below*) to assess whether the obtained PCR products were derived from monoclonal or polyclonal cell populations.

PCR amplification of the t(14;18) translocation was performed according to BIOMED-2 guidelines in three muliplex reactions covering the MBR, 3'MBR, and mcr regions.[20] This analysis was performed in all CBCL and pseudo B-cell lymphoma patients. PCR analysis for the detection of the two main types of *TAL1* gene deletions (types 1 and 2) was essentially performed as described previously.[29] All CTCL patients with a clonal T-cell population were analyzed using this PCR.

Heteroduplex (HD) analysis

The PCR products for HD analysis were denatured at 94ºC for 5 min and subsequently cooled at 4ºC for 60 min to induce duplex formation.[30] After duplex formation, 20 μL of the hetero- and/or homoduplexes were immediately loaded on 6% non denaturing polyacrylamide gels in 0.5 x Tris-Boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining. A 100 base pair (bp) size marker was used to determine the correct size of the PCR products.

Fluorescent fragment analysis (GeneScan (GS) analysis)

Two microliters of 10-fold diluted PCR products were mixed with 2.0 μL of formamide, 0.5 μL of 6-carboxytetramethylrhodamine-labeled internal standard (Genescan 500-TAMRA, Applied Biosystems), and 0.5 μL of loading buffer (blue dextran). After denaturation at 95°C for 2 min and cooling, 3 μL of the mixture were size-separated on a high-resolution polyacrylamide gel and analyzed using an automated ABI PRISM 377 fluorescent sequencer (Applied Biosystems). The size and profile of the PCR products were determined using GeneScan 672 computer software (Applied Biosystems).[31,32]

RESULTS

We investigated 154 DNA samples from 60 patients in whom a clinical diagnosis of (primary) cutaneous lymphoma was initially considered. PCR analysis of Ig/TCR gene rearrangements was performed on all samples, whereas SB analysis could be performed on 144 of these samples (i.e. all skin samples and the vast majority of non-skin samples)

(Tables 1-3). PCR and SB analysis were, in principle, performed once. In practice this means that Ig clonality was evaluated with the IGHJ6 probe and in three different FR *IGH* multiplex PCR tubes, whereas TCR clonality was analyzed with several *TCRB* probes and in *TCRB* as well as *TCRG* multiplex PCR tubes. In case of doubtful or discrepant results, PCR assays were repeated and SB was checked using *IGK* and/or *TCRG* probes. Although skin biopsies were available from all patients, in three patients the amount of isolated DNA was not sufficient for reliable molecular analysis; in these patients DNA from suspicious LN samples was analyzed.

Ig/TCR gene rearrangement analysis in CBCL

Three patients diagnosed with *primary cutaneous follicular center cell lymphoma* (*PCFCCL*) and one with *immunocytoma* (Table 1) all demonstrated clonal *IGH* rearrangements by PCR HD/GS and SB analysis. In two of the three patients with *large B-cell lymphoma of the leg*, PCR HD and PCR GS analysis showed clonal B-cell populations in skin samples (Figure 1), whereas clonally rearranged *IGH* genes were detected in all three skin samples by SB analysis. Clonal *IGH* gene rearrangements were detected in four out of five patients with a primary *nodal B-NHL and skin involvement* by PCR-based HD/GS and SB analysis. Remarkably two of the CBCL patients showed large clonal PCR products after *IGH* PCR analysis. These products were outside the expected size range of V_H-D_H-J_H couplings, and concerned amplifications of V_H-D_H-J_H rearrangements in which the consensus JH primer appeared to anneal better to the more downstream J segment. Additional *IGK* PCR analysis confirmed clonality in both cases.

Table 1. Frequency of clonal *IGH* rearrangements in the skin of CBCL and 4 pseudo B-cell lymphoma patients, classified according to histopathologic and clinical criteria, as assessed by PCR heteroduplex/GeneScan analysis and Southern blot analysis.

	Number (%) of patients with clonal *IGH* rearrangements			
	IGH			
Diagnosis (no. of patients)	HD	GS	SB	t(14;18)
Primary cutaneous follicle center cell lymphoma (n=3)[a]	3/3 (100)	3/3 (100)	3/3 (100)	0/3 (0)
Immunocytoma (n=1)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)
Large B-cell lymphoma of the legs (n=3)[a]	2/3 (67)	2/3 (67)	3/3 (100)	0/3 (0)
Nodal B-NHL with skin involvement (n=5)	4/5 (80)	4/5 (80)	4/5 (80)	2/5 (40)
All CBCL (n=12)	**10/12 (83)**	**10/12 (83)**	**11/12 (92)**	**2/12 (17)**
Pseudo B-cell lymphoma (n=4)[b]	0/4 (0)	0/4 (0)	2/4 (50)	1/4 (25)

Abbreviations: HD, heteroduplex; GS, GeneScan analysis; SB, Southern blot analysis; *IGH*, immunoglobulin heavy chain gene; ND, not done

[a] In one patient with a primary cutaneous follicle center cell lymphoma and one diagnosed with large B cell lymphoma of the legs large clonal *IGH* PCR products (see text for explanation) were detected. After additional PCR analysis of the *IGK* locus, clonal *IGK* gene rearrangements were detected in both patients.

[b] Clonal *IGK* gene rearrangements were detected in one patient.

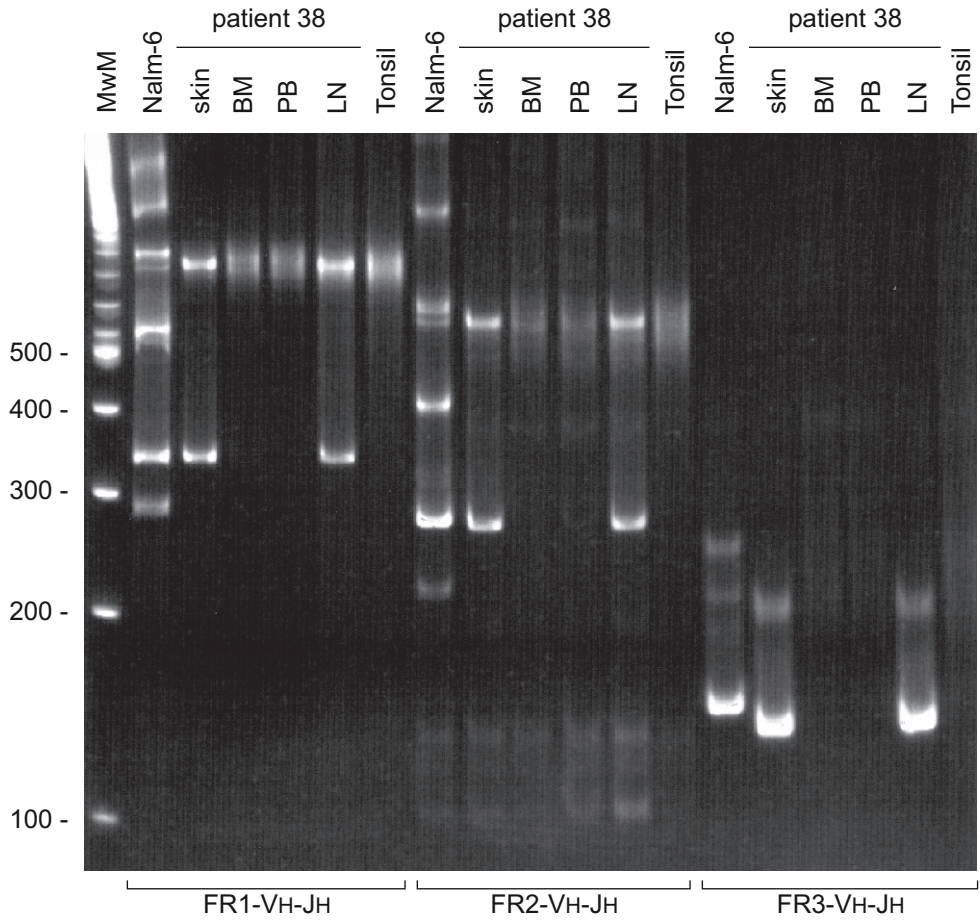

Figure 1. Heteroduplex analysis of *IGH* PCR products from a CBCL patient.
After amplification of the *IGH* gene rearrangements with FR1-VH, FR2-VH, FR3-VH and a JH consensus primer, PCR products were denatured for 5 min at 94ºC and renatured for 1 h at 4ºC. Electrophoresis was performed in 6% non-denaturing polyacrylamide gels, using a 100 bp size marker. Homoduplexes can be identified in control cell line DNA (Nalm-6) and in skin and LN DNA from patient 38, diagnosed with a large B-cell lymphoma of the leg. Heteroduplexes as identified in polyclonal tonsillar DNA and in BM and PB DNA from patient 38, suggest the absence of the B-cell clone in BM and PB.

Molecular analysis of the *IGH* locus was also performed on the skin samples of four patients with *pseudo B-cell lymphoma* (Table 1). Clonal B-cell populations were detected in two cases by SB analysis and in none by PCR analysis. In one patient, who did not show any clonally rearranged *IGH* gene rearrangements, clonal *IGK* but polyclonal *IGL* gene rearrangements were detected (data not shown).

Two out of twelve analyzed CBCL patients demonstrated a t(14;18) translocation in skin and PB samples. These two patients were diagnosed as having a primary nodal follicular

lymphoma with secondary skin involvement, whereas none of the patients with primary cutaneous CBCL were positive. Remarkably, one out of four patients diagnosed with a pseudo B-cell lymphoma showed a t(14;18) translocation in the skin sample as well. Although no clonal Ig gene rearrangements could be detected by SB and PCR analysis, this finding is suggestive of an early stage primary nodal follicular lymphoma, but requires further investigation and intensive follow-up.

Clonal TCR rearrangements were not detected in any of the skin and/or LN samples of the CBCL or pseudo B-cell lymphoma patients.

Ig/TCR gene rearrangement analysis in CTCL

Our study population included 16 patients with *mycosis fungoides (MF)* stage I to IV. Molecular analysis was performed on skin and/or LN samples from all these patients. Nine patients presented in an early stage of the disease with patch/plaque cutaneous lesions (stage I-II). Clonally rearranged *TCRB/TCRG* genes were detected in four out of eight cases (50%) by PCR HD analysis and five out of eight (63%) by PCR GS analysis. Clonal *TCRB* gene rearrangements were found in the skin samples of six out of nine stage I-II patients (67%) by SB analysis. Seven patients were diagnosed with MF stage III-IV. In the skin of four out of six of these patients (67%) clonal T-cell populations were detected by PCR-based *TCRB/TCRG* gene rearrangement analysis and SB analysis of the *TCRB* locus. In the seventh patient, from whom only LN and PB samples could be studied molecularly, a T cell clone was found in the LN sample by both PCR and SB analysis.

Molecular analysis of the skin and/or LN samples of all three patients classified as having *Sézary syndrome (SS)* showed clear clonal *TCRG* and *TCRB* gene rearrangements by PCR and SB analysis (Figures 2 and 3).

Skin samples of four patients with *anaplastic/pleomorphic CD30*$^+$ *CTCL* were analyzed molecularly. Three samples showed clonal *TCRG* gene rearrangements by PCR HD analysis and all four by GS analysis. Molecular analysis of the *TCRB* locus demonstrated clonal T-cell populations in three out of four skin samples (Figure 4). SB analysis of a LN sample of a fifth patient did not show clonal *TCRB* gene rearrangements. In one of the three patients diagnosed with *lymphomatoid papulosis (LyP)*, a clonal T-cell population could be detected by TCR PCR analysis of the skin sample.

The remaining four patients in our study were patients with T-cell lymphoma/leukemia with skin involvement: *pleomorphic small sized CTCL* (n=1), *CD8*$^+$ *CTCL* (n=1), *primary nodal T-NHL* (n=1) and *adult T-cell leukemia lymphoma (ATLL*; n=1).

PCR GS analysis of the *TCRG* locus showed clonal rearrangements in all four patients (100%). Clonal *TCRG* gene rearrangements in the LN sample of the ATLL patient were not detected by HD analysis. Clonally rearranged *TCRB* genes were only detected in skin and/or LN DNA of the CD8$^+$ CTCL and ATLL patients by PCR analysis but in all four by SB analysis.

We also investigated the presence of deletions affecting the *TAL1* gene, but were not able to detect *SIL/TAL1* fusion genes in any of the analyzed CTCL patients. Moreover clonal Ig gene rearrangements were not found in skin and/or LN samples of any of the CTCL patients either.

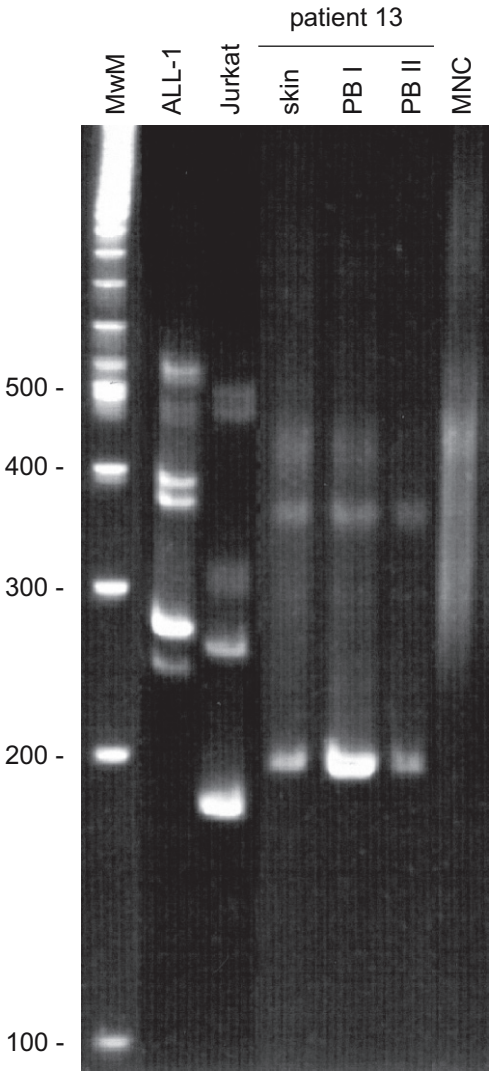

Figure 2. Heteroduplex analysis of *TCRG* PCR products from a CTCL patient.
After amplification of the *TCRG* gene rearrrangements with Vγlf, Vγ10 and Jγ1.3/2.3, Jγ1.1/2.1 primers, PCR products were denatured for 5 min and renatured for 1 h at 4°C. Electrophoresis was performed in 6% non-denaturing polyacrylamide gels, using a 100 bp size marker. Homoduplexes can be identified in control cell line DNA (ALL-1 and Jurkat) and in skin and PB DNA of patient 13, diagnosed with Sézary syndrome. The PB II sample was taken 4 years after treatment, indicating a relapse. Heteroduplexes can be identified in polyclonal MNC DNA.

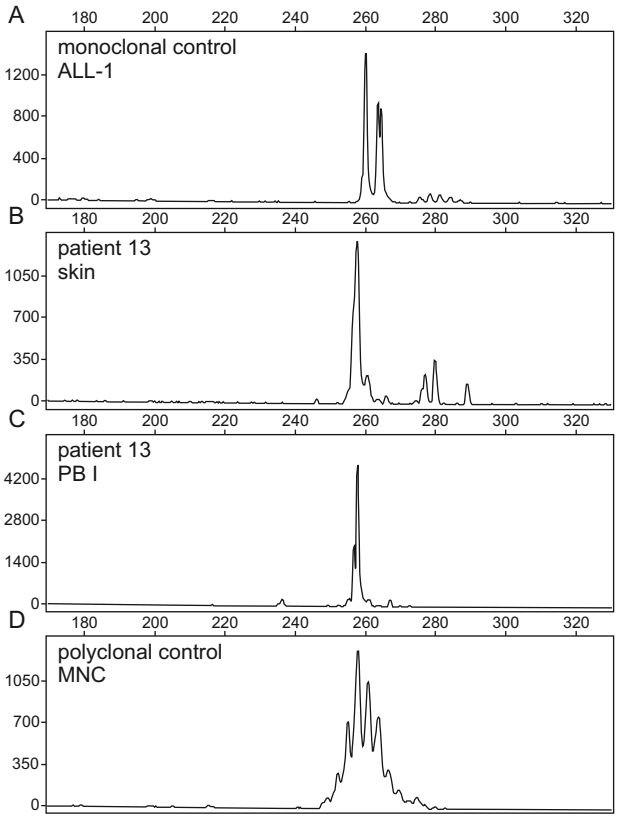

Figure 3. GeneScan analysis of PCR products of *TCRB* gene rearrangements using multiplex combination A. Monoclonal *TCRB* gene rearrangements can be identified in ALL-1 cell line DNA and skin and PB DNA from patient 13, diagnosed with Sézary syndrome. Polyclonal *TCRB* gene rearrangements can be identified in MNC DNA.

Finally one case of *NK-cell lymphoma* was included in this study. Of this patient a LN sample was analyzed (Table 2). No clonal *IGH* and TCR gene rearrangements could be detected by PCR or SB analysis. However, we did detect clonal EBV genome in the involved LN by SB analysis.

Ig/TCR gene rearrangement analysis in benign dermatoses

Our study included eight patients with *dermatitis*. Polyclonal TCR and Ig gene rearrangements were detected in cutaneous lesions in seven out of eight (88%) patients confirming the presence of reactive T- and B-cells. Remarkably one patient showed clonal *TCRB/TCRG* gene rearrangements in skin and PB by PCR HD/GS analysis, but not by SB analysis. However, on clinical, histomorphological, cytomorphological and immunphenotypical grounds there was no suspicion of a CTCL in this patient.

Furthermore, two patients diagnosed with *histiocytosis*, one with *M. Jessner* and one with *mucinosis follicularis* only showed polyclonal T- and B-cells in their skin samples (Table 3).

Table 2. Frequency of clonal *TCRB* and *TCRG* rearrangements in the skin or lymph node (LN) of CTCL, ATLL and NK-cell lymphoma patients, classified according to histopathologic and clinical criteria as assessed by PCR heteroduplex/GeneScan analysis and *TCRB* Southern blot analysis.

	Number (%) of patients with clonal TCR gene rearrangements in skin and/or LN									
	Skin					Skin and/or LN				
	TCRG		*TCRB*			*TCRG*		*TCRB*		
Diagnosis (no. of patients)	HD	GS	HD	GS	SB	HD	GS	HD	GS	SB
Mycosis fungoides (n=16)										
-early; stage I-II (n=9)	4/8 (50)	5/8 (63)	4/8 (50)	5/8 (63)	6/9 (67)	4/8 (50)	5/8 (63)	4/8 (50)	5/8 (63)	6/9 (67)
-late; stage III-IV (n=7)	4/6 (67)	4/6 (67)	4/6 (67)	4/6 (67)	4/6 (67)	5/7 (71)	5/7 (71)	5/7 (71)	5/7 (71)	5/7 (71)
Sézary syndrome (n=3)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
Large cell CD30$^+$ CTCL, Anaplastic and pleomorphic (n=5)	3/4 (75)	4/4 (100)	3/4 (75)	3/4 (75)	3/4 (75)	3/4 (75)	4/4 (100)	3/4 (75)	3/4 (75)	3/5 (60)
Lymphomatoid papulosis (n=3)	1/3 (33)	1/3 (33)	1/3 (33)	1/3 (33)	0/3 (0)	1/3 (33)	1/3 (33)	1/3 (33)	1/3 (33)	0/3 (0)
Pleomorphic small sized CTCL (n=1)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)
CD8$^+$ CTCL (n=1)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
T-NHL (with skin involvement) (n=1)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)
ATLL (with skin involvement) (n=1)	ND	ND	ND	ND	1/1 (100)	0/1 (0)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
All CTCL (n=31)	**17/26 (65)**	**19/26 (73)**	**15/26 (58)**	**16/26 (62)**	**20/29 (69)**	**19/29 (66)**	**22/29 (76)**	**18/29 (62)**	**19/29 (66)**	**21/31 (68)**
NK-cell lymphoma (n=1)[a]	ND	ND	ND	ND	ND	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)

Abbreviations: HD, heteroduplex analysis; GS, GeneScan analysis; SB, Southern blot analysis; *TCRB*, T-cell receptor beta gene; *TCRG*, T-cell receptor gamma gene; ND, not done

[a] Clonal (episomal) EBV genome was observed.

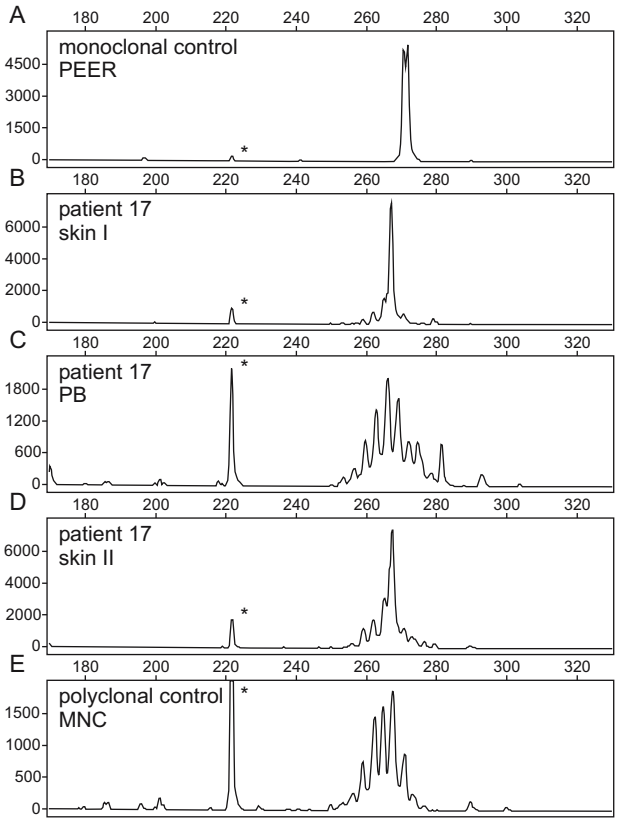

Figure 4. GeneScan analysis of PCR products of *TCRB* gene rearrangements using multiplex combination B. Monoclonal *TCRB* gene rearrangements can be identified in PEER cell line DNA and skin DNA from patient 17, diagnosed with $CD30^+$ CTCL. Skin samples were taken from two different sites. Polyclonal *TCRB* gene rearrangements can be identified in PB DNA from patient 17 and MNC DNA. Asterisks indicate an aspecific peak.

Detection of identical clonal Ig/TCR rearrangements in extracutaneous tissues

The presence of identical clonal Ig/TCR gene rearrangements in LN, PB, BM, ascites and/or synovium specimens was investigated in patients with a clonal B-/T-cell population in the skin (Table 4).

Table 4 shows that gene rearrangement analysis of the draining LN is of additional diagnostic value, because an identical clonal B- or T-cell population could be detected in 9/12 (75%) CBCL/CTCL cases. Identical clonal TCR gene rearrangements were detected in 4/7 (57%) BM samples of CTCL patients and clonally rearranged *IGH* genes as identified in the skin, were detected in one out of two BM samples of CBCL patients. In these three patients with clonal rearrangements in the BM samples, suspicious atypical cells were identified by cytomorphologic analysis of the BM biopsy specimen.

Identical clonal TCR gene rearrangements were found in the PB of two out of three patients with late stage MF and in all patients with SS. In these patients, data of molecular clonality analysis and cytomorphologic analysis were concordant. Clonally rearranged *IGH* genes were detected in the PB of four out of five B-NHL patients with secondary skin involvement. However, cytomorphologic analysis of the PB of these patients did not show any atypical lymphocytes.

Remarkably, clonally rearranged *IGH* genes were repeatedly found in PB samples of a patient with MF stage I, suggesting that this patient was not only suffering from MF (in the skin), but probably also from an indolent chronic B-cell leukemia. Dual genotypes in the same patient have recently been described to be a quite frequent event.[33] On the other hand, clear clonal *TCRG*/*TCRB* gene rearrangements were detected in the PB sample by PCR HD/GS analysis in a patient diagnosed with a nodal B-NHL with secondary skin involvement.

Although unexpected, clonal *TCRG* and *TCRB* gene rearrangements were found in PB and BM by PCR analysis in one patient diagnosed with dermatitis (Table 3). Cytomorphologic analysis did not reveal any atypical lymphocytes in these samples.

One MF IV patient showed ascites according to CT scanning. Gene rearrangement analysis of the ascites demonstrated clear clonal *TCRG* and *TCRB* gene rearrangements, identical to those found in skin, LN, BM and PB (Figure 5). From another MF IV patient, suffering from arthritis deformans of the hands,[34] a synovium biopsy was taken. Gene rearrangement analysis demonstrated clonal *TCRB* gene rearrangements that were identical to the rearrangements observed in the skin lesion of this patient.

Table 3. Frequency of clonal *IGH*/TCR rearrangements in the skin of patients with benign dermatoses, according to histopathologic and clinical criteria, as assessed by PCR heteroduplex/Genescan analysis and Southern blot analysis.

	Number (%) of patients with clonal *IGH*/TCR rearrangements		
	Skin		
	IGH/TCR		
Diagnosis (no. of patients)	HD	GS	SB
Dermatitis[a] (n=8)	1[a]/8 (13)	1[a]/8 (13)	0/7 (0)
Histiocytosis[b] (n=2)	0/2 (0)	0/2 (0)	0/2 (0)
M. Jessner (n=1)	0/1 (0)	0/1 (0)	0/1 (0)
Mucinosis follicularis (n=1)	0/1 (0)	(0/1)	0/1 (0)
Benign lymphoproliferations (n=12)	**1/12 (8)**	**1/12 (8)**	**0/12 (0)**

Abbreviations: HD, heteroduplex analysis; GS, GeneScan analysis; SB, Southern blot analysis; *IGH*, immunoglobulin heavy chain gene; *TCR*, T-cell receptor gene

[a] The clonal *TCRG* and *TCRB* gene rearrangements were observed by PCR analysis only.

[b] In one case of histiocytosis clonal *TCRG* and *TCRB* gene rearrangements were observed in BM by PCR analysis and in PB by PCR and SB analysis.

Table 4. Detection of identical clonal Ig/TCR rearrangements in extracutaneous tissues in CBCL/CTCL patients, as assessed by PCR and/or Southern blot analysis.

Diagnosis	LN	BM	PB	Other
CBCL				
Primary cutaneous follicle center cell lymphoma	n.a.	n.a.	0/3	n.a.
Large B-cell lymphoma of the legs	1/1	0/1	0/2	n.a.
Immunocytoma	n.a.	n.a.	n.a.	n.a.
B-NHL with skin involvement[a]	n.a.	1/1	4/5	n.a.
Pseudo B-cell lymphoma	n.a.	n.a.	1/2	n.a.
CTCL				
Mycosis fungoides				
-early; stage I-II[b]	0/1	0/2	0/6	n.a.
-late; stage III-IV	2/3	1/1	2/3	2/2[c]
Sézary syndrome[d]	3/3	2/2	3/3	n.a.
Anaplastic pleomorphic $CD30^+$ CTCL	n.a.	0/1	0/3	n.a.
Lymphomatoid papulosis	0/1	n.a.	0/3	n.a.
Pleomorphic small sized CTCL	1/1	1/1	n.a.	n.a.
$CD8^+$ CTCL	n.a.	n.a.	0/1	n.a.
T-NHL (with skin involvement)	1/1	n.a.	n.a.	n.a.
ATLL (with skin involvement)	1/1	n.a.	1/1	n.a.

Abbreviation: n.a.; not applicable

[a] Clonal *TCRG/TCRB* gene rearrangements were found by PCR analysis in the PB of one patient as well.

[b] Clonal *IGH* gene rearrangements were repetitively detected in PB samples of one MF I patient.

[c] Rearrangement analysis was performed on an ascites and a synovium sample.

[d] A fourth SS patient showed identical T-cell clones in PB and BM samples, but no skin/LN samples were available.

DISCUSSION

We studied a group of 60 patients with an initial suspicion of (primary) cutaneous lymphoma to evaluate the contribution of molecular analyses in the diagnostic process of this special group of non-Hodgkin's lymphomas (NHL). A series of 144 (tissue) DNA samples was analyzed by SB analysis. The same samples and ten additional ones were analyzed by two PCR-based strategies (PCR HD and PCR GS analyses). In these strategies, we used well-defined sets of primers and PCR protocols, optimized and standardized in a recently finished European BIOMED-2 Concerted Action.[20] The results of all three techniques were compared and related to the clinico-pathologic diagnosis.

In skin samples of patients with histomorphologically proven CBCL clear clonal *IGH* rearrangements were observed in 92% of patients as assessed by SB analysis, but in 67% by PCR analysis. However, PCR analysis of the FR1 *IGH* locus demonstrated clonal products of a larger size (*see explanation in Results section*) in two additional patients, which was confirmed by *IGK* clonality in both patients. This raised the overall PCR-based clonality detection to 83% among CBCL patients. Thus, despite the additional *IGK* analyses, a 100% score was not reached by PCR analysis. This can partly be explained by somatic hypermutation in the V_H-D_H-J_H gene rearrangements of primary CBCL,[35] which may hamper the annealing of V_H-FR primers and, to a lesser extent, of J_H primers.[21,36] Polyclonal *IGH* PCR results in clinically

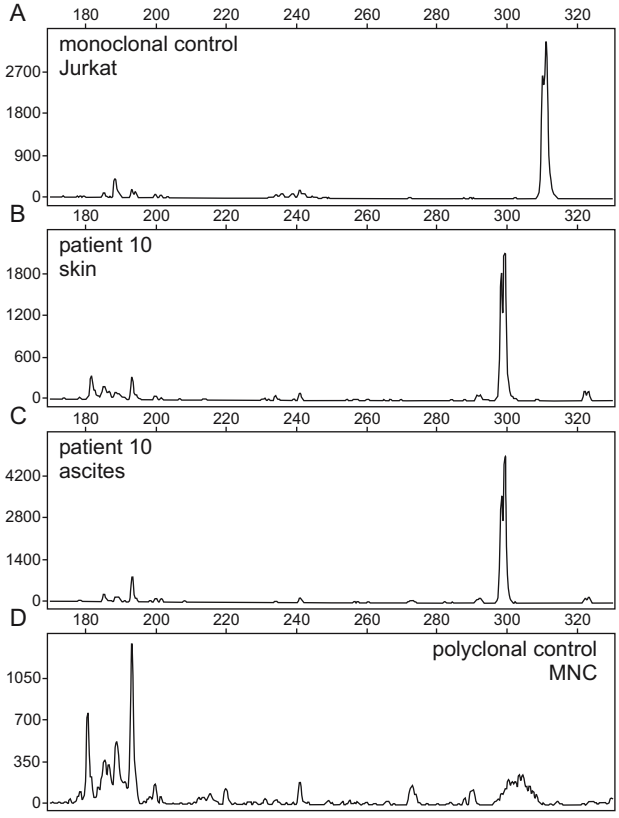

Figure 5. GeneScan analysis of PCR products of *TCRB* gene rearrangements using multiplex combination C. Monoclonal *TCRB* gene rearrangements can be identified in Jurkat cell line DNA and skin and ascites DNA from patient 10, diagnosed with mycosis fungoides, stage IV. Polyclonal *TCRB* gene rearrangements can be identified in MNC DNA.

suspicious skin samples should therefore be interpreted with caution and should at any time be checked by additional PCR assays using *IGK* and *IGL* primers, or by performing SB analysis. Two patients diagnosed with primary nodal follicular lymphoma with secondary skin involvement, but none of our primary CBCL patients, demonstrated a t(14;18) translocation in skin and PB. This confirms earlier findings that the t(14;18) translocation does not occur in primary CBCL.[37]

Interestingly, two out of four patients with pseudo B-cell lymphoma in our study had clonal *IGH* rearrangements in the skin lesions detectable by SB analysis, but not by PCR analysis. In one of these, clonal *IGK* gene rearrangements were detected by PCR analysis. Although pseudo B-cell lymphomas should be considered as benign lymphoid infiltrates, clonal B-cell populations have been found in a significant number of cases.[38,39] This may

support the concept that pseudo B-cell lymphomas and primary B-cell lymphomas are part of a continuous and progressive spectrum of CBCL.[38] In addition, a t(14;18) translocation was detected in skin samples of a patient with pseudo B-cell lymphoma without clonal Ig gene rearrangements. Regular follow-up of these three patients with clonality is therefore recommended.

One of the 31 patients with histomorphologically proven CTCL (ATLL included, NK-cell lymphoma excluded) in our study, clonal *TCRG* gene rearrangements were detected in 66% (PCR HD analysis) and 76% (PCR GS analysis) in skin and/or LN biopsies. Gene rearrangement analysis of the *TCRB* locus showed dominant T-cell clones in 68% by SB analysis, in 62% by PCR HD analysis, and in 66% by PCR GS analysis in skin and/or LN samples. Overall GS analysis had a slightly higher detection rate than HD analysis. Combination of *TCRG* and *TCRB* gene rearrangement analyses resulted in a slightly higher PCR HD clonality detection rate (69%), but the detection rate reached by PCR GS analysis did not change. Previous studies reported the involvement of translocation t(2;5) in a small number of primary CTCL.[9,40] In our study no involvement of t(2;5) could be detected (*data not shown*).

Molecular analysis of TCR genes appears to be a very sensitive technique for detecting clonal T cell populations. Previous studies reported higher TCR clonality detection rates by PCR analysis.[8,11,41] However, our series included many patients who were diagnosed with early stage MF (29%), a condition at least partly known for its oligoclonal nature.[42] The PCR clonality detection rate in early stage MF I-II patients was relatively low in our series (Table 2). PCR GS analysis reached the highest rate of detecting clonality (63%), this rate being comparable to that found by others.[43] Because of the oligoclonal nature, polyclonal/oligoclonal profiles have been detected in early MF skin lesions.[44] Data in the literature report infiltration of numerous reactive non-malignant lymphocytes in early MF lesions.[45,46] The ratio of clonal tumor cells to reactive polyclonal cells might, therefore, be lower than the detection threshold of the PCR analysis.[47] In as many as 38% of early forms of MF no clonal T cell population could be detected after PCR analysis of the *TCRG* locus.[47] Therefore, the finding of polyclonality/oligoclonality in patients with a cutaneous lymphoproliferation does not completely exclude a CTCL. Our study also included three patients with LyP. Only some patients diagnosed with LyP have a monoclonal T-cell proliferation.[48] When early stage MF and LyP were not considered, *TCRG* clonality was detected in 78% (PCR HD) and 89% (PCR GS), whereas *TCRB* clonality was 72% (PCR HD and GS) and 79% (SB).

In patients with an initial suspicion of cutaneous T- or B-cell lymphoma, the finding of a molecularly confirmed clonal/polyclonal B- or T-cell population in skin and/or other tissues can be of additional diagnostic value. PCR and SB analysis can give relevant information, especially in patients who cannot be diagnosed by conventional methods, such as histomorphology, immunophenotyping, and cytological analysis. Detection of a clonal B- or T-cell population can be an early sign of malignancy which should therefore result in careful follow-up of the involved patients. The finding of a molecularly confirmed polyclonal/oligoclonal lymphocyte proliferation, especially in patients without true signs of malignancy, makes a benign process more likely.

One out of twelve patients (8%) with benign dermatoses showed clonal T-cell populations by PCR analysis of the *TCRG* and *TCRB* genes in the skin sample, but not by SB analysis of *TCRB* genes. Also, the same clonal TCR rearrangements were demonstrated after

independently repeated *TCRG* and *TCRB* PCR analysis, thereby excluding the possibility of *pseudoclonality* due to a low frequency of polyclonal T-lymphocytes.[49] Although *clonal dermatitis* is known to progress to overt CTCL in 25% of cases,[50] monoclonality does not necessarily imply malignancy and molecular results should therefore always be correlated with the clinical, histopathologic and phenotypic data of the individual patient.[51] Clinical and histologic follow-up of the skin lesions of this and similar patients are thus strongly recommended. Unfortunately, our patient died of heart failure preventing further molecular and clinical follow-up in order to evaluate possible progression of the clonal skin lesions to overt CTCL.

Our BIOMED-2 multiplex PCR approach is a rapid, cheap, and simple procedure, which is therefore very suitable in a diagnostic setting. Because of the large number of concordant results between the histopathologic diagnosis and the PCR analyses, we can conclude that both HD and GS analyses of Ig/TCR rearrangements are very helpful in diagnosing patients with cutaneous lymphoproliferative disorders.

In general GS analysis might be slightly favorable over HD analysis because of the former's speed, accuracy, sensitivity, and easy interpretation.[21-23] However, the latter can be a cheaper and reliable alternative in a diagnostic setting, because expensive automated sequencing equipment and fluorochrome-labeled oligonucleotides are not necessary.

Extracutaneous presence of identical clonal T-cell populations is not exceptional in primary CTCL.[13,52-54] In our patients with early stage MF no clonal TCR rearrangements were identified in samples from extracutaneous sites. However, identical clones were detected in PB, LN and BM samples of 67% of patients with late stage MF. Furthermore, the same clonal B- or T-cell populations as those identified in the skin were detected in regional LN samples of nine out of twelve CBCL/CTCL cases. In two MF stage IV cases, gene rearrangement studies demonstrated the presence of the same clonal T-cell populations in skin and in uncommon extracutaneous locations, such as ascites and synovium. Dissemination of clonal lymphocytes in extracutaneous tissues can be easily detected by Ig/TCR analysis and is very helpful for staging.

In conclusion, our results show that the rate of clonality detection by the novel standardized BIOMED-2 based PCR analysis of rearranged *IGH* genes in CBCL is 83%, which is only slightly lower than the SB detection rate (92%), and can be further increased by *IGK* PCR analysis. However, because of the relatively low number of CBCL patients the small difference in sensitivity between SB and PCR analysis did not reach statistical significance. Furthermore, our study shows that BIOMED-2 PCR-based GS analysis of rearranged *TCRG* genes enables clonality detection in a rather high percentage of CTCL (76%). Because of this high detection rate and its speed, this technique is recommended for clonality analysis of tissue samples from patients initially suspected of having primary CTCL.

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Chapter 3.3

Clonal identity between skin and synovial tissue in a case of mycosis fungoides with polyarthritis

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ABSTRACT

Polyarthritis in the presence of a cutaneous T-cell lymphoma is a rare phenomenon. We describe a case of mycosis fungoides with development of a symmetric erosive polyarthritis of the small hand joints and feet, diagnosed as rheumatoid arthritis. An identical monoclonal T-cell population in the skin and in the synovium was detected by T-cell receptor gene rearrangement analysis, illustrating articular dissemination of lymphoma cells. Differentiating mycosis fungoides-associated arthritis from rheumatoid arthritis may have important implications for treatment. Based on this case, the relevant literature and the newest disease concepts, pathogenic mechanisms and therapeutic options of mycosis fungoides-associated arthritis are discussed.

INTRODUCTION

Mycosis fungoides (MF) is an uncommon mature T-cell lymphoma, presenting primarily in the skin with patches and plaques. In early-stage MF the main features are eczematous or psoriasiform skin lesions. In later stages these lesions might progress to nodules, tumors, and even erythroderma can be seen. In addition to skin, the lymph node (LN), peripheral blood, bone marrow, and almost any visceral organ can also be involved. In late-stage MF, extracutaneous dissemination of monoclonal cell populations can be easily detected by molecular T-cell receptor (TCR) gene rearrangement analysis.[1,2]

Polyarthritis in the presence of malignant cutaneous T-cell lymphoma has been described before,[3-12] but this combination of entities still is a rare phenomenon. However, defining the possible cause of the arthritis is important, even when the patient already has MF. If infiltration of malignant clonal T lymphocytes into the synovium was responsible for the arthritis, the staging of MF and, thereby, the treatment would be altered.[3,7,12] Typically, such polyarthritis is rheumatoid factor- negative, nonerosive, symmetric and mimics rheumatoid arthritis (RA).[9,11] If RA has developed in the absence of extracutaneous localization of MF, both disease entities can be treated separately.

In a meeting report, we previously mentioned a case of MF-associated polyarthritis.[13] In the current article this illustrative case will be described more extensively, using conventional diagnostic modalities like histomorphology, immunophenotyping, and cytologic analysis extended with molecular clonality tools with high specificity and sensitivity. The pathogenesis of polyarthritis in the presence of MF will be discussed in the context of the newest disease concepts together with the relevant literature.

CASE REPORT

A 57-year-old man was admitted to our hospital with a 12-year history of psoriasiform skin lesions. The patient was known to have had tuberculosis, seborrhoic eczema and a basal cell carcinoma of the right eyelid. A skin biopsy specimen of one of the psoriasiform lesions had been taken before and the histopathologic diagnosis of eczema was made. However, because of the clinical presentation of progressive skin lesions, the diagnosis of

MF was considered. A new biopsy specimen demonstrated dense lymphocytic infiltration with some atypical lymphocytes. Routine immunologic examination was not conclusive. Oral psoralen-UVA treatment was started.

Shortly after therapy onset, the patient presented with generalized dry and purple-red colored skin with several scattered necrotic nodules and enlarged inguinal LNs. In addition to a large plaque on the lower aspect of his abdomen, another large indurated plaque (15 × 20 cm) with ulcerations extended from the upper aspect of the right leg to the abdomen (Figure 1, A). Histopathologically, the skin biopsy demonstrated a dense infiltrate of atypical cells with epidermotropism (Figure 1, B). Electron microscopy confirmed the presence of atypical lymphocytes with cerebriform nuclei (Figure 1, C). This case was discussed in the Dutch Cutaneous Lymphoma Working Group (DCLWG), a panel of pathologists and dermatologists. A consensus of the definite diagnosis (stage II MF) was reached. Topical treatment with class II corticosteroid creams, and UVB therapy had a positive effect on the skin lesions. Symmetrical polyarthritis of wrists, small hand joints, and feet developed at age 62 years (Figure 2, A). He had morning stiffness, which lasted about 30 minutes.

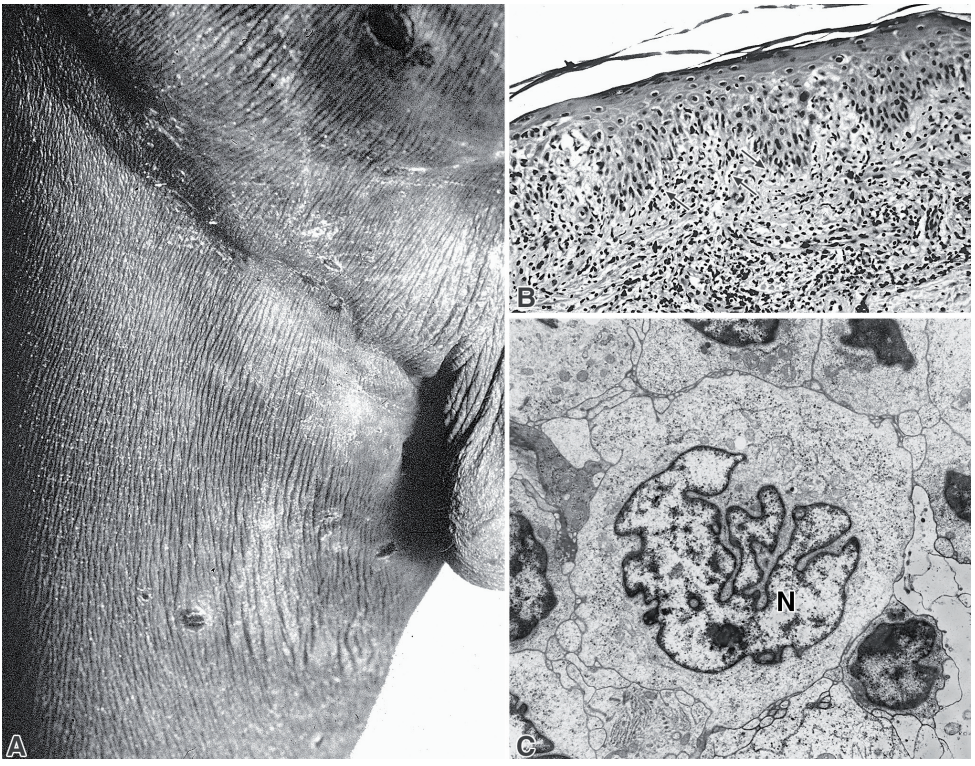

Figure 1.
A. Large indurated plaque with ulcerations and underlying lymphadenopathy of upper aspect of right leg and groin. **B.** Histology of skin. Skin biopsy specimen demonstrates an infiltrate of atypical lymphocytes with alignment along the basal layer of the epidermis (arrows) and epidermotropism consistent with diagnosis of mycosis fungoides (Hematoxylin-eosin stain; original magnification x 100). **C**. Electronmicroscopic image of atypical lymphocyte isolated from skin lesion. Note typical cerebriform nucleus (N).

Laboratory investigations revealed an erythrocyte sedimentation rate of 54 mm/h. Hemoglobin was 9.5 g/dL, white blood cell count was 3.9 x 10^9/L with 62% neutrophils, 7% eosinophils, 0.5% basophils, 14% lymphocytes and 16.5% monocytes. Rheumatoid factor, antinuclear antibody, extractable nuclear antigen antibodies, and antihuman T-cell lymphotrophic virus type I antibody were all negative.

Cytologic examination of peripheral blood and bone marrow did not reveal any abnormalities. In particular, no Sézary cells could be detected. Immunophenotyping of peripheral blood showed an abnormal T-cell population (15% of lymphocytes) inconsistent with normal T-cell

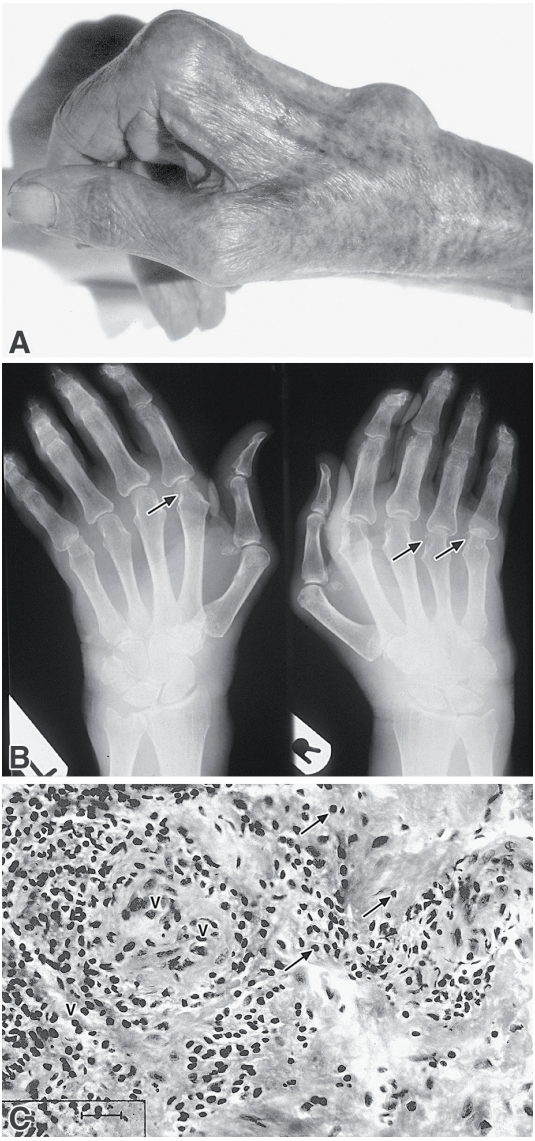

Figure 2.
A. Hand, showing signs of arthritis deformans. **B.** Roentgenogram of hands, demonstrating radiar deviation of carpus and ulnar deviation of fingers, periarticular osteoporosis, joint space narrowing, and joint erosions (arrows). **C**. Histology of synovium. Localization of an infiltrate composed of predominantly small round nucleated mature lymphocytes (arrows) with slight propensity for alignment along vessels (V). (Hematoxylin-eosin stain; original magnification x 400).

Table 1. Patients given the diagnosis of mycosis fungoides and associated polyarthritis or arthritis: review of the English-language literature.

Case No.	Age (y)/sex	MF stage[a]	Arthritis	Laboratory investigations	Radiography	Cytology/histology[b]		Clonality analysis[c]	Therapy target	
						PB/BM/LN	SF/synovium		MF	Joint symptoms
1 [3]	47/M	Ic	Arthritis of right knee and digits of left hand	ESR 119 ANA ND RF ND	Osteolytic defects/ destruction/ fractures of multiple bones, no erosive joint disease	ND/-/-	-/ND note: atypical lympho-cytes in tibial tumor	ND	Polychemo-Therapy/Rx for tibial mass	Prednisone
2 [4]	30/M	Ib	Symmetric poly-arthritis of hands, elbows, tempero-mandibular joints, knees and ankles	ESR ND ANA - RF -	No erosive joint disease	-/-/ND	ND/ND	ND	EBT	
3 [5]	74/M	IV	Symmetric poly-arthritis, including hands and feet	ESR ND ANA - RF - HTLV-I -	No erosive joint disease	+/+/+	-/chronic nonspecific synovitis	ND	PUVA/Rx	Methylprednisolone/ prednisone/NSAID/ MTX
4 [6,7]	41/F	II	Symmetric poly-arthritis of hands and ankles	ESR ND ANA - RF +	No erosive joint disease	ND/+/ dermatopathic lymphadenopathy	95% $CD4^+$ T-cells/ lymphocytic infiltrate	SF + PB -	Nitrogen mustard/EBT/ PUVA/retinoid/ anti-CD4 Ab	NSAID/hydrochlo-roquine/prednisone/ MTX
5 [8]	66/M	Ib	Monoarthritis of right ankle	Unknown	Destructive arthritis	Unknown	ND/chronic reactive changes without lymphocytic infiltrate	ND	Unknown	Surgery
6 [11]	64/F	Ib	Symmetric poly-arthritis including hands and feet	ESR 60 ANA - RF -	Erosive destruction of the left carpus and distal interphalangeal joints	ND/-/ND	Cell rich fluid/no indication of malignancy, infection, inflammation	ND	Methylpredni-solone/EBT	Steroids/EBT/aza-thioprine/cyclophos-phamide
7 [12]	31/F	II	Monoarthritis of right knee	ESR 31 ANA - RF + HTLV-I -	No erosive joint disease	ND/ND/ dermatopathic lymphadenopathy	+/ND	SF +	Polychemo-therapy	Polychemotherapy
8 (current study)	57/M	II	Symmetric poly-arthritis of hands and feet	ESR 54 ANA - RF - HTLV-I -	Erosive joint disease, compatible with RA	-/-/dermatopathic lymphadenopathy	ND/lymphocytic infiltrate indicative of chronic synovitis	Skin + SF ND Synovium +	PUVA/MTX	NSAID/ DMARD/ MTX

Ab: Antibody; ANA, antinuclear antibody; BM, bone marrow; DMARD, disease-modifying antirheumatic drug; EBT, total body electron beam therapy; ESR, erythrocyte sedimentation rate; F, female; HTLV, human T-cell lymphotropic virus; LN, lymph node; M, male; MF, mycosis fungoides; MTX, methotrexate; ND, not done; NSAID, nonsteroidal anti-inflammatory drug; PB, peripheral blood; PUVA, psoralen-UVA; RA, rheumatoid arthritis; RF, rheumatoid factor; Rx, radiotherapy; SF, synovial fluid.

[a] Clinical staging system according to van Doorn *et al.* [17]

[b] + Presence of atypical lymphoid cells ,-: no atypical cells could be detected.

[c] + Clonal *TCRB* and/or *TCRG* rearrangements, -: no clonal *TCRB* and/or *TCRG* rearrangements.

differentiation, but it was impossible to make a distinction between a malignant and a reactive cell population. Immunohistochemical bone marrow examination revealed no abnormalities. Histologic analysis of an inguinal LN revealed signs of dermatopathic lymphadenopathy.

Radiographs of the hands and wrists demonstrated radiar deviation of the wrists and ulnar deviation of the fingers, joint space narrowing, periarticular osteoporosis and multiple erosions (Figure 2, B). Radiographs of the forefeet showed erosions of the caput of fifth metatarsal bones. A synovial biopsy specimen of a swollen metacarpophalangeal joint of the left hand showed a large dense infiltrate of small mature lymphocytes with some perivascular infiltrates (Figure 2, C). Immunologic examination demonstrated a lymphocytic infiltrate that almost exclusively contained T cells, which were $CD45RO^{+}$ and $CD20^{-}$ (data not shown). The estimated CD4/CD8 ratio was 3. A large amount of fibroblasts, sporadic plasma cells, and macrophages were seen. Atypical cells were not detected. The whole picture was indicative of chronic synovitis, which could be compatible with a diagnosis of RA.

An RA diagnosis was made based on symmetric erosive polyarthritis of hands and feet. Treatment with both a nonsteroidal anti-inflammatory drug (naproxen) and a disease-modifying antirheumatic drug (sulfasalazine) was started. Because of therapeutic ineffectiveness, methotrexate was started. This showed subjective improvement, but the patient developed a severe anemia on this medication. His condition exacerbated and he developed widespread tumor-stage disease. A computed tomography scan of the abdomen revealed para-aortic lymphadenopathy. Multiagent chemotherapy was, therefore, indicated according to the treatment protocol of the DCLWG.[14,15] However, the patient refused more aggressive therapy.

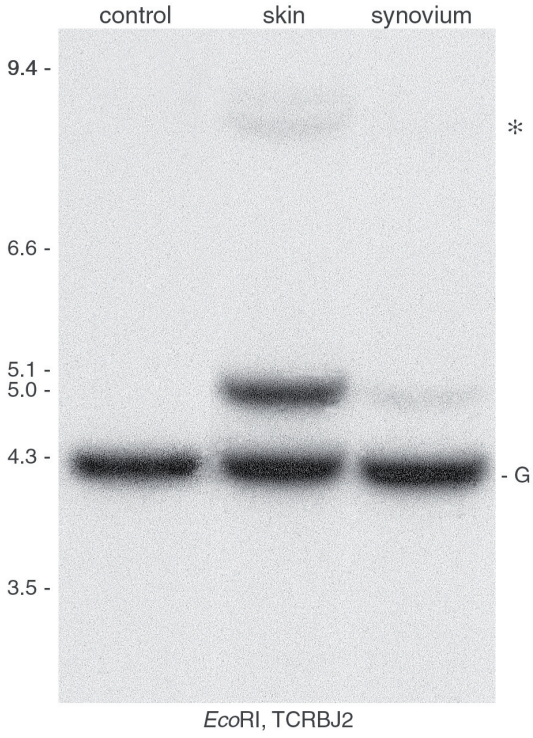

Figure 3. Southern blot analysis of *TCRB* genes of skin and synovial tissue DNA.
High molecular weight DNA (15 mg) was digested with *Eco*RI, electrophoresed in 0.7% agarose gels, and vacuum transferred to nylon filters.[16] On hybridization with the TCRBJ2 probe in EcoRI digests, clear clonal nongermline band (in kilobases, y-axis) is observed in the skin sample; same band in synovial DNA indicates clonal identity of the T-cell population in these tissues. Faint additional bands (asterisk) also represent germline and rearranged bands and result from incomplete digestion at an EcoRI site in between the Jβ2 and Cβ gene segments. Also in BglII digests identical bands were seen in skin and synovial tissue (data not shown).

The patient became progressively systemically ill with B symptoms (fever, weight loss, night sweats). Histologic examination of two skin lesions of both arms again confirmed the diagnosis of MF. His disease progressed, and he died 11 years after the initial admission to our hospital. Permission for autopsy was not given.

Shortly before the patient died, DNA extracted from both skin and synovial tissue was analyzed by conventional Southern blot (SB) analysis using the TCRBJ1, TCRBJ2 and TCRBC probes (DAKO Corp, Carpinter, California) in *Eco*RI and *Bgl*II digests.[16] Analysis of skin biopsy specimen showed two clonal *TCRB* rearrangements using both digests, one involving the Jβ1 and one involving the Jβ2 region. Identical clonal *TCRB* gene rearrangements were detected in synovial tissue DNA (Figure 3). A small proportion (approximately 5%) of the total cell infiltrate in the synovium consisted of the malignant clonal T-cell population but no polyclonal background of other T cells could be seen. These results indicate that the T cells that had infiltrated the synovial tissue were derived from the same malignant clonal T-cell population as found in the skin and that this population was a dominant clone within the infiltrate.

DISCUSSION

This case illustrates the prolonged clinical course of MF through many stages.[17] The diagnosis of MF was confirmed by panel review in the DCLWG. Five years after the clinical diagnosis of MF was made, the patient developed a chronic erosive polyarthritis that could be classified as RA according to the 1987 revised American Rheumatism Association criteria.[18] In a large study of 144 patients diagnosed with MF, no joint involvement was found on autopsy.[19] Nevertheless, arthritis or polyarthritis has been reported as a rare manifestation of MF. Because of the rarity of a combination of MF and polyarthritis, we can only speculate on the pathogenic mechanisms on basis of the literature and the observations we made in our case. Review of the literature demonstrated that MF-associated arthritis tends to be nonerosive and rheumatoid factor-negative (Table 1). Differentiating MF-associated polyarthritis from RA can be troublesome. The chronic polyarthritis in association with MF is sometimes accompanied by deformities of joints, morning stiffness, and eventually subcutaneous nodules, which are features mimicking RA. To our knowledge only two other cases of erosive arthritis in the presence of MF have been described. One patient with destructive monoarthritis, the other patient with destructive arthritis of the left carpus and the distal interphalangeal joints.[8,11] However, the erosive arthritis in both patients was not compatible with the clinical picture of RA.

Molecular analysis of synovial fluid cells of patients with MF and polyarthritis has been performed and revealed monoclonal TCR gene rearrangements.[6,7,12] As such, the finding of an (oligo)clonal T-cell population in the synovial tissue of patients with RA or other inflammatory rheumatic diseases even in absence of a T-cell malignancy can occur.[20-22] Tachibana *et al*[12] reported the detection of an identical clone in synovial fluid and LN by SB analysis in a patient with T-zone lymphoma. However, up until now no primary skin lesions of patients with MF have been analyzed for direct comparison. In our case, SB analysis demonstrated the presence of two rearranged bands of exactly the same size in both skin and synovial tissue DNA.

This means that both rearrangements used identical V, D, and J segments in both samples. This is highly suggestive of clonal identity, taking into account the extensive combinatorial diversity of the *TCRB* locus.[23] For final proof of clonal identity, sequencing of the clonal *TCRB* rearrangements would have to be performed. Unfortunately, this was not possible because of lack of synovial DNA. Nevertheless, based on the SB findings we have strong indications for the same malignant process with two different localizations. To our knowledge the finding of an identical clone of T cells in skin and synovial tissue by TCR gene rearrangement analysis has not been described before.

In general, there are 3 plausible explanations for MF-associated polyarthritis. The first explanation might be that of a cancer-related polyarthritis, either by direct invasion of bone and joints (tumor metastasis) or indirectly (paraneoplastic syndrome). Arthritis as a result of leukemic infiltration can develop both in adults and children with acute lymphoblastic leukemia.[24] This arthritis is often pauciarticular and preferentially involves large joints.[24] In the presence of MF, direct invasion of the synovium by malignant clonal $CD4^+$ cells has been described to result in a reactive cellular inflammatory response by the synovial tissue, in which the patients had a symmetric nonerosive polyarthritis.[6,7,12] Paraneoplastic arthritis is rare, of unknown origin, and has been reported in different types of neoplasms, including acute lymphoblastic leukemia.[25-27] Seleznick *et al.*[5] suggest that reactive phenomena directed to neoplastic T-cell proliferation may play a role in MF-associated polyarthritis. The joint symptoms are commonly asymmetric, nonerosive, and tend to spare the small joints of the hands and wrists.[26,28] Because the symmetric polyarthritis in our case demonstrated joint erosions compatible with RA, it did not fit the descriptions of the cancer-related polyarthritides.

A second explanation is that RA has developed independently of MF. Although the finding of RA in a patient with MF is very rare, the overall prevalence of RA is 1%.[29] Thus it is to be expected that 1% of patients with MF are likely to also have RA. However, because in our patient malignant T cells could be detected in the synovial tissue and because it is known that these cells are able to induce an immunologic response, an interaction between these diseases seems more logical.

We, therefore, favor a third explanation, namely that malignant lymphocytes are attracted to the synovium and modulate the RA. In MF, the tumor cells characteristically are $CD3^+$, $CD4^+$, $CD45RO^+$, $CD8^-$, and $CD30^-$.[14] Extravasation of circulating lymphocytes is regulated by lymphocyte-homing receptors, recognizing adhesion molecules. Synovial tissue of patients with RA contains activated endothelial cells expressing increased numbers of adhesion molecules.[30,31] We speculate that circulating malignant $CD4^+$ lymphocytes are recruited to the sites of inflammation and in RA preferentially to the inflamed joints. ”Homed” malignant T cells could further amplify inflammation by cytokine production. In MF, production of the proinflammatory cytokines IFN-γ and IL-2 has been reported *in vivo* by the lymphoma cells both at the messenger RNA as at the protein level.[32,33] IFN-γ and IL-2 are able to augment the inflammatory process in RA.[31,34]

Because of the important implications for treatment, we propose that in patients with MF and polyarthritis a synovial biopsy specimen might be considered in the diagnostic process, because even in RA lymphoma cells can be detected in the synovial tissue.

A combined diagnosis of MF and RA can also have consequences for the RA treatment. Some immunosuppressives, like cyclosporine and biologicals directed at tumor necrosis

factor-α, are of great benefit in the treatment of RA, but might worsen MF. Furthermore, because in this report it is hypothesized that MF is able to deteriorate the disease activity in RA, treating the MF will probably also influence the activity of the RA. Late-stage cutaneous T-cell lymphoma requires polychemotherapy, but it has recently been described that extracorporeal photochemotherapy might be a good treatment alternative for advanced-stage cutaneous T-cell lymphoma in combination with polyarthritis,[10] when available.

ACKNOWLEDGEMENTS

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Chapter 3.4

Late relapse of primary cutaneous CD30$^+$ anaplastic large cell lymphoma confirmed by T-cell receptor (TCR) PCR analysis

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Age: 47 years Sex: M

Clinical features: A 47 year-old Mediterranean man presented in November 1985 with an ulcerating skin tumor on the left cheek. Histopathological evaluation of a skin biopsy demonstrated a diffuse infiltrate of large polymorphic lymphoid cells, with many mitotic figures and no epidermotropism. Neoplastic cells were strongly positive for CD3 and CD8, and partially $CD4^+$. Complete regression occurred after local radiotherapy (20 x 2 Gy). Patient did not have any other complaints and dermatological examination did not demonstrate other skin lesions.

DIAGNOSIS

Primary cutaneous anaplastic large cell lymphoma

Follow up: During follow up patient reported waxing and waning of a few small skin lesions. Spontaneous regression of these lesions occurred in 2-3 months. At the end of 2003, 18 years after initial diagnosis, patient suffered from multiple progressive skin lesions. Dermatological examination demonstrated multiple fleshy nodules and erosive hemorrhagic tumors on the left arm, leg and buttock and partially regressed lesions on the right leg. Multiple skin tumor biopsies all showed localizations of an anaplastic large cell lymphoma. Immunohistochemistry was positive for CD3, CD4, CD8 and CD30 and negative for ALK-1. Molecular clonality analysis demonstrated identical monoclonal *TCRB* gene rearrangements in current skin biopsy specimens and in the initial diagnostic sample, illustrating that the current lesions concern relapses and not secondary or transformed tumors. Immunohistochemical analysis of original biopsy demonstrated large atypical cells, strongly positive for CD30. Staging did not reveal progression to extracutaneous stage of disease. Skin lesions were successfully treated with local radiotherapy (10 x 2 Gy).

COMMENT

Although it is known that in patients with primary cutaneous $CD30^+$ anaplastic large cell lymphoma skin lesions may spontaneously regress,[1] a full-blown relapse 18 years after initial diagnosis is extremely rare. This patient illustrates the spectrum of clinical features of $CD30^+$ lymphoproliferative disorders. Although the CD8/CD30 co-expression is rare and is associated with less favorable prognosis,[2] our patient appeared to be in good condition 19 years after initial diagnosis.

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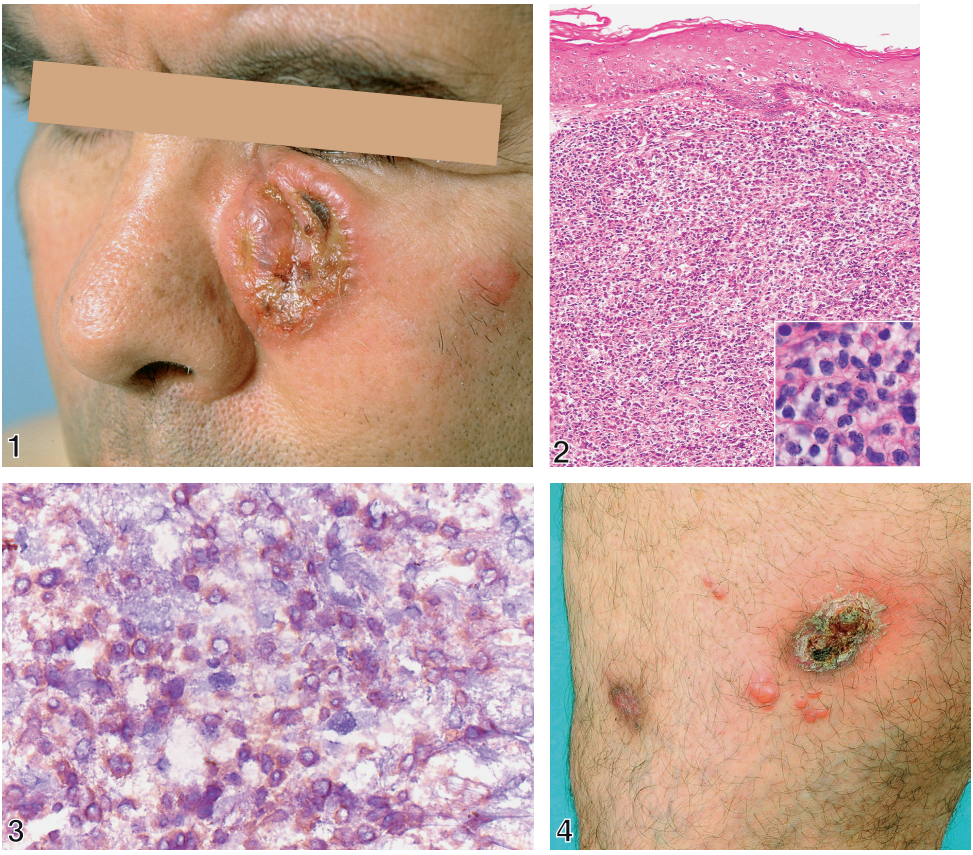

Figure 1. Initial diagnosis.
Large ulcerating skin tumor on the left cheek.

Figure 2. Histology of skin at initial diagnosis.
Diffuse infiltrate of large pleomorphic lymphoid cells in the dermis without epidermotropism. HE, microscopic magnification 100x; insert, high magnification.

Figure 3. Neoplastic cells are positive for CD8.
Microscopic magnification 200x.

Figure 4. Relapse.
Ulcerating tumor with central crust on the left leg, surrounded by multiple fleshy nodules.

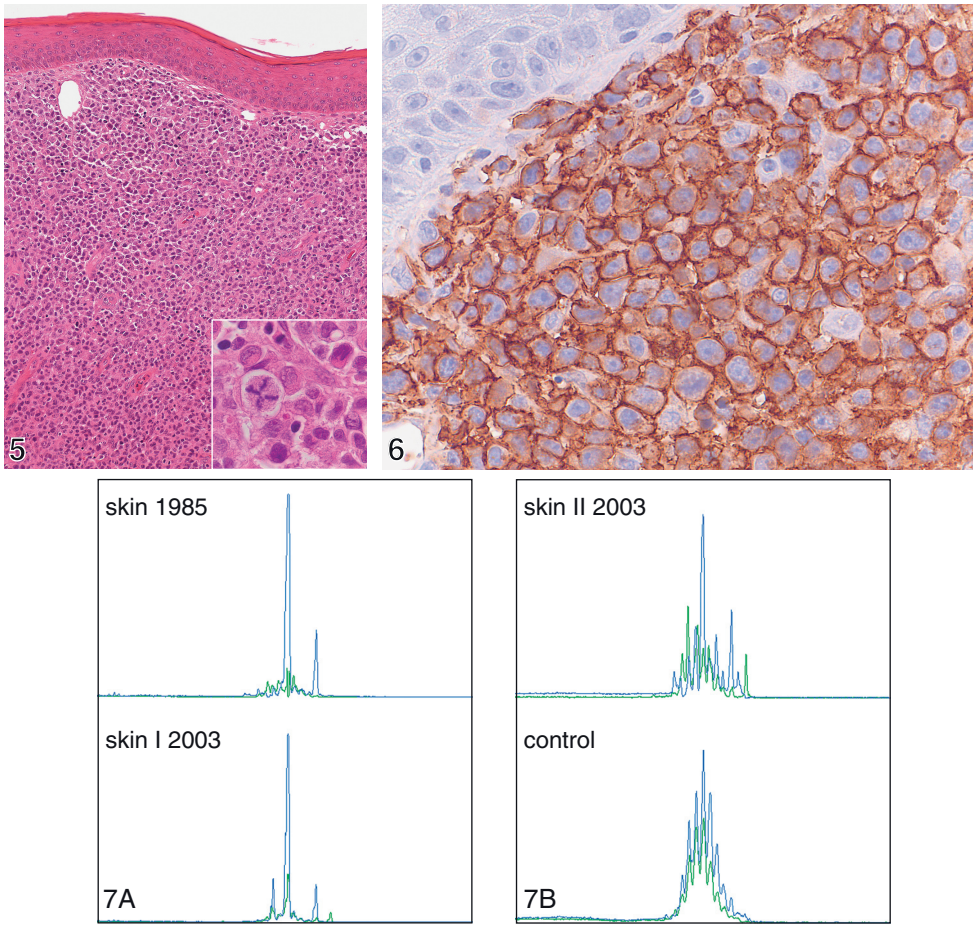

Figure 5. Histology of skin at relapse.
Diffuse dermal infiltrate of large atypical lymphoid cells with nuclear pleomorphism. HE, microscopic magnification 100x; inset, high magnification.

Figure 6. The neoplastic cells are strongly positive for CD30.
Microscopic magnification 200x.

Figure 7. PCR based GeneScan analysis.
Identical monoclonal *TCRB* gene rearrangements were detected in initial diagnostic sample (skin 1985) and recent skin biopsy specimen from tumor (skin I 2003) (**A**) as well as in skin biopsy from nodule (skin II 2003) Polyclonal *TCRB* gene rearrangements can be identified in control sample (**B**).

Chapter 4

MATURE T-CELL PROLIFERATIONS

4.1 Mature (post-thymic) T-cell leukemias

4.2 Clinically and genetically atypical T-cell prolymphocytic leukemia underlines the relevance of a multidisciplinary diagnostic approach
Haematologica 2007; 92: ECR 15

4.3 Spectrum of T-large granular lymphocyte lymphoproliferations: ranging from expanded activated effector T cells to T-cell leukemia
Br J Haematol 2003; 123: 561-562

4.4 Monoclonal TCR-Vβ13.1^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+dim}$ T-LGL lymphocytosis: evidence for an antigen-driven chronic T-cell stimulation origin
Blood 2007; Feb 15; [Epub ahead of print]

4.5 TCR-Vβ and TCR-Vα usage in CD8^{+}/TCR$\alpha\beta^{+}$ T-cell large granular lymphocyte leukemia

4.6 TCR$\gamma\delta^{+}$ large granular lymphocyte leukemias reflect the spectrum of normal antigen-selected TCR$\gamma\delta^{+}$ T-cells
Leukemia 2006; 20: 505-513

4.7 Clonal T- and NK-cell large granular lymphocyte proliferations in a single patient established by array-based comparative genomic hybridization analysis
Leukemia 2006; 20: 2212-2214

Chapter 4.1

Mature (post-thymic) T-cell leukemias

The World Health Organization (WHO) classification of hematopoietic and lymphoid tumors recognizes five types of mature T-cell neoplasms that commonly involve the peripheral blood and bone marrow at presentation. These include adult T-cell leukemia/lymphoma (ATLL), Sézary syndrome (SS), T-cell prolymphocytic leukemia (T-PLL), T-cell large granular lymphocyte (T-LGL) leukemia and hepatosplenic T-cell lymphoma (HSTCL).[1] Previously, classification of mature T-cell malignancies was inconsistent and T-PLL and T-LGL leukemia have been reported as T-cell chronic lymphocytic leukemia (T-CLL). However, the T-CLL entity is not included in the WHO classification anymore.[2-6] Mature T-cell leukemias clearly represent distinct clinicopathologic entities, although overlapping features may exist.[1] In Western countries, mature T-cell leukemias concern only 5% of lymphoid malignancies. T-PLL and T-LGL leukemia currently encompass most post-thymic T-cell leukemias in the West, whereas ATLL is extremely rare. However, in human T-cell lymphotropic virus I (HTLV-I) endemic areas such as Japan and the Caribbean, HTLV-I associated ATLL occurs at an essentially higher frequency.[7, 8] Most prominent features of all five mature T-cell leukemias are given in Table 1, whereas the characteristics of T-PLL and T-LGL leukemia, which have been the main subject of study in this thesis (Chapter 4), are described in more detail below. Sézary syndrome has been discussed in detail in Chapter 3.1.

T-cell prolymphocytic leukemia (T-PLL)

T-PLL is a rare clonal lymphoproliferative disorder of mature post-thymic lymphocytes. In general, T-PLL is an aggressive disease with poor response to chemotherapy and short overall survival.[6] It is characterized by the proliferation of small to medium-sized prolymphocytes involving the peripheral blood, bone marrow, lymph nodes, liver, spleen, and skin. T-PLL is derived from prolymphocytes with a mature T-cell immunophenotype: $TdT^-/CD1a^-/CD2^+/CD3^+/CD5^+/CD7^+/TCR\alpha\beta^+$. The most common phenotype is $CD4^+/CD8^-$ (60% of cases), but in 25% of cases the cells are $CD4^+/CD8^+$. The most common cytogenetic aberration of T-PLL involves chromosome 14 with breakpoints at 14q11 (*TCRA/D* locus) and 14q32.1 (*TCL1* locus).[9, 10] These abnormalities include inv(14)(q11q32) and t(14;14)(q11;q32), resulting in activation of the *TCL1* gene and can be detected in ~80% of cases.[4, 11, 12]

T-cell large granular lymphocyte (T-LGL) leukemia

Clonal disorders of large granular lymphocytes (LGLs) represent a biologically heterogeneous spectrum of lymphoid proliferations. LGLs have abundant cytoplasm and contain cytoplasmic cytotoxic granules and can be either of T- or NK-cell lineage.

LGL leukemia comprises 2%-5% of all T-cell/NK-cell malignancies with T-LGL leukemia being the most common subtype. T-LGL leukemia has an indolent clinical course and is characterized by a persistent (>6 months) increase in the number of T-cell large granular lymphocytes (T-LGLs).[13-17] Approximately two thirds of the patients with indolent T-cell LGL leukemia develop cytopenias, recurrent bacterial infections, autoimmune disorders, and/or splenomegaly during the course of their disease. One third of patients is asymptomatic.[15, 18] The therapeutic strategy is largely aimed at improving cytopenias and includes various agents amongst which immunosuppressive drugs. The most common immunophenotype of the malignant LGLs is $CD2^+/CD3^+/CD8^+/CD16^+/CD57^+/TCR\alpha\beta^+$. Although $CD3^+/CD4^+/TCR\alpha\beta^+$ and $CD3^+/TCR\gamma\delta^+$ cases have been reported; only little

Table 1. Major characteristics of T-cell malignancies frequently involving peripheral blood and bone marrow.

	Adult T-cell leukemia/ lymphoma	Sézary syndrome	T-cell prolymphocytic leukemia	T-cell large granular lymphocyte leukemia	Hepatosplenic T-cell lymphoma
Clinical features	Skin rash, lymphadenopathy	Erythroderma, generalized lymphadenopathy, alopecia, pruritus	Hepatosplenomegaly, generalized lymphadenopathy, B symptoms, skin infiltration	Splenomegaly, autoimmune features, neutropenia, anemia	Hepatosplenomegaly, thrombocytopenia, anemia
Cytomorphology	Medium-sized to large cells, nuclear pleomorphism ("flower cells")	Mixed small/large cells with convoluted (cerebriform) nuclei (Sézary cells)	Small to medium-sized cells, non-granular basophilic cytoplasm, irregular nuclei	Large cells, abundant cytoplasm containing coarse azurophilic granules	Medium-sized, rim of pale cytoplasm, small nucleoli
Immunophenotype	$CD2^{+}/CD3^{+}/CD4^{+}/CD7^{-}/CD25^{++}/TCR\alpha\beta^{+}$	$CD2^{+}/CD3^{+}/CD4^{+}/CD5^{+}$ $CD7^{+/-}/CD26^{+/-}/TCR\alpha\beta^{+}$	$CD2^{+}/CD3^{+}/CD4^{+}/CD5^{+/-}/CD7^{++}/TCR\alpha\beta^{+}$	$CD2^{+}/CD3^{+}/CD8^{+}/CD5^{+/-}/CD7^{+/-}/CD16^{+/-}/CD56^{+/-}/CD57^{+}/CD11b^{+}/TCR\alpha\beta^{+}$	$CD2^{+/-}/CD3^{+}/CD4^{-}/CD8^{-}/CD5^{-}/CD7^{+}/TCR\gamma\delta^{+}$
TCR gene rearrangement	Clonal	Clonal	Clonal	Clonal	Clonal
Chromosome aberration	Clonally integrated HTLV-I	Many, no recurrent one known	inv(14)(q11;q32)/ t(14;14)(q11;q32)	-	isochromosome 7q
Clinical course	Aggressive	Aggressive	Aggressive	Indolent	Aggressive
Relative frequency within mature T-cell leukemia (%)	<5*	~15	25-35	35-45	~10

*This percentage reflects the rough frequency observed in Western countries. In HTLV-I endemic regions (Japan, the Caribbean) the frequency is much higher

is known about differences in clinical characteristics and etiopathogenesis between these three T-LGL types. In T-LGL leukemia, TCR genes by definition are clonally rearranged. In contrast, in chronic NK-LGL leukemia TCR genes are in germline configuration. Leukemic T-LGLs most frequently have a normal karyotype and currently no recurrent cytogenetic abnormalities have been described. Although the etiology of LGL leukemia is unknown, it has been hypothesized that chronic activation of T cells with autoantigen or viral antigen is involved in the pathogenesis of the disease.[19-21] Antigen-driven expansions of oligoclonal cytotoxic T lymphocytes could precede the occurrence of secondary oncogenic events leading to neoplastic transformation and/or dysregulation of growth/apoptosis resulting in leukemia.[20,22,23] Since well-defined clonal chromosomal aberrations have been reported in few cases only,[24] the pathologically relevant secondary genetic events are probably more subtle and remain to be identified with novel molecular techniques.

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Chapter 4.2

Clinically and genetically atypical T-cell prolymphocytic leukemia underlines the relevance of a multidisciplinary diagnostic approach

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Prolymphocytic leukemia (PLL) is a rare clonal lymphoproliferative disorder of mature lymphocytes. The disease originates from B lymphocytes in approximately 80% of cases and from T lymphocytes in 20% of cases.[1] B-PLL and T-PLL are both characterized by an aggressive clinical course with splenomegaly, and a high WBC count. However, generalized lymphadenopathy and cutaneous involvement are rare in B-PLL, while present in ~25% of cases with T-PLL.[1,2] We report a patient with T-PLL with an unusual clinical presentation and relatively indolent clinical course in whom a translocation was found, which was previously described in B-PLL.

A 61-year-old man presented with a 2-year history of asymptomatic red raised skin lesions, starting with a few lesions on the back, and gradually progressing until it became almost generalized. On a lesional skin biopsy taken at another clinic 4 months after the first symptoms developed, an initial diagnosis of lichen nitidus was suggested. The patient had no history of skin diseases. There was no pruritus, fever, night sweats, or weight loss. Dermatological examination at our hospital demonstrated numerous erythematous non-scaling papules on the trunk, extremities and behind the ears (Figure 1). The face, palms,

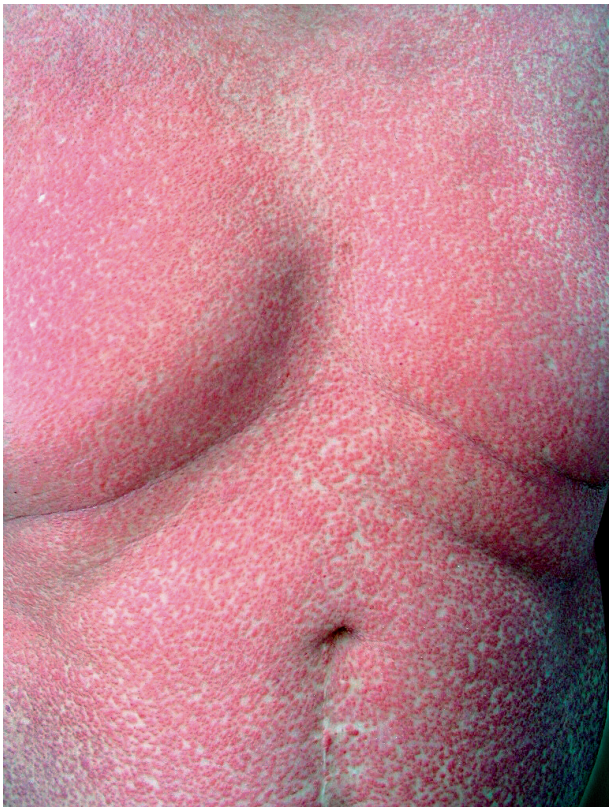

Figure 1.
Photograph of the thorax and abdomen of the patient showing disseminated erythematous papules.

and soles were not involved. General physical examination demonstrated cervical and inguinal lymphadenopathy but no hepatosplenomegaly. Computed tomography (CT) showed generalized lymphadenopathy and mild splenomegaly.

Laboratory examination revealed a white blood cell (WBC) count of 61 x 10^9/L, with 85% lymphocytes, 11% neutrophils, 1% eosinophils, and 3% monocytes. The hemoglobin (Hb) level was 15.2 g/dL and platelet count 98 x 10^9/L. Biochemistry revealed a slightly

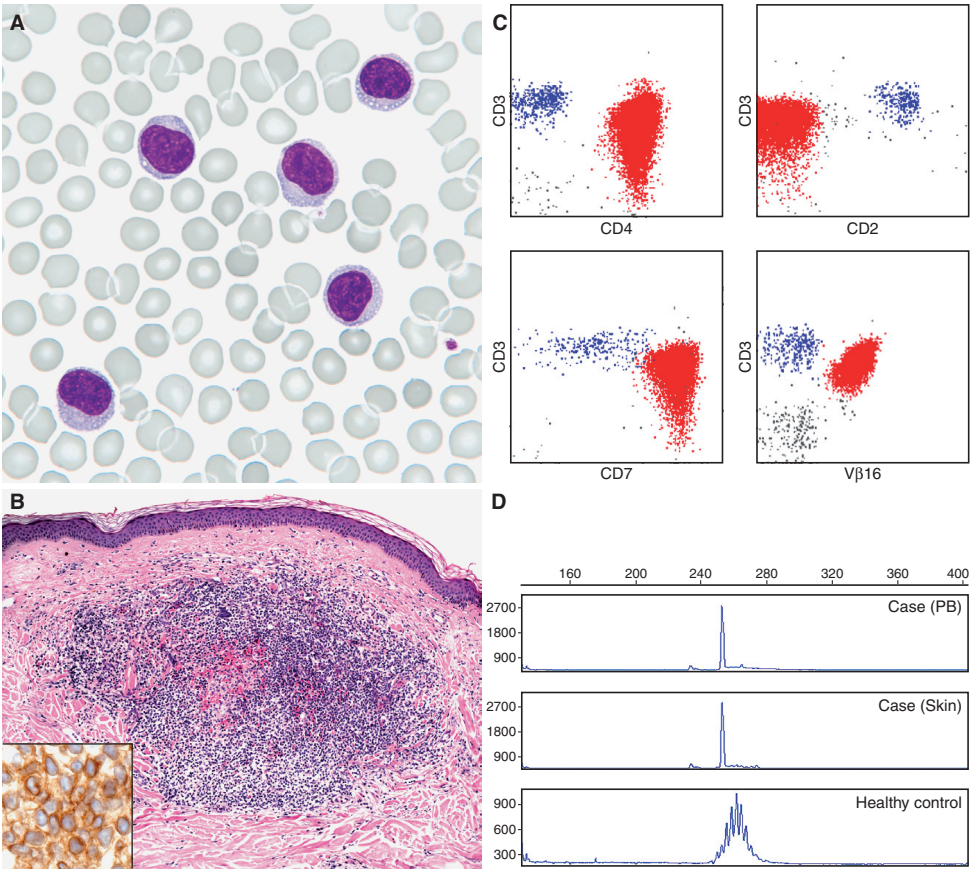

Figure 2.
A. Peripheral blood (PB) smear demonstrating medium-sized lymphoid cells with light grey, non-granular vacuolated cytoplasm (MGG; magnification x 630) **B**. Histology of skin lesion. Skin biopsy specimen demonstrates the patchy dermal infiltrate of lymphoid cells admixed with histiocytes and Langerhans cells (HE; magnification x 100). The vast majority of cells are positive for CD3 (insert) (Magnification x 400). **C**. Immunophenotype of T-PLL cells (shown in red) and normal T-cells (shown in blue) present in PB at diagnosis. The T-PLL cells are $CD2^-/CD3^{+\ dim}$/strong $CD7^+/V\beta16^+$. **D**. GeneScan analysis of PCR products. *TCRB* gene rearrangement analysis was performed using multiplex tube B of the BIOMED-2 multiplex PCR protocol.[11] Identical monoclonal *TCRB* gene rearrangements were detected in skin and PB samples. Polyclonal *TCRB* gene rearrangements can be detected in a PB sample from a healthy donor.

elevated lactate dehydrogenase (LDH) level of 567 U/L. Serological analysis of human T cell leukemia virus type 1 (HTLV-I) was negative. Peripheral blood smear showed 91% medium sized lymphoid cells with light grey, sometimes vacuolated cytoplasm, but without cytoplasmic granules (Figure 2A). The nucleus was round or oval shaped and some nuclei showed indentation. An occasional nucleolus was present. The bone marrow smears showed all hematopoietic cell lineages at different stages of maturation and infiltration with lymphoid cells, morphologically similar to the peripheral blood lymphoid cells.

Histologic examination of a later skin biopsy showed various patches of non-epidermotropic dermal infiltrates, composed of lymphocytes admixed with Langerhans cells and histiocytes (Figure 2B). The lymphocytes were only slightly enlarged and barely demonstrated atypical features such as nuclear polymorphism. Immunohistochemistry demonstrated that the infiltrate was mainly composed of $CD2^-$, $CD3^+$, $CD4^+$, $CD5^+$, $CD7^+$, $CD8^-$, $CD56^-$, $CD30^-$, $ALK1^-$, $TCL1^-$ T cells (Figure 2B; insert). Examination of a bone marrow biopsy demonstrated focal infiltration of small to medium-sized T cells with similar morphology and immunophenotype as those found in skin and peripheral blood.

Immunophenotypic analysis of peripheral blood revealed a large population (83% of total leukocytes) of aberrant T cells with the immunophenotype $CD3^{+\,dim}$/$CD2^-$/$CD4^+$/$CD5^+$/$CD7^{++}$/$CD8^-$/$CD25^-$/$TCR\alpha\beta^+$/$CD16^-$/$CD56^-$/$CD57^-$/$CD30^-$/$CD52^+$/$CD11b^-$/$CD1a^-$/$CD10^-$/TdT^- (Figure 2C; red dots). The normal T-cell population represented 1% of total T lymphocytes (Figure 2C; blue dots). B-cell markers (CD19, CD20, CD79a) were negative. TCR Vβ flowcytometric analysis demonstrated that 98% of $CD3^{+\,dim}$ cells reacted with a TCR-Vβ16 antibody (Figure 2C; red dots). Flowcytometric analysis confirmed bone marrow localization of this aberrant T-cell population. The immunophenotypical findings in peripheral blood and bone marrow were most compatible with a diagnosis of T-cell prolymphocytic leukemia (T-PLL). PCR based GeneScan analysis of T-cell receptor (TCR) genes demonstrated identical clonal TCRγ (*TCRG*) and TCRβ (*TCRB*) gene rearrangements in peripheral blood and skin biopsy samples (Figure 2D), while the immunoglobulin heavy-chain (*IGH*) genes were polyclonally rearranged. These results indicate the presence of clonally identical T-cell populations in peripheral blood and skin.

Cytogenetic analysis of PB cells using standard cytogenetic techniques and classification according to the International System for Human Cytogenetic Nomenclature ISCN 2005, revealed an aberrant karyotype: 46,X,-Y,t(6;12)(q15;p13),-12,+mar1,+mar2[7]/ 46,XY[15] (Figure 3A). Fluorescence in situ hybridization (FISH) using whole chromosome paints (WCP) demonstrated the presence of Y sequences in the large marker chromosome as suggested by the QFQ banded karyogram (Figure 3A; top). FISH also showed that chromosome 12 sequences were present on the large marker chromosome harbouring the Y sequences (described as: der(?)t(?;Y)t(?;12)) and on the small marker chromosome (Figure 3B; arrows). FISH with WCP for chromosome 6 (green), and 12 (red) confirmed the t(6;12), with the 6q breakpoints being more centromeric than in the karyotypic results (Figure 3B; arrowheads). Based on the banding pattern, the breakpoint on the long arm of chromosome 6 was determined to be in 6q15. FISH using WCP for chromosome 6 and 12 showed that there was hardly any chromosome 6 WCP present below the centromeric region of the der(6) showing that the estimated breakpoint on 6q was in fact more centromeric than determined by banding.

FISH analysis with ETV6 (located on 12p13) exon 1a and 5-8 probes showed only

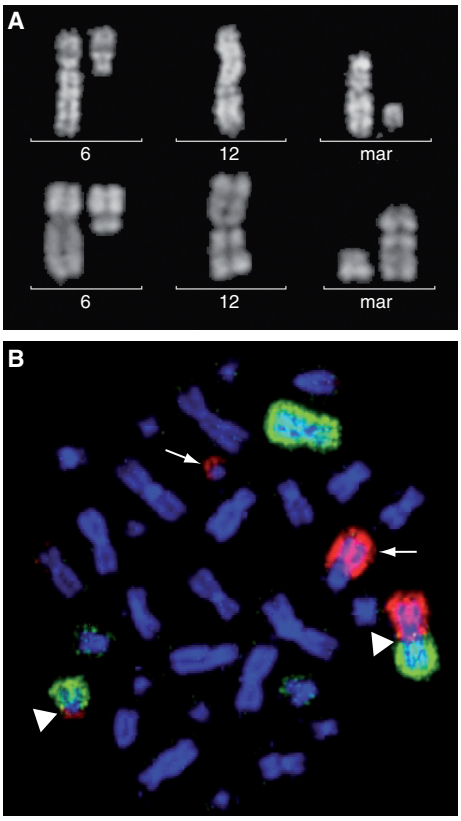

Figure 3.
A. Partial karyogram showing chromosome 6, 12 and markers present in QFQ-banding (top), or RBA-banding (bottom) in PB cells. **B**. Partial metaphase spread hybridized with WCP 6 and WCP12, showing both derivatives of the t(6;12) (arrowheads). The large marker and the small marker are shown to contain chromosome 12 sequences (arrows). The dim green fluorescent signals are secondary signals.

one ETV6 signal, present on the very tip of the long arm of the large marker chromosome, suggesting further intrachromosomal rearrangements. The absence of the other signal is indicative for an ETV6 deletion, which has been reported as a recurrent genetic event in T-PLL.[3] This also indicates that the seemingly balanced t(6;12) is in fact accompanied by a small deletion of chromosome 12p13 sequences.

In the Mitelman Database (http://cgap.nci.nih.gov/Chromosomes/Mitelman) eleven cases with various hematological malignancies were found to contain translocations involving the 6q1 and 12p1 regions. Interestingly, five of these had been diagnosed with B-cell prolymphocytic leukemia (B-PLL) and t(6;12)(q15;p13).[4] As the chromosomes of the case presented here look exactly like the ones detected in these B-PLL cases, t(6;12)(q15;p13) seems not specific for B-PLL, but may rather be considered as a marker for PLL in general. Notably, the most

common cytogenetic aberrations in T-PLL, i.e. (inv(14)(q11q32) and t(14;14)(q11;q32),[5] could not be detected in our patient.

Based on histo- and cytomorphology, immunophenotypical, and molecular genetic analysis, the diagnosis of T-PLL was made according to WHO Classification criteria.[6] Revision of the initial skin biopsy demonstrated a lymphocytic infiltrate with non-specific reactive changes, likely to be compatible with early manifestation of T-PLL. Revision of a lymph node (LN) biopsy (at first suggested to contain a reactive lymphocytic infiltrate), showed a slightly disturbed architecture and involvement with neoplastic T-PLL cells.

The clinical presentation and the so far indolent clinical course of our patient are remarkable. T-PLL is considered to be an aggressive leukemia with a poor prognosis and a median overall survival of less than 7.5 months.[2] Interestingly, our patient is in good clinical condition 32 months after onset of the erythematous papules. He has a mild thrombopenia, and a moderate but stable leukocytosis. Because of the indolent course of his disease, no treatment was initiated. The patient is monitored closely. Although patients diagnosed with T-PLL may present with an indolent clinical course,[7] cutaneous manifestation of the disease at presentation generally is a feature of initially progressive disease.[7,8] Moreover, one of the characteristics of the progression phase of patients with initially indolent disease is the development of skin lesions.[7, 9]

This case demonstrates that skin lesions can be the initial presentation of T-PLL and that a skin biopsy is essential in revealing the underlying disease. However, as prolymphocytoid features of T-PLL cells can be difficult to detect in routinely stained sections of extramedullary biopsy specimens,[10] extensive histopathological examination, cytomorphological, flow cytometric, and molecular (cyto)genetic analysis all contribute to reach the diagnosis. This case therefore exemplifies the importance of a multidisciplinary team approach in correctly diagnosing mature T-cell malignancies.

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Chapter 4.3

Spectrum of T-large granular lymphocyte lymphoproliferations: ranging from expanded activated effector T cells to T-cell leukemia

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CORRESPONDENCE

Lymphoproliferations of large granular lymphocytes (LGL) are generally divided into $CD3^+$ T-LGL and $CD3^-$ $CD16^+$ natural (NK)-LGL types.[1] Contrary to NK-LGL leukemia, which shows a rather aggressive clinical course, T-LGL leukemia is generally considered to be an indolent disease.[1] Clinical symptoms typically consist of neutropenia and autoimmune characteristics and the lymphocytosis is often limited.[1] Detection of a monoclonal LGL cell population is an important criterion for the diagnosis of T-LGL leukemia.[1] In the World Health Organization classification, LGL cell counts of >5 x 10^9/l and certainly >10 x 10^9/l are considered a sign of leukemia, although it is suggested that LGL counts of >2 x 10^9/l can be consistent with a diagnosis of T-LGL leukemia as well.[2] On the contrary, LGL cell expansions <0.5 x 10^9/l and <40% of T lymphocytes can be found in healthy controls and thus represent true reactive proliferations.[1,3] However, the clinical importance of LGL proliferations ranging from ~0.5 x 10^9/l to 2-5 x 10^9/l, which can also be clonal in nature, is often less clear. These are sometimes termed T-cell clonopathy of undetermined significance (TCUS) in analogy to monoclonal gammopathy of undetermined significance.[4]

Recently, it was shown that T-LGL leukemia cells in patients are indistinguishable from the small (oligo)clonal $CD8^+$ LGL effector cell expansions in asymptomatic individuals, given the common $CD3^+$ $CD8^+$ $CD57^+$ phenotype and the expression of FasL, granzyme, and activating CD94/NKG2 molecules.[5] Antigen stimulation of LGL effector cells and triggering of effector molecules may dysregulate immune mechanisms and thereby cause clinical problems.[5] In line with the idea of antigen-activated cytotoxic effector cells, we have previously shown that T-LGL proliferations often display a polyclonal to oligoclonal Vβ repertoire, with many cases showing one or more dominant clones with restricted Vβ usage or even clear monoclonal Vβ usage.[6] We strongly believe that this clonal heterogeneity reflects a continuous spectrum of T-LGL proliferations from reactive to clinically malignant (Figure 1).

To further illustrate this spectrum, we describe three representative patients showing LGL proliferations (see also legend to Figure 1): (i) patient 1, presenting with seropositive rheumatoid arthritis and neutropenia and finally diagnosed as having Felty syndrome, only showed ~ 0.1 x 10^9/l reactive LGL effector cells; (ii) patient 2 showed anemia, disturbed erythropoiesis, and a limited LGL clone and was diagnosed with TCUS; and (iii) patient 3 presented with arthritis, but developed T-LGL leukemia with a high cell count. These patients fit into a continuous spectrum of T-LGL proliferations ranging from limited or relatively mild lymphocytoses to cases that show a large monoclonal expansion and display signs of clinically malignant behaviour, justifying the term leukemia.

The idea of true leukemic cases is further strengthened by a recent description of CD26 as marker of a clinically more aggressive form of T-LGL disease.[7] Consistent chromosomal abnormalities, which might elude to oncogenic events in the true leukemic and clinically aggressive cases, have only rarely been reported so far; deletion of chromosome 6q and inversion 14 and 7 are among the aberrations described to date.[8] T-LGL proliferations need better discrimination between TCUS and true T-LGL leukemia by searching for additional markers that identify disease progression, and for oncogenic events involved in transformation and leukemogenesis.

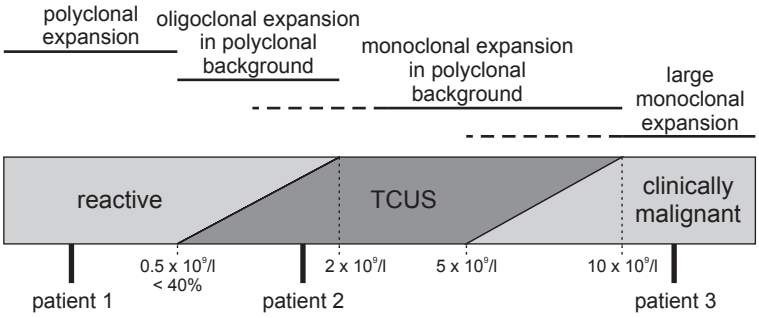

Figure 1.
The spectrum of T-large granular lymphocyte (LGL) lymphoproliferations, ranging from polyclonally expanded reactive T-cell proliferations with LGL cell populations up to 0.5 x 10^9/l or <40% of T lymphocytes to clinically malignant monoclonal T-cell leukemias with LGL cell counts >5 x 10^9/l and certainly those >10 x 10^9/l. In the intermediate area, i.e. cell counts of 0.5-5 x 10^9/l, oligoclonal or monoclonal expansions can be seen in a polyclonal background, which (because of the unclear clinical significance) are referred to as T-cell clonopathy of undetermined significance (TCUS). The discussed patients are plotted in the scheme, based on their clonality features and absolute and relative sizes of the LGL population. Patient 1 (53-year-old female: white blood cell (WBC) count 1.96 x 10^9/l; $CD3^+$ $CD4^-$ $CD5^{+/-}$ $CD7^{+/-}$ $CD8^+$ $CD16^-$ $CD56^-$ $CD57^{+/-}$ $HLA\text{-}DR^+$; ~ 0.1 x 10^9/l LGL cells, representing <10% of T lymphocytes and no apparent Vβ MoAb restriction) was diagnosed with Felty syndrome showing activated effector T lymphocytes; the patient was treated with granulocyte colony-stimulating factor and cyclosporine. Patient 2 (42-year-old female: WBC 5.6 x 10^9/l; $CD3^+$ $CD4^-$ $CD5^{+/-}$ $CD7^-$ $CD8^+$ $CD16^-$ $CD56^-$ $CD57^+$ $HLA\text{-}DR^-$; 1.6 x 10^9/l LGL cells; 43% of T lymphocytes; undefined Vβ MoAb restriction and clonal T-cell receptor (TCR) rearrangements) had TCUS; the patient was treated with cyclosporine for her red cell aplasia. Patient 3 (56-year-old female: WBC 36 x 10^9/l; $CD3^+$ $CD4^-$ $CD5^+$ $CD7^+$ $CD8^+$ $CD16^-$ $CD56^-$ $CD57^+$ $HLA\text{-}DR^-$; 28.4 x 10^9/l effector cells representing 95% of T lymphocytes and showing Vβ12 MoAb restriction and clonal TCR rearrangements) was diagnosed as having T-cell leukemia and was treated for the progressive lymphocytosis using chlorambucil.

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Chapter 4.4

Monoclonal TCR-Vβ13.1^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+dim}$ T-LGL lymphocytosis: evidence for an antigen-driven chronic T-cell stimulation origin

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ABSTRACT

Monoclonal TCRαβ$^+$/CD4$^+$ T-large granular lymphocyte (T-LGL) lymphocytosis is a T-cell disorder with a restricted TCR-Vβ repertoire. In the present study we explored the potential association between the expanded TCR-Vβ families, the CDR3 sequences of the TCR-Vβ gene, and the HLA genotype of patients with monoclonal TCRαβ$^+$/CD4$^+$ T-LGL lymphocytosis. For that purpose, 36 patients with monoclonal TCRαβ$^+$/CD4$^+$ T-LGL lymphocytosis (15 TCR-Vβ13.1 versus 21 non-TCR-Vβ13.1) were selected. For each patient, both the HLA (class I and II) genotype and the DNA sequences of the VDJ rearranged TCR-Vβ were analyzed. Our results show a clear association between the TCR-Vβ repertoire and the HLA genotype, all TCR-Vβ13.1$^+$ cases being HLA-DRB1*0701 (*P*=.004). Interestingly, the HLA-DR7/TCR-Vβ13.1 restricted T-cell expansions displayed a highly homogeneous and strikingly similar TCR, arising from the use of common TCR-Vβ gene segments, which shared (1) unique CDR3 structural features with a constantly short length, (2) similar combinatorial gene rearrangements with frequent usage of the Jβ1.1 gene, and (3) a homolog consensus protein sequence at recombination junctions. Overall, these findings strongly support the existence of a common antigen-driven origin for monoclonal CD4$^+$ T-LGL lymphocytosis, with the identification of the exact peptides presented to the expanded T cells deserving further investigations.

INTRODUCTION

Monoclonal chronic T-cell lymphocytosis and T-cell leukemias/lymphomas are a heterogeneous group of disorders, whose diagnosis and classification have been hampered by their relatively low frequency and variable clinical and histopathological behavior,[1-4] the lack of easily applicable clonality markers for T cells and the substantial clinical overlap with non-malignant inflammatory disorders.[1,5,6] Although the pathogenetic mechanisms involved in the development of clonal T-cell disorders remain largely unknown, in recent years significant advances have been made in this regard.[7-9] Among other observations, an association between chronic inflammatory and infectious processes and the occurrence of (mono)clonal expansions of lymphoid cells has recurrently been reported, particularly for chronic B-cell malignancies,[10] but also for mature T-cell neoplasias.[1,8,9] Accordingly, different viruses (eg human T-cell lymphotropic virus type I (HTLV-I), Epstein-Barr virus (EBV), and cytomegalovirus (CMV) and bacterial superantigens (i.e. staphylococci-derived superantigens) have been associated with the pathogenesis of specific mature T-cell malignancies, either because they infect tumor cells[11-14] or because they could induce an antigen-driven expansion of neoplastic T cells.[9,15,16] In line with the latter hypothesis, recent reports suggest that T-cell receptor (TCR)-associated signals could contribute to tumor development, particularly in T-cell large granular lymphocyte (T-LGL) leukemia.[5, 9] In these cases, antigen-driven expansions of cytotoxic T lymphocyte (CTL) clones could precede the occurrence of oncogenic events leading to neoplastic transformation and/or dysregulation of growth/apoptosis resulting in T-LGL leukemia[9] with a restricted TCR-Vβ/Vα usage.[17] This notion is supported by the observation that TCRαβ$^+$/CD8$^+$ T-LGL leukemia often occurs in the context of specific autoimmune diseases[9,16]. Also in about one third of cases TCRαβ$^+$/CD4$^+$ T-LGL leukemia/lymphocytosis is associated with neoplasias other than this T-LGL, with a preferential usage of the TCR-Vβ13.1 family.[18]

In addition, the reactive versus neoplastic nature of some (mono)clonal expansions of T-LGL remains a matter of debate,[15] particularly in cases where it is associated with viral infection, severe immune disturbances, or in the elderly.[19,20] In this regard, the search for the potential involvement of common antigens in driving the development of monoclonal T-cell disorders through the analysis of complementary determining region 3 (CDR3) sequences of TCR genes, has provided controversial findings. Accordingly, while CDR3 sequences from CD8^{+} T-LGL leukemia did not show any apparent structural homology,[21,22] in nearly half of all TCRγδ^{+} T-LGL neoplasias, clonal T cells express the same TCR-Vδ/Vγ family members (TCR-Vγ9/Vδ2) and share common TCR sequences. This is reflected by the systematic presence of the antigen-selected invariant T nucleotide in the first codon of the Vδ2-Jδ1 junctional region from all patients.[23] Such apparent discrepancy could be related to the fact that antigen-driven TCRαβ^{+}/CD8^{+} T-LGL leukemias would depend not only on the complementary sequences and specific binding of the TCR to the antigen, but also on the individual HLA haplotypes, while for TCRγδ^{+} T cells this HLA restriction would not apply.

In the present study, we have analyzed a large series of 36 patients with monoclonal TCRαβ^{+}/CD4^{+} T-LGL lymphocytosis grouped according to TCR-Vβ13.1^{+} usage versus other TCR-Vβ families. Our aim was to explore the potential existence of an association in these patients between the expanded TCR-Vβ families, the CDR3 sequences of the TCR-Vβ gene and the HLA genotype. Our results indicate that all patients with monoclonal expansions of TCR-Vβ 13.1^{+}/CD4^{+} T cells display a common HLA-DRB1*0701^{+} genotype and express identical motifs in the CDR3-TCR-Vβ sequence, suggesting a common antigen-driven origin.

PATIENTS, MATERIALS AND METHODS

Peripheral blood (PB) samples from human patients were obtained after informed consent was given by the patients (all of them over 18 years old), in accordance with the local ethics committee of the University Hospital of Salamanca and the Declaration of Helsinki.

Patients and samples

A total of 161 T-LGL cases were referred to the Cytometry Service of the University Hospital of Salamanca (63 TCRαβ^{+}/CD8^{++}/CD4^{-}, 55 TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+dim}$, 40 TCRγδ^{+} and 3 TCRαβ^{+}/CD8^{-}/CD4^{-} cases), between September 1999 and March 2006. Out of these cases, 36 individuals with monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+dim}$ lymphocytosis (19 males and 17 females; mean age 63±12 years, ranging from 36 to 81 years) were selected and included in this study. Peripheral blood (PB) samples from the included cases were collected into tubes containing K3-EDTA, according to the Local Ethics Committees recommendations. The major clinical and laboratory features of this group of patients, according to the type of TCR-Vβ family expressed (TCR-Vβ13.1 *vs* non-TCR-Vβ13.1), in comparison with those of a randomly-selected series of 24 patients with monoclonal TCRαβ^{+}/CD8^{+}/CD4^{-} lymphocytosis are shown in Table 1. At the closing of the study, the median follow-up of the patients with monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+dim}$ lymphocytosis was 72 months (ranging from 10 to 233 months).

Table 1. Clinical and laboratory characteristics of monoclonal TCRαβ$^+$/CD4$^+$ LGL lymphocytosis according to the type of TCR-Vβ family expressed (Vβ13.1 vs non-Vβ13.1), compared with monoclonal TCRαβ$^+$/CD8$^+$ LGL lymphocytosis.

Characteristic	Monoclonal TCRαβ$^+$/CD4$^+$ LGL lymphocytosis			Monoclonal TCRαβ$^+$/CD8$^+$ LGL lymphocytosis	*P*
	TCR-Vβ13.1	Non-TCR-Vβ13.1	Total cases		
No. of patients	15	21	36	24	
Mean age ±1 SD, y (range)	64±8 (52-80)	61±13 (36-81)	63±12 (36-81)	56±15 (31-79)	NS
% male/% female	25 / 75	61 / 39	47 / 53	37 / 63	NS
Reason for consulting, %					
Routine blood analysis	100	83	90	83	NS
Skin lesions	0	11	7	0	NS
Abdominal distension	0	6	3	0	NS
Fever	0	0	0	4	NS
General symptons	0	0	0	13	NS
Physical examination, %					
Adenomegalies	11	6	7	0	NS
Hepatomegaly	0	0	0	4	NS
Splenomegaly	0	6	3	4	NS
Skin lesions	0	11	7	0	NS
Associated neoplasias	25	22	23	26	NS
Associated autoimmune diseases	18	0	13	33	.05
Laboratory parameters, %					
Leukocytosis (>10x10^9/L)	64	61	63	25	.01
Lymphocytosis (>5x10^9/L)	70	67	68	42	.09
Neutropenia (<1.5x10^9/L)	14	0	3	61	<.001
Anemia (<10g/dL)	0	0	0	29	.003
Thrombocytopenia (<100x10^9/L)	10	0	3	8	NS
Increased lactic dehydrogenase level (>460U/L)	10	0	3	22	NS
Increased β2-microglobulin level (>2mg/dL)	0	7	3	63	.001
Cases requiring treatment because of the lymphocytosis or the associated autoimmune disease, %	0	0	0	37	.001
Outcome: stable disease, %	100	100	100	87	NS
Total deaths, %	10	18	15	8	NS
Deaths related to the TCRαβ$^+$ LGL lymphocytosis, %	0	0	0	4	NS
Mean follow-up ± 1 SD, mo (range)	68±48 (13-136)	74±56 (10-233)	72±53 (10-233)	44±40 (1-152)	.03

P value corresponds to comparisons between monoclonal TCRαβ$^+$/CD4$^+$ LGL and TCRαβ$^+$/CD8$^+$ LGL lymphocytosis. NS indicates no statistically significant differences: *P*>.1).
No statistically significant differences (*P*>.05) were found between TCR-Vβ13.1 and non-TCR-Vβ13.1 clonal CD4$^+$ LGL lymphocytosis.
A detailed description of the clinical characteristics of patients with monoclonal TCRαβ$^+$/CD4$^+$ LGL lymphocytosis is provided by Lima *et al*.[18]

A total of 930 PB samples from unrelated healthy subjects were used as controls to establish the frequency of the different HLA haplotypes in the healthy population, while PB samples from 15 adult individuals (older than 50 years) were used as controls to establish the TCR-Vβ repertoire usage in TCRαβ^{+}/CD4^{+} T cells.

Immunophenotypic studies

For the analysis of the TCR-Vβ repertoire of CD4^{+}/CD8$^{-/+dim}$ LGL T lymphocytes a panel of 24 monoclonal antibodies (MAb) directed against an identical number of members of 21 different TCR-Vβ families (TCR-Vβ repertoire Kit; Immunotech, Marseille, France) was used in 4-color stainings. Further phenotypic characterization of CD4^{+}/CD8$^{-/+dim}$ LGL T-cells was performed using the following 4-color combinations of MAbs: fluorescein isothiocyonate (FITC)/phycoerythrin (PE)/PE-cyanin 5 (PC5) or peridinin chlorophyll protein (PerCP)/allophycocyanin (APC): CD2/CD7/CD4/CD8, CD5/CD7/CD4/CD8, CD38/CD11b/CD4/CD8, CD57/CD11c/CD4/CD8, CD16/CD56/CD4/CD8, CD122/CD25/CD4/CD8, CD45RA/CD45RO/CD4/CD8, CD62L/CD28/CD4/CD8, CD11a/HLA-DR/CD4/CD8, CD16/NKB1/CD4/CD8, CD158a/CD161/CD4/CD8, CD57/CD8/CD56/CD4 and cytoplasmic (Cy) perforin/Cy granzyme B/CD56/CD4. The source and specificity of each MAb reagent used has been previously described in detail.[18]

Cell staining was performed using a whole blood "stain-and-then-lyse" method (FACS lysing solution; Becton Dickinson Biosciences [BDB], San Jose, CA) and a direct immunofluorescence technique, as previously reported in detail.[18] For the cytoplasmic staining, the Fix & Perm reagent kit (Caltag Laboratories, San Francisco, CA) was used according to the recommendations of the manufacturer.

Data acquisition was performed immediately after completion of sample preparation, in a FACSCalibur flow cytometer (BDB), using the CellQUEST software program (BDB). The Paint-A-Gate Pro software program (BDB) was used for data analysis. In each case, the aberrant T-cell population was defined as CD4^{+}/CD8$^{-/+dim}$ and/or CD4^{+}/CD56^{+} large granular - intermediate sideward light scatter (SSC^{intermediate}) - events (Figure 1) for its further phenotypic characterization.

Preparation of DNA and HLA typing

High molecular weight DNA was prepared from 200 μl PB using the QIAGEN bloodmicro kit (Qiagen, Hilden, Germany). HLA genotyping for HLA-ABC and both HLA-DRB1 and HLA-DQB1 was performed by sequence-specific oligonucleotide-polymerase chain reaction (SSPO-PCR) techniques using the Dynal Reli SSO kit (DYNAL Biotech, Bromborough, United Kingdom). Ambiguous results were resolved by sequence based typing (SBT). DNA samples were amplified by PCR using the Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing was performed on an ABI 377 DNA sequencer (Applied Biosystems) and the data obtained were analyzed using the Match Tools v 1.0 Sequencing Analysis software program (Applied Biosystems). The ancestral haplotypes are putative because it was not possible to verify their segregation from family studies.

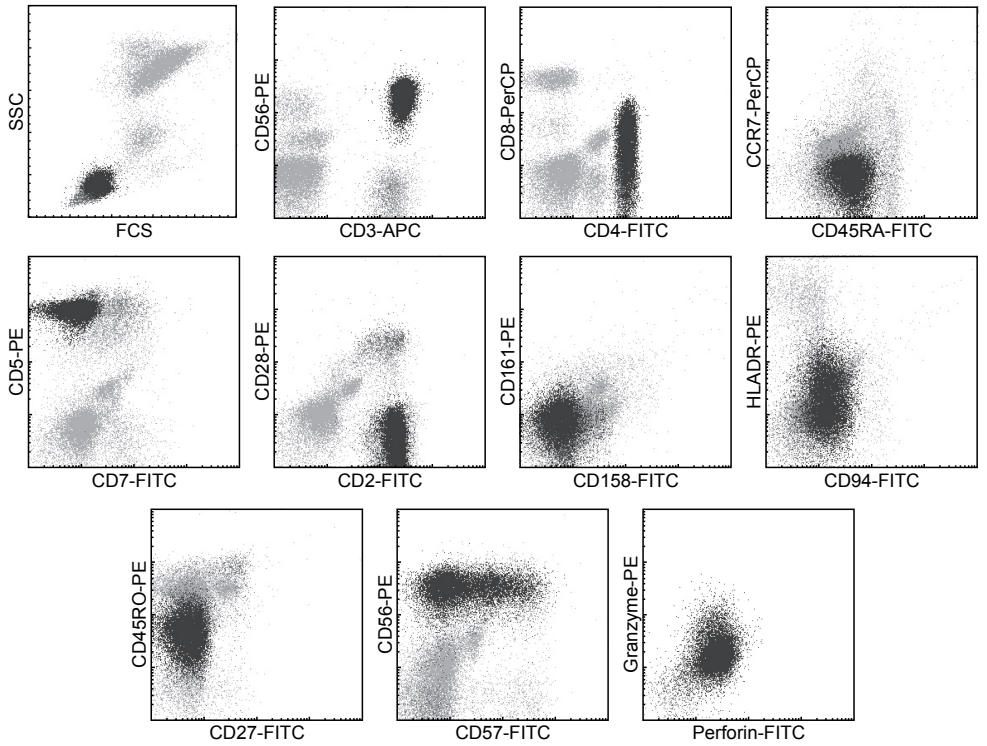

Figure 1. Immunophenotypic features of monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL.
Representative dot plots illustrate the phenotypic patterns shown by monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL. Black dots correspond to monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGLs, dark grey dots correspond to normal residual non-LGL CD4^{+} T cells, while light grey dots correspond to PB leukocytes other than CD4^{+} T cells.

PCR amplification and nucleotide sequence analysis of TCR

High-molecular-weight DNA was prepared from freshly-frozen PB samples using standard protocols including proteinase K treatment. In addition, total RNA was isolated from fluorescence-activated cell sorter (FACS)-sorted CD4^{+} and CD8^{+} T-cell populations (purity more than 95%) from pooled PB mononuclear cells (MNC of 5 non-HLA-DR*0701 adult healthy donors and from PB MNCs of 1 HLA-DR*0701 healthy individual and reverse transcribed into cDNA. TCR Vβ13.1 gene family-specific PCR was performed using specific primers as previously described.[24] PCR products were cloned into pGEM-T easy vector, and single colony PCR was performed on positive clones. Single colony PCR products were directly sequenced.

DNA was amplified using a mixture of sense primers annealing to the TCR-Vβ13 sequence in conjunction with a mixture of antisense primers complementary to the germ line J regions, as previously reported in detail.[24] Most samples, clonal products from the Vβ gene PCR were sequenced directly using the BigDye Terminator Cycle Sequencing Reaction Kit. In fact, the amplified sequences exhibited identical rearranged monoclonal TCR sequences, even in the VDJ junctional hypervariable regions, indicating that these expanded regions were

clonal. To confirm the validity of the sequences obtained (thereby avoiding the possibility of either contamination or sequencing mistakes), a more detailed analysis of the sequences obtained was performed in some cases. For that purpose, PCR products were inserted into the PCR2.1-TOPO vector (Invitrogen, Barcelona, Spain), which was followed by transformation into competent *Escherichia coli* cells; on average, 5 colonies were randomly selected for sequencing using the BigDye Terminator Cycle Sequencing Reaction Kit. A total of 98 Vβ13.1 clones (69 from the non-HLA-DR*0701 donors and 29 from the HLA-DR*0701 donor) in the CD4^{+} T-cell fraction and 53 Vβ13.1 clones (38 from the non-HLA-DR*0701 donors and 15 from the HLA-DR*0701 donor) in the CD8^{+} T-cell fraction were sequenced and analyzed. All sequence reactions were analyzed using an automated DNA sequencer (ABI 377; Applied Biosystems).

Database searches

Sequences obtained were aligned using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, Bethesda, MD) and ImMunoGeneTics (IMGT) databases.

Statistical methods.

For all clinical and laboratory parameters included in table 1, mean, standard deviation and range were calculated using the SPSS program (SPSS 12.0, Chicago, IL). In order to establish the statistical significance of the differences observed between groups, either the Pearson's $\chi 2$ test or Fisher's exact test, or the Mann-Whitney U non-parametric test were used (SPSS 12.0, Chicago, IL), for categorical and continuous variables, respectively. *P* values below .05 were considered to be associated with statistical significance.

RESULTS

Immunophenotype of the expanded CD4^{+} LGL T cells

In all cases studied, expanded CD4^{+} LGL T cells showed relatively high SSC features as compared with normal PB CD4^{+} T lymphocytes and common phenotypic characteristics, consisting of TCRαβ^{+}/CD4^{+}/CD8$^{-/+dim}$ cells with a typical cytotoxic (granzyme B^{+}, CD56^{+}, CD57^{+}, CD11b$^{+/-}$) activated, memory/effector T-cell phenotype (CD2$^{+bright}$, CD7$^{-/+d}$, CD11a$^{+bright}$, CD28^{-}, CD62L^{-}, HLA-DR^{+}) (Figure 1). In about half (47%) of the cases, clonal T cells co-expressed CD45RA and CD45RO, while in the other cases they had a CD45RA^{+}/CD45RO^{-} phenotype. Other NKa markers (CD11c, CD16, CD94, CD158a, CD161 and NKB1) and T-cell activation-related antigens (CD25, CD38, and CD122) were absent on the CD4^{+} LGL T cells. Overall, these cells represented 47% ± 23% of all PB lymphocytes, with a mean (± 1 SD) absolute number of 6.6 x 10^{9} ± 3.3 x 10^{9} PB TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+dim}$ T lymphocytes per liter.

Flow cytometric analysis of the TCR-Vβ repertoire of CD4^{+}/CD8$^{-/+dim}$ LGL T-cells was consistent with a (mono)clonal expansion in all cases studied, which accounted for 72% ± 21% of all PB CD4^{+} T-cells. In 27 cases the expanded TCR-Vβ family was identified with the panel of TCR-Vβ reagents used, corresponding to TCR-Vβ13.1 in 15 cases (42%),

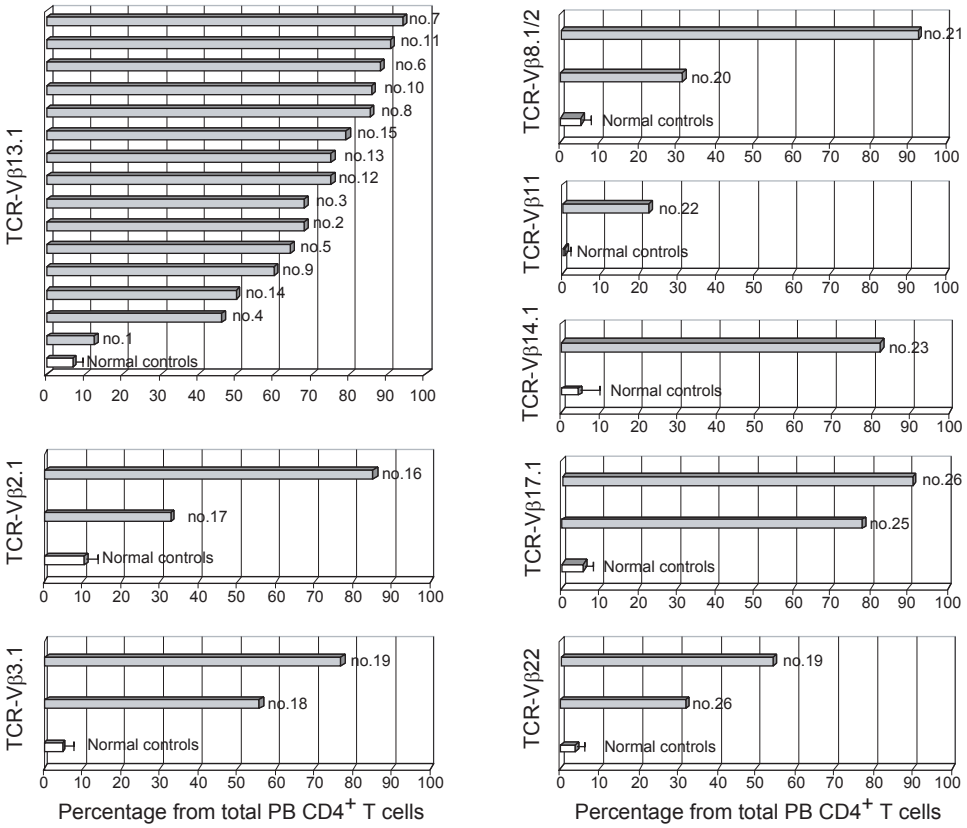

Figure 2. Illustrative representation of the size of the actual identifiable TCR-Vβ expansion present in each patient (as percentage of total PB CD4$^+$ T cells) in comparison with the size of the corresponding TCR-Vβ family observed in a cohort of age-matched healthy subjects (n= 15).

Grey bars correspond to patients, each one identified by the corresponding case number, while white bars and horizontal lines correspond to the mean value and 1 SD found in healthy controls, respectively.

TCR-Vβ2.1 in 2 (5.6%), TCR-Vβ3.1 in 2 (5.6%), TCR-Vβ8.1+ Vβ8.2 in 2 (5.6%), TCR-Vβ17.1 in 2 (5.6%), TCR-Vβ22 in 2 (5.6%) and TCR-Vβ11 or TCR-Vβ14.1 in 1 case each (2.8%). In the remaining 9 patients, the expanded TCR−Vβ family was not identified (25%) with the panel of MAbs used. Figure 2 shows the nominal size of the TCR-Vβ expansion present in each patient (as percentage of the total PB CD4$^+$ T-cells) in comparison with the size of the corresponding TCR-Vβ family observed in a cohort of age-matched healthy subjects; the proportion of TCR-Vβ13.1$^+$ cells represented 6.7%±2.5% of total PB CD4$^+$ T cells from healthy subjects, while in the patient group it represented between 12.5% and 94.1% (median, 75%).

Table 2. HLA genotype of patients with clonal TCRαβ⁺/CD4⁺ expansions.

Case no.	Expanded TCR-Vβ family	HLA-DRB	HLA-DQB	HLA-A	HLA-B	HLA-C
1	13.1	0701/1103	0301/0303	2301/3201	3501/5002	0401/0401
2	13.1	0701/1404	0202/0503	0101/2601	1401/5701	0602/0802
3	13.1	0701/0102	0303/0501	0201/0201	5101/5701	0102/0701
4	13.1	0701/1401	0303/0503	0201/2402	4402/5701	0501/0701
5	13.1	0701/0101	0202/0501	0201/0201	3501/4901	0401/0701
6	13.1	0701/0101	0202/0501	0301/0301	1302/3501	0401/0602
7	13.1	0701/0401	0202/0301	2601/2902	4402/4403	0501/1601
8	13.1	0701/0701	0202/0202	0101/2402	1801/5001	0501/0602
9	13.1	0701/0101	0202/0501	0101/2902	1401/4403	0802/1601
10	13.1	0701/1501	0303/0602	0201/0201	0702/5701	0602/0702
11	13.1	0701/0301	0201/0202	0301/2902	1801/4403	0501/1601
12	13.1	0701/0301	0201/0202	0101/2301	0801/4403	0401/0701
13	13.1	0701/0301	0201/0202	2301/3002	1801/5801	0501/0701
14	13.1	0701/0101	0201/0501	1101/2902	3501/4403	0401/1601
15	13.1	0701/1501	0202/0602	2902/3002	0702/4101	0701/1701
16	2.1	0301/1317	0201/0603	2402/2402	0801/3508	0401/0701
17	2.1	0701/1301	0202/0609	3002/6801	4403/5101	0701/1402
18	3.1	0403/1501	0302/0602	0301/3202	0702/3501	0401/0702
19	3.1	1501/1602	0502/0602	0201/0201	3701/4402	0501/0602
20	8.1+8.2	1101/1101	0301/0301	0201/6901	1801/3508	0701/1203
21	8.1+8.2	0301/1501	0201/0602	0201/0201	1518/3501	0401/0704
22	11	1101/1601	0301/0502	0101/0201	3701/4002	0602/1204
23	14.1	0701/0701	0202/0303	0103/2902	4403/5701	0701/1601
24	17.1	0802/1101	0301/0402	0102/2301	1401/3501	0701/0801
25	17.1	0701/1401	0202/0503	0201/2902	4403/5101	1502/1601
26	22	0102/0801	0402/0501	2402/3301	1402/3503	0401/0802
27	22	1103/1301	0301/0603	0201/3201	1509/4002	0202/0704
28	NI	0701/1104	0201/0301	1101/2902	4001/4403	0304/1601
29	NI	0701/1101	0201/0301	0201/0301	3503/5001	0401/0501
30	NI	0701/1301	0202/0604	3201/6801	1401/5301	0401/0802
31	NI	0701/1602	0202/0502	0301/2402	0702/3801	0702/1203
32	NI	1101/1401	0301/0503	0201/2603	4002/3501	0401/0501
33	NI	0101/1301	0501/0604	1101/1101	4004/5601	0102/0202
34	NI	0101/1101	0301/0501	0201/2402	1801/3501	0401/1203
35	NI	0101/1301	0501/0604	1101/3101	3501/5101	0401/0501
36	NI	1302/1305	0301/0604	0201/0205	3508/4101	0401/0701

Extended/ancestral haplotypes are underlined.
NI indicates TCR−Vβ family not identified by immunophenotyping.

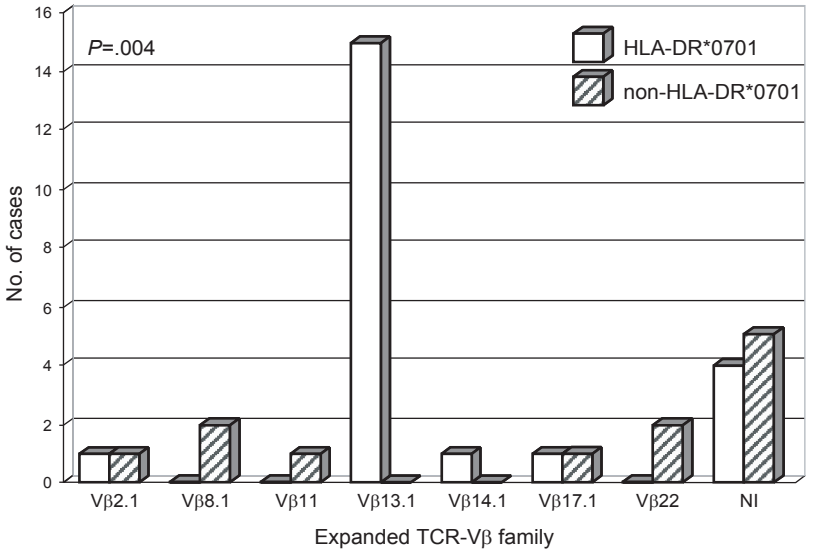

Figure 3. Frequency of the HLA-DRB1*0701 genotype in patients with monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL lymphocytosis grouped according to the expanded TCR-Vβ family.
NI indicates that the exact TCR-Vβ family expanded was not identified with the panel of anti-TCR-Vβ MAbs used.

Association between the HLA haplotypes and the TCR-Vβ repertoire of clonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+dim}$ T-LGL

A significant association (P=.004) was found between the TCR-Vβ repertoire and the HLA genotype of the studied cases (Table 2). Accordingly, all 15 patients who showed expansion of TCR-Vβ 13.1^{+} CD4^{+} T cells were HLA-DRB*0701^{+} (Figure 3). In turn, the frequency of the HLA-Cw*0401 allele was slightly higher among those patients in whom the expanded TCR-Vβ family was not contained in the panel of MAb reagents used than among both TCR-Vβ13.1^{+} patients and cases expressing a known TCR-Vβ other than 13.1 (67% versus 33%; P=.1). In addition, the frequency of cases with an HLA-DRB1*03 and HLA-DRB1*04 genotype was lower among CD4^{+} T-LGL patients than in the control group (14% versus 29% and 6% versus 22%, respectively; $P \leq .04$).[25] Interestingly, 4 of the 15 HLA-DRB*0701 cases included the 44.2 ancestral haplotype, (HLA-A*2902, B*4403, C*1601, DRB*0701, and DQB1*0202) (cases 7, 9, 11 and 14) (Table 2). Another 2 cases (cases 2 and 10) were related to the 57.1 ancestral haplotype (HLA-A*01, B*5701, C*0602, DRB1*0701, and DQB1*0303). The ancestral haplotypes 35.2 (HLA-A*1101, B*3501, C*0401, DRB*0101, DQB1*0501) and 35.1 (HLA-C*0401, B*3501, DRB1*11, DQB1*0301) were also found in 3 (cases 5, 6 and 14) and 1 patients (case 1), respectively; however, among these patients, only case 14 showed a complete 35.2 haplotype, with a 4-locus haplotype being detected in the other 3 cases. Cases 8, 13, and 15 were not considered to be related with ancestral haplotypes, although they showed haplotypes that are frequently present in the Spanish population.[26]

Expanded clonal CD4^{+}/CD8$^{-/+dim}$ T cells from HLA-DRB1*0701 patients exhibit conserved TCRβ chain motifs

Molecular analysis of the length of CDR3 of CD4^{+}/CD8$^{-/+dim}$T cells from those cases expressing TCR-Vβ13.1 showed a pattern consistent with monoclonality based on both TCR-Vβ usage and CDR3 length (Table 3). Further comparison of CDR3 size distribution in clonal CD4^{+}/CD8$^{-/+dim}$T cells from the same patients showed a highly restricted usage of $V_{\beta}D_{\beta}J_{\beta}$ gene segments and shared CDR3 configurations. Accordingly, 11 of 13 patients used the same J1.1 and V segments and they had highly similar CDR3 configurations (Table 3). Although 3 different J segments were used in the clonal expansions derived from CD4^{+}/CD8$^{-/+dim}$T lymphocytes, similar VDJ junctional region sequences were found (Table 4). Remarkably, in 5 of the 11 Vβ13.1-Dβ1-Jβ1.1 cases, the combinatorial process involved the deletion of 1 to 3 nucleotides from the 5' end of Vβ13.1 gene and the insertion of a variable number of nontemplate nucleotides (Table 4). Accordingly, in all cases analyzed (n=14) the mean length of CDR3 was considerably shortened (4 or 5 codons), the distance between the CASS and FG motifs constantly being of 9 codons. Interestingly, shared motifs consisting of at least 2 identical amino acids were found within the VDJ junctional regions of the expanded CD4^{+} T cells derived from different patients, a consensus XQGX motif being shared by all cases (Table 3). For 7 cases the GGG codon yielded a glycine; in contrast, in 2 other cases the glycine was generated by the GGT and in another case by a GGA codon. In turn, glutamine (Q) was yielded in all cases by the CAG codon. One of the motifs (YQGA)

Table 3. VDJ protein sequences of clonally expanded TCRαβ^{+}/CD4^{+} T-LGL.

Case no.	V –J usage	CDR2	3' end of Vβ	CDR3: N-Dβ1-N	5' end of Jβ
1	Vβ13S1-J1.1	SVGAGI	CASS	KQGV	TEAFFG
2	Vβ13S1-J1.1	SVGAGI	CASS	KQGA	TEAFFG
3	Vβ13S1-J1.1	SVGAGI	CAS	RKQGA	TEAFFG
4	Vβ13S1-J1.1	SVGAGI	CASS	YQGA	TEAFFG
5	Vβ13S1-J1.1	SVGAGI	CASS	SQGT	TEAFFG
6	Vβ13S1-J1.1	SVGAGI	CAS	RHQGS	TEAFFG
7	Vβ13S1-J1.2	SVGAGI	CASS	HQGA	NGYTFG
8	Vβ13S1-J1.5	SVGAGI	CASS	YQGA	QPQHFG
9	Vβ13S1-J1.5	SVGAGI	CASS	YQGS	QPQHFG
10	Vβ13S1-J1.1	SVGAGI	CAS	RRQGY	TEAFFG
12	Vβ13S1-J1.1	SVGAGI	CASS	YQGA	TEAFFG
13	Vβ13S1-J1.1	SVGAGI	CAS	RRQGA	TEAFFG
14	Vβ13S1-J1.1	SVGAGI	CAS	NLQGS	TEAFFG
15	Vβ13S1-J1.1	SVGAGI	CASS	YQGSA	EAFFG
# 10	Vβ13S1-J1.1	ND	CASS	Y	NTEAFFG
# 52	Vβ13S1-J1.1	ND	CASS	WQGVD	TEAFFG
# 29*	Vβ13S1-J1.1	ND	CAS	NRGLY	TEAFFG

Comparison of the CDR2 and CDR3 of clonal TCR Vβ13S1 gene rearrangements from 14 CD4^{+} T-LGL cases (this analysis could not be performed in case no. 11 due to sample shortage).

Segments are aligned according to the conserved motifs (CASS for the Vβ and FG for the Jβ segment). Nucleotide sequences were aligned to TCR sequences according to the Basic Local Alignment Search Tool (BLAST) and ImMunoGeneTics (IMGT) databases. ND indicates not determined.

VDJ protein sequence of the 3 out of 98 clones using the Jβ1.1 segment found among PB Vβ13.1^{+} CD4^{+} T-cells from adult healthy donors; *this clone corresponds to a HLA-DR*0701 donor.

Table 4. Sequence of the VDJ junctional TCRβ regions of the clonally expanded TCRαβ$^+$/CD4$^+$ T-LGL showing Vβ13S1-J1.

Case no.	V-J usage	3`end of Vβ13.1	N	Dβ1	N	5`end of Jβ
1	Vβ13S1-J1.1	AGCAGT	AA	gggACAGGGggc	AGT	aaCACTGAAGCTTTC
2	Vβ13S1-J1.1	AGCAGT	AA	gggACAGGGGGC		aaCACTGAAGCTTTC
3	Vβ13S1-J1.1	AGCAG	AAA	gggACAGGGGGC		aaCACTGAAGCTTTC
4	Vβ13S1-J1.1	AGCAGT	TAC	gggaCAGGGGGc	CG	aacACTGAAGCTTTC
5	Vβ13S1-J1.1	AGCAGT	TCC	gggaCAGGGGgc	AC	aaCACTGAAGCTTTC
6	Vβ13S1-J1.1	AGCAG	ACAT	gggaCAGGGggc	TAG	aaCACTGAAGCTTTC
10	Vβ13S1-J1.1	AGC	CGGC	ggGACAGGGGgc	AAA	aacACTGAAGCTTTC
12	Vβ13S1-J1.1	AGCAGT	TAT	gggaCAGGGGGC		aCACTGAAGCTTTC
13	Vβ13S1-J1.1	AGCAG	GC	ggGACAGGGGGC		aacACTGAAGCTTTC
14	Vβ13S1-J1.1	AGCA	ATCT	gggACAGGGggc	TAG	aaCACTGAAGCTTTC
15	Vβ13S1-J1.1	AGCAGT	-	TACCAAGGCTCGG		aacaCTGAAGCTTTC
#10	Vβ13S1-J1.1	AGCAGTTAC	-	-	-	AACACTGAAGCTTC
#52	Vβ13S1-J1.1	AGCAGTT	GG	gggaCAGGGGGc	TGG	aACACTGAAGCTTTC
#29*	Vβ13S1-J1.1	AGCA	-	gggACAGGGGGc	TTGT	aACACTGAAGCTTTC

Sequence of the 3 clones using the Jβ1.1 segment found among PB Vβ13.1$^+$ CD4$^+$ T cells from adult healthy donors. * This clone corresponds to a HLA-DR*0701 donor.

was identical among the clonally expanded CD4$^+$/CD8$^{-/+dim}$ T-cells derived from 3 patients, and the XQGA motif was detected in 7 individuals. In contrast, only 2 (2.9%), 8 (11.6%) and 2 (2.9%) of 69 clones of PB CD4$^+$ T cells from non-HLA-DR*0701 healthy adults were found to use the Jβ1.1, Jβ1.2 and Jβ1.5 gene segments, respectively; similarly, from the 29 clones of CD4$^+$ T cells sequenced from the HLA-DR*0701 adult healthy donor, only 1 (3.4%) and 3 (10%) of them were found to use Jβ1.1 and Jβ1.2 gene segments, respectively. Interestingly, of these 16 clones of CD4$^+$ T cells, only 1 of those 3 clones using the Jβ1.1 gene segment was highly similar in the CDR3 configuration to those detected in the patients analyzed (Tables 3-4); this clone was sequenced from the pooled non-HLA-DR*0701 MNCs. In addition, the XQGX configuration could not be detected in other clones of PB CD8$^+$ T cells from healthy adults using the Jβ1.1, Jβ1.2, and Jβ1.5 gene segments (0 of 9 clones from the 38 CD8$^+$ T-cell clones sequenced from non-HLA-DR*0701 donors and 0 of 3 from the 15 CD8$^+$ T-cell clones sequenced from the HLA-DR*0701 donor). Finally, we searched GenBank for VDJ rearrangements with similar XQGX aminoacid sequences, but no close TCR matches were found.

DISCUSSION

Monoclonal TCRαβ$^+$/CD4$^+$/NKa$^+$/CD8$^{-/+d}$ T-LGL lymphocytosis is a subgroup of monoclonal LGL lymphoproliferative disorders, different from both the CD8$^+$ TCRαβ$^+$ T-LGL, TCRγδ$^+$ T-LGL and natural killer (NK) cell-type LGL leukemias.[18] Noteworthy, in the present study, the former subgroup of clonal T-LGL lymphocytosis was found at a higher frequency than both TCRγδ- and NK-LGL leukemias, whereas it was slightly less common than TCRαβ$^+$ CD8$^+$ T-LGL. In contrast to TCRαβ$^+$ CD8$^+$ T-LGL, monoclonal TCRαβ$^+$/CD4$^+$/

NKa^{+}/CD8$^{-/+d}$ T-LGL cases have been only sporadically reported in the literature, while they were relatively frequent in our series. According to the present study, such discrepancy might be related to the fact that TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL cases usually display a more indolent clinical course - although rare cases with aggressive disease have also been reported in the literature - are associated with a significantly lower frequency of neutropenia, anemia and other associated autoimmune diseases, in addition to a lower percentage of cases requiring treatment, as compared to TCRαβ^{+}/CD8^{+} T-LGL lymphocytosis. However, the apparently high frequency of CD4 LGL cases found in our series could also be due to the fact that we actively searched for these cases. Recently, we showed that in patients with monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL lymphocytosis the expanded clonal T cells display a restricted usage of a limited number of TCR-Vβ families,[18] from which TCR-Vβ13.1 was particularly overrepresented in comparison to its frequency in the PB counterpart of these cells from normal healthy individuals.[27] These observations suggest the potential involvement of a common antigen in driving the expansion of clonal T cells in these patients. In such a situation, shared HLA haplotypes, as well as common motifs in the CDR3 sequences of the TCR-Vβ genes, could be expected. Upon comparing TCR-Vβ13.1^{+} cases with all non-TCR-Vβ13.1 individuals, a clear association was found between the expanded TCR-Vβ family and the HLA genotype, all TCR-Vβ13.1^{+} cases displaying an HLA-DRB1*0701 allele. The random chance that both events coincide is about 2%, versus 42% in our patients. In line with these observations, it has recently been reported[28] that most CD4^{+} T cells from an HIV-1^{+}/CMV^{+-} infected patient with lytic granules containing cytotoxic proteins (such as granzymes and perforin) displayed a clear HLA class II –and not class I– restricted lytic activity. Accordingly, after specific blocking of HLA class II, CMV-specific CD4^{+} LGL T cells from this patient were completely inhibited in their in vitro ability to produce cytokines. In addition to the strong association between the expanded TCR-Vβ and HLA class II, all (unrelated) HLA-DRB1*0701^{+} patients showing TCR-Vβ13.1 expansions had a common CDR3 amino acid motif (XQGX) in the expanded T lymphocytes. Interestingly, this common "XQGX" CDR3 amino acid motif could not be found among the TCR-VDJβ sequences of T lymphocytes from healthy individuals deposited in GenBank, and it was detected only at very low frequencies among the few clones using the Jβ1.1, Jβ1.2, and Jβ1.5 gene segments identified in both purified CD4^{+} (1 of 16 clones) and CD8^{+} (0 of 12 clones) PB T cells from healthy adults. In addition, in a normal T-cell repertoire, different T cells have distinct CDR3 lengths that result in a gaussian distribution, while in our series virtually all expanded monoclonal CD4^{+} T-LGL cases expressing TCR-Vβ13.1 showed the presence of TCRβ chains characterized by a unique CDR3 length. Alltogether, the association between monoclonal expansions of TCR-Vβ13.1 T-LGL, the HLA-DRB1∗0701 genotype, and a common XQGX motif in the CDR3 sequence strongly suggests that monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL lymphocytosis from these unrelated patients has been selected by a specific common antigen and that they could be the result of a chronic, long-term, antigen-driven process, as previously reported for TCRγδ^{+} LGL leukemias[23] and B-cell chronic lymphocytic leukemias using the VH3-21 gene, based on their CDR3 homology.[29] Because the expanded CD4^{+}/CD8$^{-/+d}$ T-LGL clones expressed TCR-Vβ13.1 with restricted antigen-binding sites in the context of HLA-DR*0701, it could be suggested that they result from an exogenous peptide-driven T-cell stimulation. Furthermore, if this selection involves antigen binding and triggering through the TCR, the

antigenic epitope would most likely be restricted in its nature and structure,[30] although some differences in the amino acid sequences of the CDR3 region were noted. The overlapping phenotypes of the expanded cells between different (unrelated) patients would further reinforce an underlying common pathogenesis. As previously reported,[18] monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL cases show a remarkably uniform cytotoxic T-cell phenotype, as reflected by a common pattern of expression of NKa surface markers and cytotoxic proteins (CD56^{+}, CD57^{+}, Cy granzyme B^{+}) in the absence of expression of other (CD16^{-}, CD94^{-}, CD158a^{-}, CD161^{-}, NKB1^{-}) NK-associated receptors.

Recent reports provide strong accumulating evidence for a role of chronic antigen stimulation in clonal selection and progression of B-cell lymphomas[10] as well as T-LGL leukemias.[9,22,23] Although identical TCR gene rearrangements are typically identified in LGL leukemia, indicating a (mono)clonal proliferative disease, demonstration of monoclonality does not necessarily imply either neoplastic or malignant transformation.[9,23] In fact, in the present study we were unable to demonstrate the presence of any genetic alteration in the patients studied, either by conventional karyotyping or by fluorescence in situ hybridization (FISH) (data not shown). Accordingly, the most probable pathogenetic mechanism leading to an increased survival and/or proliferation of specific T-cell clones in CD4^{+} T-LGL patients is more likely to be related to chronic antigenic stimulation than to a cytogenetic-associated neoplastic transformation. TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T cells have been found in increased proportions in humans in different disease conditions where chronic antigen stimulation may occur, such as neoplasias, chronic viral infections, autoimmune disorders and allografts.[28,31-34] Unfortunately, no study has been reported in which CDR3 sequences of the expanded cells have been analyzed in such disease conditions; an exception would be graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT), where the expanded CTL clones (including both CD4^{+} and CD8^{+} T cells) have been clonotyped.[35,36] Accordingly, a variable but frequently high degree of CDR3 homology within a given Vβ family has been reported in patients undergoing allo-HSCT[35] whereby the extent of the alteration of the T-cell repertoire is significantly higher in PBMCs from patients with acute GVHD than it is in cases without GHVD.[36] Based on these results, it has been hypothesized that such abnormalities could reflect multiple antigen-driven T-cell clonal expansions against alloantigens. Altogether, the evidence of oligoclonal expansions of TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T cells in several pathological conditions, interpreted as a specific T-cell response against tumor cells, virus, and autoantigens or alloantigens, clearly suggests that clonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL lymphocytosis represents a dysregulated reaction to exogenous antigens. As a result, a wide and complex spectrum consisting of different clinical entities (from transient immune reaction to LGL leukemia) could be expected, similar to that described for clonal TCRαβ^{+}/CD8^{++} T-LGL lymphocytosis.[9] Although the exact identity of such antigen(s) remains unknown, based on our results we may conclude that monoclonal TCRαβ^{+}/CD4^{+}/NKa^{+}/CD8$^{-/+d}$ T-LGL lymphocytosis cases are not random, because they do not reflect the expected Vβ-J physiological frequencies. In addition, the diverse geographic origin of our patients would suggest that the potential antigen involved in these processes is widely distributed. We can also rule out the involvement of a superantigen, due to the clear major histocompatibility complex (MHC)-TCRVβ-restricted association here observed.[37] Finally, the HLA-II restriction found for these clonal expansions of CD4^{+} T cells supports the involvement of a peptide with an exogenous

origin leading to a repetitive and chronic engagement of the TCR of the expanded CD4$^+$ T-LGL proliferations.

Another interesting observation is that monoclonal expansions of CD4$^+$ T-LGL have only rarely been reported in the literature[18] despite the fact that HLA-DRB1*0701 is frequently observed in the Caucasian population (about 30%).[25] These observations further support the role of factors other than the HLA genotype in leading to the dysregulation of the immune response and clonal expansion of CD4$^+$ T-LGL. In this sense, the presence of common extended haplotypes among the TCR-Vβ13.1$^+$ patients suggests that a genetic influence cannot be ruled out. In particular, polymorphisms in genes within the MHC (ie. MICA, cytokines) should be considered with regard to dysregulation of CD4$^+$ cytotoxic T cells.

In summary, in the present study we show that patients with monoclonal expansions of TCR-Vβ13.1$^+$/CD4$^+$ T-LGL display a common HLA-DRB1*0701 genotype and express identical motifs in a consistently shorter-length CDR3-TCR-Vβ sequence, supporting a common antigen-driven origin for these T-cell disorders. Further identification of the short peptides bound to HLA molecules preferentially expressed by clonal TCRαβ$^+$/CD4$^+$ T-LGL would provide new insight into the pathogenesis of the disease; at the same time it could facilitate the identification and establishment of novel preventive and/or therapeutic strategies in individuals with monoclonal CD4$^+$ T-LGL lymphocytosis at risk for transformation.

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Chapter 4.5

TCR-Vβ and TCR-Vα usage in $CD8^+$/$TCR\alpha\beta^+$ T-cell large granular lymphocyte leukemia

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ABSTRACT

Clonal $CD8^+/TCR\alpha\beta^+$ T-cell large granular lymphocyte (LGL) proliferations arise from their normal cytotoxic T-cell counterparts. It is the most common subtype of LGL leukemia and is a well-recognized disease entity in the World Health Organization (WHO) classification of hematopoietic malignancies. In the present study we explored the potential association between the expanded TCR-Vα and TCR-Vβ families, the CDR3 sequences of the *TCRB* and *TCRA* gene rearrangements, and the HLA-A and HLA-B genotypes of patients diagnosed with $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia. TCR-Vβ clonotypes from leukemic T-LGL populations and sorted $CD8^+$ T-cell populations from healthy adult donors were compared. No complete identical TCR-Vα CDR3/Vβ CDR3 motifs were found in our patient series. However, we identified 1 patient with an immunodominant Vβ24 CDR3 sequence, which was nearly identical to three LGL associated clonotypes reported previously. The physiologic TCR repertoire of $CD8^+$T lymphocytes appeared to be highly diverse and no significant clonal sharing was found in healthy donors. We therefore believe that our data could still point to non-random selection, possibly driven by a common pathogen.

INTRODUCTION

Lymphoproliferations of large granular lymphocytes (LGLs) range from activated polyclonal expansions to clinically malignant leukemias. Clonal LGL proliferations are assumed to be derived from their normal LGL counterparts, which comprise 10-15% of peripheral blood (PB) mononuclear cells (MNCs).[1,2] The majority of LGLs (85%) are of NK-cell origin, and a minority is derived from mature (post-thymic) T lymphocytes. T-cell LGL (T-LGL) leukemia is the most common subtype, representing approximately 85% of all cases diagnosed in Western countries. It is a rare and heterogeneous disorder and most cases have an indolent clinical course. The main clinical manifestations are related to chronic neutropenia and/or anemia. There is a frequent association with autoimmune diseases and other malignancies.[2-6] Its diagnosis is based on finding a persistent (>6 months) morphologically and/or immunophenotypically increased $CD3^+/CD57^+$ LGL population in PB, usually $>2 \times 10^9$/L, though the most recent criteria for diagnosing T-LGL leukemia do not require a minimum number of circulating T-LGLs.[7]

T-LGL leukemias can be divided into three groups on basis of their immunophenotypical and molecular characteristics. Most leukemic T-LGL proliferations are positive for CD3, CD8, and TCRαβ. Although $CD3^+/CD4^+/CD8^{-/+dim}TCR\alpha\beta^+$ T-LGL leukemia and $CD3^+/TCR\gamma\delta^+$ T-LGL leukemia are far less common, both disease entities have recently been described in detail.[8,9] Clonality assessment studies are essential to discriminate true T-LGL leukemia from other reactive proliferations. In T-LGL proliferations, clonality can easily be detected by PCR analysis of T-cell receptor (TCR) genes. However, the finding of clonality does not imply malignancy since most patients with clonal T-LGL proliferations have an indolent clinical course, not requiring therapy. These patients are generally diagnosed as having T-cell clonopathy of undetermined significance (TCUS).[6,10,11]

Although the etiology of T-LGL leukemia is unknown, it has been hypothesized that chronic

antigenic stimulation contributes to the pathogenesis of this disorder. This is in line with the activation-related phenotype and the skewed T-cell receptor expression pattern found in T-LGL leukemia. An exaggerated response to an immunodominant autoantigen or viral antigen might be the initial step in the development of this disorder.[12-15] On top of that, secondary molecular events are assumed to be required to establish the full leukemic phenotype of the chronically antigen stimulated T-LGL population.[16,17]

In order to further substantiate the potential involvement of a common antigen in driving development of clonal T-LGL proliferations, the complementarity determining region 3 (CDR3) sequences of TCRδ (*TCRD*), TCRγ (*TCRG*), and TCRβ (*TCRB*) genes can be analyzed. The CDR3 region of the TCR molecule has the highest antigenic specificity and directly binds to the antigenic peptide presented in the context of HLA.[18] The analysis of CDR3 regions in T-LGL leukemia has resulted in controversial findings. Although no clear single structural homologic motif could be detected in CDR3 sequences of *TCRB* genes in $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia, non-random clonal selection has still been suggested.[15,19] In the presence of identical HLA restriction element, TCRs with identical Vβ CDR3 regions would strongly suggest recognition of the same antigenic peptides. The lack of such identical TCR specificities is most probably explained by the diversity of the HLA background of patients. Interestingly, common CDR3 sequences could be detected in *TCRG* and *TCRD* genes in nearly half of patients diagnosed with $TCR\gamma\delta^+$ T-LGL leukemia, supporting a common antigen-driven origin of this disorder.[9] Moreover, Garrido *et al*. recently demonstrated strikingly similar motifs in CDR3 TCR-Vβ13 sequences in 42% of $CD4^+/TCR\alpha\beta^+$ T-LGL leukemia cases and a clear association with a HLA-DRB1*0701 genotype (Garrido *et al*., *Blood 2007, in press).*

Although the TCR-Vβ CDR3 region appears to interact with the antigenic peptide, the TCR-Vα chain may also play an important role, especially in the initial phase of high-affinity clonal TCR selection.[20] However, the CDR3 regions of the TCR-Vα chains have not extensively been studied in both subtypes of $TCR\alpha\beta^+$ T-LGL leukemia.

In the present study we have analyzed a large cohort of 26 patients diagnosed with $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia. We report on the clinical and hematological features of these patients and explored the potential existence of an association between CDR3 sequences of the expanded TCR-Vα and TCR-Vβ clonotypes and the HLA genotype, which would be in favour of common antigenic stimuli.

MATERIALS AND METHODS

Patients, controls and cell samples

Peripheral blood (PB) and/or bone marrow (BM) samples from 26 patients with $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia were obtained. The diagnosis of T-LGL leukemia was established by clinical and laboratory parameters as defined previously.[5,21] Patients with a persistent (>6 months) and increased (>1 $\times 10^9$/L) monoclonal $CD3^+/CD8^+/TCR\alpha\beta^+$ T-LGL proliferation in PB were included. PB/BM mononuclear cells (MNCs) isolated by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) centrifugation were used for immunophenotyping, DNA isolation, and RNA isolation. Cytomorphological May-Grünwald-Giemsa staining of PB smears was used for detection of LGLs. HLA genotyping for HLA-A and HLA-B was

performed by Luminex-based SSOP-PCR techniques (One Lambda Inc.).

As controls, PB samples from five healthy adults were obtained after informed consent was given. All patient and control samples were obtained according to the guidelines of the Medical Ethics Committee of Erasmus MC, University Medical Center (Rotterdam, The Netherlands).

Immunophenotypical analysis

MNCs were analyzed for cell membrane expression using a routine panel of monoclonal antibodies, including CD2, CD3, CD4, CD5, CD7, CD8, CD16, CD56, CD57, anti-TCRαβ (BMA031 and WT31), anti-TCRγδ (11F2), and anti-HLA-DR. Immunofluorescence stainings were performed as described[22] and evaluated on a FACSCalibur (BD Biosciences) flow cytometer. Data analysis was performed using CellQuest and Paint-A-Gate Pro software (BD Biosciences)

The PB and/or BM samples were studied in more detail for Vβ domain expression to quantitate the contribution of each Vβ family to the $CD8^+$ lymphocyte population. Flowcytometric analysis was performed using the IO Test Beta Mark kit (Beckman Coulter) as described.[22] Samples in which the Vβ restriction of the expansion could not be identified by flow cytometry, were analyzed by *TCRB* RT PCR as described.[23]

DNA and RNA isolation and cDNA synthesis

High molecular weight DNA from fresh or frozen PB MNCs was extracted using a phenol-chloroform extraction-based protocol, followed by ethanol precipitation and resolution in TE buffer.[24] Alternatively, DNA was isolated using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. In addition, total DNA was isolated from sorted $CD8^+$ T-cell populations from pooled PB-MNCs of five healthy adult donors. Total RNA was extracted from fresh or frozen PB and/or BM MNCs from patients and reverse transcribed into cDNA as previously described.[25] cDNA quality was checked using *ABL* as control gene.

***TCRB* and *TCRA* gene rearrangement analysis**

TCRB gene rearrangement analysis was performed according to the BIOMED-2 multiplex PCR protocol.[26] All BIOMED-2 multiplex PCR kits were obtained from InVivoScribe Technologies (San Diego, CA, USA; http://www.invivoscribe.com). Amplification reactions were performed in an automated thermocycler (model ABI 2700; Applied Biosystems, Foster City, CA, USA). *TCRB* multiplex PCR products from healthy donors were cloned into pGEM-T easy vector and single colony PCR was performed on positive clones. For *TCRA* gene rearrangement analysis, cDNA was amplified using a constant region antisense primer (Cα) and 54 different Vα family-specific sense primers, which were distributed in 5 different multiplex tubes. In each 50 µl PCR reaction, 2µl of cDNA, 10 pmol of 5' and 3' oligonucleotide primers, 0.2 mmol/L dNTP, 5 µL 10 x buffer II, and 1-2 U Ampli*Taq* Gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. The concentration of $MgCl_2$ was 3 mmol/L.

Sequence analysis

After PCR amplification of *TCRA* and *TCRB* gene rearrangements, products were subjected to heteroduplex (HD) and GeneScan (GS) analysis.[27] Products found to be monoclonal in HD analysis were directly sequenced except for cases with more than one clonal product. In such cases homoduplexes were excised from the polyacrylamide gel, eluted, and directly sequenced. Sequencing was performed either on the ABI377 fluorescent sequencer (Applied Biosystems), using the dye terminator cycle sequencing kit and Ampli*Taq*FS DNA polymerase (Applied Biosystems), as previously described[28] or on an ABI 3100 Genetic Analyzer. Single colony PCR products from healthy donors were directly sequenced. Assignment of Vβ, Dβ, Jβ, Vα, and Jα gene segments and reading frames of the involved *TCRB* and *TCRA* gene rearrangements was done using the IMGT database.[29]

RESULTS

Clinical and laboratory features of CD8⁺/TCRαβ⁺ T-LGL leukemia patients

Table 1 summarizes the most relevant clinical and hematological findings at diagnosis of the 26 CD8⁺/TCRαβ⁺ T-LGL leukemia patients enrolled in this study. The median age was 58 years (range 31-86 years) and there was no male or female predominance. 21 patients (81%) were symptomatic at presentation. Nine of the 26 patients (35%) had an episode of bacterial infection or B symptoms (fever, night sweats, weight loss). T-LGL leukemia cases most frequently (62%) presented with neutropenia and/or anemia. Also some T-LGL leukemias presented with neutropenia and thrombocytopenia (12%). Thrombocytopenia with coexistent anemia was found in one case (4%). Splenomegaly was found in 2 cases (8%) and lymphadenopathy in one (4%).

An associated disease was found in 11 cases (42%) (Table 1). In our series, 7 patients (27%) had a co-existent autoimmune disorder. The most common autoimmune manifestation was rheumatoid arthritis (RA), which was diagnosed in three patients (12%). Co-existence of a second hematological malignancy was demonstrated in three patients (12%).

Examination of PB smears showed an increased number of T-LGLs with abundant cytoplasm containing azurophilic granules in the majority of patients.

Therapy and follow-up of CD8⁺/TCRαβ⁺ T-LGL leukemia

At closing of the study, the median follow-up of the patients was 34 months (range 6-122 months). Therapeutical approaches differed per patient and are demonstrated in Table 1.
Two thirds of patients required therapy with one or more agents. The therapeutic strategy was largely aimed at improving cytopenias and included red blood cell transfusions and various immunosuppressive drugs. We observed one disease related death in these 26 patients (case 86-041) which is in line with an indolent clinical course.

Immunophenotypical characteristics of CD8⁺/TCRαβ⁺ T-LGL leukemia

The T-LGL cells of all 26 cases showed membrane coexpression of CD3, CD8, and TCRαβ molecules. Furthermore, the T-LGL cells of all cases showed expression of one or more LGL-associated markers (CD16, CD56, CD57) with CD57 being the most consistently expressed

Table 1. Characteristics, clinical presentation and immunophenotype of 26 patients diagnosed with CD8$^+$/TCRαβ$^+$ T-LGL leukemia.

Case no.	Sample	Age, sex	Immunophenotype	Main clinical presentations[1]	Associated disease	LGL x10^9/L	Therapy
1	86-041	58, F	CD2/3/8/5/7	Anemia	PRCA	21.6	RBC transfusion, leukeran
2	96-013	58, F	CD2/3/8/16/56/57	Anemia/neutropenia	-	13.0	Unknown
3	96-043	73, F	CD2/3/8/5/7/16/56/57	Neutropenia/ thrombocytopenia	Oligo arthritis	9.9	MTX
4	92-050	50, M	CD2/3/8/5/7/16neg/ 56neg/57/HLA DR	Neutropenia	RA (RF+), DLBCL	1.9	None
5	98-126	73, M	CD2/3/8/5/7/16/56/57/ HLA DR	Anemia/ thrombocytopenia	HCC	1.0	Leukeran
6	92-024	55, F	CD2/3/8/2/5/7/16/56neg/57/HLA DR	Neutropenia/anemia	AIHA, ITP, bronchus carcinoma	7.4	CSA
7	93-027	53, M	CD2/3/8/5/7/16neg/ 56neg/57/HLA DR	Anemia	AIHA	2.0	Unknown
8	96-067	39, F	CD2/3/8/5/16/57	Neutropenia	-	5.9	None
9	97-064	71, M	CD2/3/8/16neg/56neg/57/HLA DR neg	Neutropenia	-	1.2	Unknown
10	99-100	49, M	CD2/3/8/5/7/16/56neg/ 57/HLA DR neg	Anemia	-	5.5	Unknown
11	98-194	38, F	CD2/3/8/5/7/16/56/ 57neg/HLA DR	Neutropenia	-	6.6	None
12	05-060	70, M	CD2/3/8/5/7/16neg/ 56neg/57/HLA DR	Neutropenia/ thrombocytopenia	-	3.1	Unknown
13	05-100	31, F	CD2/3/8/5/7/57	Neutropenia/anemia	-	2.0	Corticosteroids, CSA
14	05-191	73, M	CD2/3/8/5/7/16neg/ 56neg/57/HLA DR	Neutropenia/recurrent infections	Chronic NK-LGL leukemia	4.4	MTX
15	03-030	41, F	CD2/3/8/5/7neg/57/ HLA DR	Anemia/B symptoms	PRCA	2.5	CSA

Table 1. Characteristics, clinical presentation and immunophenotype of 26 patients diagnosed with CD8$^+$/TCRαβ$^+$ T-LGL leukemia (continued).

Case no.	Sample	Age, sex	Immunophenotype	Main clinical presentations[1]	Associated disease	LGL x10^9/L	Therapy
16	03-086	39, F	CD2/3/8/5/7/16/56neg/ 57	Fatigue	M. Hodgkin	2.0	None
17	00-113	72, M	CD2/3/8/5neg/7/16neg/ 56neg/57/HLA DRneg	Neutropenia/ B symptoms	-	3.5	None
18	93-054	62, M	CD2/3/8/7/16neg/57	Anemia/ splenomegaly/B symptoms	chronic osteomyelitis, melanoma	4.4	Leukeran, RBC transfusion
19	02-047	76, F	CD2/3/8/5/7/57/HLA DR	B symptoms	-	12.0	None
20	91-004	86, F	CD2/3/8/7/16neg/56neg	Neutropenia/anemia/ lymphadenopathy	-	3.8	None
21	05-281	59, M	CD2/3/8/5/7neg/16neg/ 56/57/HLA DRneg	B symptoms	-	2.7	None
22	06-026	67, M	CD2neg/3/8/5/7/16/56neg/57/HLA DRneg	Neutropenia/B symptoms/ thrombocytopenia	(Oligo) RA	2.0	Plaquenil
23	06-038	69, M	CD2/3/8/5neg/7/16neg/ 56neg/57/HLA DR	Neutropenia	RA	3.2	MTX
24	06-127	35, F	CD2/3/8/5/7/16/56neg/ 57/HLA DR	Neutropenia	-	3.7	None
25	06-131	61, F	CD2/3/8/5/7/16neg/56/ 57/HLA DR	Recurrent infections	-	1.2	None
26	06-246	47, F	CD2/3/8/5/7/16/56/57/ HLA DR	Neutropenia/ splenomegaly	-	10.2	CSA

[1] Neutropenia was defined as absolute neutrophil count (ANC) <1.5 x10^9 neutrophils/L; anemia was defined as hemoglobin level <10 g/dL; thrombocytopenia was defined as platelet count <150 x 10^9/L anemia; CSA, cyclosporin A; DLBCL, diffuse large B-cell lymphoma; HCC, hepatocellular carcinoma; ITP, idiopathic thrombocytopenic purpura; MTX, methotrexate; PRCA, pure red cell aplasia; RA, rheumatoid arthritis; RF, rheumatoid factor

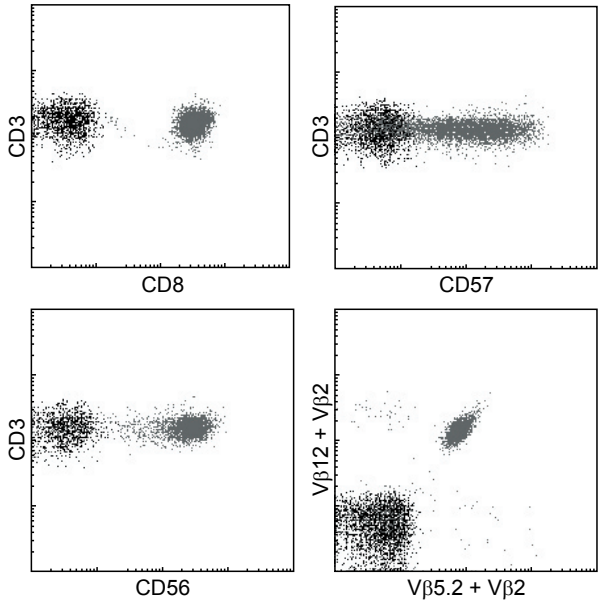

Figure 1. Immunophenotype of blood mononuclear cells.
Immunophenotype of the T-LGL cells (shown in grey) present in the peripheral blood at diagnosis. There is a large expansion of $CD3^+/CD8^+$ T-cells expressing CD56 and heterogeneous expression of CD57. Nearly all T-cells (90%) are positive for Vβ2.

antigen. Heterogeneous expression of CD57 could be demonstrated in 23 out of 24 evaluable cases (96%) (Figure 1). Out of 22 cases that were analyzed for expression of CD16, 11 were positive (50%). Expression of CD56 was seen in 7 out of 20 cases analysed (35%). The vast majority of leukemic LGLs expressed CD2 (100%), CD5 (77%), and CD7 (81%) (Table 1).

Immunophenotypical and/or molecular Vβ and Vα analysis of $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia

The *TCRB* repertoire was studied using specific anti-TCR-Vβ domain MoAbs, with a dominant TCR-Vβ protein being detectable in 22 out of 26 cases (Table 2; Figure 1). All cases, including the four cases without detectable TCR Vβ expression, demonstrated clonal in-frame gene rearrangements, as detected by BIOMED-2 multiplex PCR based HD/GS analysis and/or by *TCRB* RT PCR analysis (Figure 2) (Table 3). Results of sequence analysis of *TCRB* gene rearrangements and Vβ protein/mRNA expression were concordant in all cases. Usage of Vβ and Jβ gene segments (Table 3), appeared to be largely random. Nevertheless, there was a slight predominance of some Vβ families with Vβ2, Vβ5, and Vβ12 being the most frequently expressed ones. The Jβ2 family was used more frequently than the Jβ1 family (62% vs 38%) and the proportion using Jβ2.1 was the highest (15%). This seems to reflect the non-random Jβ gene segment distribution as seen in mature peripheral blood $TCR\alpha\beta^+$ T cells from healthy individuals.

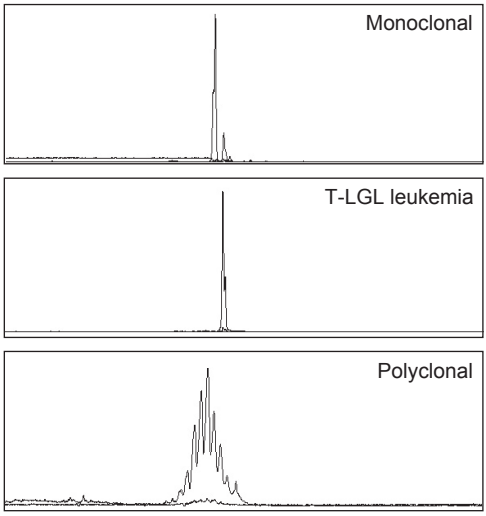

Figure 2. PCR-based GeneScan analysis.
Clear clonal *TCRB* gene rearrangements could be identified in T-cell line DNA and PB DNA from a patient diagnosed with CD8⁺/TCRαβ⁺ T-LGL leukemia. Polyclonal *TCRB* gene rearrangements can be identified in PB MNC DNA from a healthy donor.

Clonal *TCRA* gene rearrangements could be demonstrated in all 22 cases analyzed (Table 2). In the remaining four cases no RNA could be isolated due to lack of material. Similar to Vβ usage, there was also no common Vα gene segment usage among the 22 patients studied. Gene segments from the Vα19, Vα8, and Vα12 families were used most frequently and were expressed in three cases each. The Jα gene segment usage was also highly diverse (Table 4). In addition to the expanded Vβ and Vα families, Table 2 also shows the HLA genotypes of the studied cases. No clear association was found between a particular TCR-Vβ/Vα specificity and the HLA genotypes (Table 2).

TCR Vβ and Vα CDR3 motifs of immunodominant T-LGL clones

Because detection of identical clonotypes shared by patients with TCRαβ⁺ T-LGL leukemia could suggest non-random transformation of clonotypes, we analyzed the *TCRB* and *TCRA* CDR3 sequences in more detail. Translation of nucleotide sequences into amino acid code resulted in 27 T-LGL TCR Vβ CDR3 clonotypes from 25 patients and 24 T-LGL TCR-Vα CDR3 clonotypes from 22 patients. Detailed information concerning the configuration of *TCRB* and *TCRA* genes and the CDR3 Vβ/Vα amino acid sequence of each CD8⁺/TCRαβ⁺ T-LGL leukemia patient is summarized in Tables 3 and 4. The CDR3 motifs of the immunodominant T-cell clones were compared, but no identical sequences could be detected. No obvious association between TCR-Vβ CDR3/Vα CDR3 and HLA genotypes was found either. In addition, the sequences of our cohort were compared to the Vβ CDR3

Table 2. HLA genotype and Vα/Vβ usage in CD8$^+$/TCRαβ$^+$ T-LGL leukemia patients.

Case no.	Sample no.	Expanded TCR-Vβ family[a]	Expanded TCR-Vα family[b]	HLA-A	HLA-B
1	86-041	Vβ1	Vα19	02, 11	35, 50
2	96-013	Vβ12	Vα19	02	40, 44
3	96-043	Vβ22	Vα29	01, 25	07, 08
4	92-050	Vβ23	Vα17	02, 02	13, 15
5	98-126	Vβ2	Vα19	ND	ND
6	92-024	Vβ7.2	Vα12.2	01, 24	08, 40
7	93-027	Vβ8.1/8.2	Vα6	02	40, 40
8	96-067	No reactivity (Vβ6 in PCR)	Vα26	02, 31	27, 58
9	97-064	Vβ13	Vα35	24, 29	45, 49
10	99-100	Vβ17	Vα23	01, 02	08, 41
11	98-194	No reactivity (Vβ6/Vβ12 in PCR)	Vα6/Vα21	02, 03	07, 27
12	05-060	Vβ17	Vα30/Vα26	03, 32	41, 55
13	05-100	Vβ16	Vα12.2	02, 03	18, 51
14	05-191	Vβ5	Vα1	02	15, 35
15	03-030	No reactivity (Vβ6/Vβ16 in PCR)	ND	02	08
16	03-086	Vβ14	Vα3	02, 03	08, 35
17	00-113	Vβ8.2	Vα8	01, 26	38, 44
18	93-054	Vβ12/Vβ15	Vα12.3	ND	ND
19	02-047	Vβ7.2	Vα8	ND	ND
20	91-004	Vβ5/Vβ6	Vα41	ND	ND
21	05-281	Vβ2	Vα1.2	ND	ND
22	06-026	No reactivity (Vβ24 in PCR)	Vα29	ND	ND
23	06-038	Vβ2	Vα12.1	01, 24	08, 18
24	06-127	Vβ13	ND	01, 11	27, 35
25	06-131	Vβ12	ND	ND	ND
26	06-246	Vβ5.1	ND	ND	ND

ND; not done
[a] TCR-Vβ family usage defined by immunophenotyping and molecular analysis
[b] TCR-Vα family usage defined by molecular analysis

Table 3. Amino acid sequences of CDR3 motifs of in-frame *TCRB* rearranged alleles in patients with CD8+/TCRαβ+ T-LGL leukemia.

Case no.	Sample no.	Rearrangement	V						N-(D)-N										J				
1	86-041	Vβ1-Jβ2.1	C	A	S	S			L	S	G	R	A	L							N	E	Q	F	F
5	98-126	Vβ2-Jβ1.3	C	S	A				S	L	G	G	R	P	T	I	A				G	N	T	I	Y	F
21	05-281	Vβ2-Jβ1.4	C	A					V	P	T	G	R								N	E	K	L	F	F
23	06-038	Vβ2-Jβ2.5	C	S	A				R	N	G	P	N	Y							Q	E	T	Q	Y	F
14	05-191	Vβ5.1-Jβ1.4	C	A	S	S	L		A	K	G	K	G	A						T	N	E	K	L	F	F
26	06-246	Vβ5.1-Jβ1.2	C	A	S	S	L		G	S	G											Y	G	Y	T	F
20	91-004	Vβ5.6-Jβ2.1	C	A	S	S			L	D	N										Y	N	E	Q	F	F
		Vβ6.5-Jβ1.1	C	A	S	S			F	S	P	Y	T	R	P								E	A	F	F
8	96-067	Vβ6.2-Jβ2.1	C	A	S	S			L	A	H										S	Y	N	E	Q	F	F
11	98-194	Vβ6.4-Jβ1.1	C	A					R	S	F	S	P	S	L	D	T			S	S	L	F	V	E	A	F	F
		Vβ12.3-Jβ2.3	C	A	S	S			C	Y	Q	P	G	L	D					L	P	R	A	D	T	Q	Y	F
6	92-024	Vβ7.2-Jβ1.5	C	A	S	S	Q		D	V	R	P	P	P	E	D	R	P	Y			S	N	Q	P	Q	H	F
19	02-047	Vβ7.2-Jβ2.2	C	A	S				G	G	V	G	G											G	E	L	F	F
7	93-027	Vβ8.2-Jβ2.5	C	A	S	S	L		G	T	G	G	M											E	T	Q	Y	F
17	00-113	Vβ8.2-Jβ1.5	C	A	S	S			W	G	G											N	Q	P	Q	H	F
2	96-013	Vβ12.1-Jβ2.2	C	A	I	S	E		G	S	G	P												G	E	L	F	F
18	93-054	Vβ12.1-Jβ2.3	C	A	I	S			G	R	L	A	G	G	R	T						S	T	D	T	Q	Y	F
25	06-131	Vβ12.2-Jβ1.2	C	A	S	S			P	K	G													Y	G	Y	T	F
9	97-064	Vβ13.3-Jβ2.7	C						G	S	L	G	Q	G	A	W								Y	E	Q	Y	F
24	06-127	Vβ13.6-Jβ1.5	C	A	S	S			Y	G	S	P	L	D	I	D	S	A	I	S					P	Q	H	F
16	03-086	Vβ14-Jβ2.1	C	A	S				N	N	R	G	S										Y	N	E	Q	F	F
13	05-100	Vβ16-Jβ2.5	C	A	S	S			P	V	G	A	Y	P	K									E	T	Q	Y	F
10	99-100	Vβ17-Jβ2.7	C	A	S				S	P	E	S	L	F									S	Y	E	Q	Y	F
12	05-060	Vβ17-Jβ2.7	C	A	S	S			I	F	R	G	N												E	Q	Y	F
3	96-043	Vβ22-Jβ2.6	C	A	S				G	G	D	R	G	T							G	A	N	V	L	T	F
4	92-050	Vβ23-Jβ1.5	C	A	S	S	L		G	G	R	Y									S	N	Q	P	Q	H	F
22	06-026	Vβ24-Jβ2.7	C	A	T	S			R	D	L	L	T											Y	E	Q	Y	F

A L I V G (P)	neutral side chain	D E	acidic side chain
S T	aliphatic side chain	N Q	amide side chain
K R H	basic side chain	C M	sulphur-containing side chain
F Y W	cyclic side chain		

Table 4. Amino acid sequences of CDR3 motifs of in-frame *TCRA* rearranged alleles in patients with CD8$^+$/TCRαβ$^+$ T-LGL leukemia.

Case no.	Sample no.	Rearrangement	V					N-(D)-N							J										
1	86-041	Vα19-Jα49	C	A	L	S	E	S	G										G	N	Q	F	Y	F
2	96-013	Vα19-Jα26	C	A	L	S	E	G	S	R	F							Y	G	Q	N	F	V	F
3	96-043	Vα29-Jα52	C					G	R	V									A	G	G	T	S	F
4	92-050	Vα17-Jα20	C	A				T	L	S							S	N	D	Y	K	L	S	F
5	98-126	Vα19-Jα37	C	A	L	S	E	A	E	G	S						S	N	T	G	K	L	I	F
6	92-024	Vα12.2-Jα53	C	A				V	T							G	G	S	N	Y	K	L	T	F
7	93-027	Vα6-Jα21	C					V	G										F	N	K	F	Y	F
8	96-067	Vα26.2-Jα20	C	I				P	S	P	S							N	D	Y	K	L	S	F
9	97-064	Vα35-Jα49	C	A				G	F									T	G	N	Q	F	Y	F
10	99-100	Vα23-Jα52	C	A				A	P	V						G	G	T	S	Y	G	K	L	T	F
11	98-194	Vα8.6-Jα56	C	A	V	S		L									T	G	A	N	S	K	L	T	F
		Vα21-Jα57	C	A	V			K										G	G	S	E	K	L	V	F
12	05-060	Vα26.2-Jα24	C	I	L	R	D	V	E	S	W	G	K	F	Q										F
		Vα30-Jα44	C	G				T	P	G	N							G	T	A	S	K	L	T	F
13	05-100	Vα12.2-Jα23	C	A	V														Q	G	G	K	L	I	F
14	05-191	Vα1.1-Jα10	C	A				V	D	P	G	L	A	A					G	G	N	K	L	T	F
16	03-086	Vα3-Jα36	C	A				S	D								Q	T	G	A	N	N	L	F	F
17	00-113	Vα8.1-Jα28	C	A	V			M							Y	S	G	A	G	S	Y	Q	L	T	F
18	93-054	Vα12.3-Jα9	C	A	M	S		A	V	M	R								G	F	K	T	I	F
19	02-047	Vα8.1-Jα39	C	A	V			M	S	D								A	G	N	M	L	T	F
20	91-004	Vα41-Jα48	C	A	V			N																	F
21	05-281	Vα1-2-Jα33	C	A				T	L									D	S	N	Y	Q	L	I	W
22	06-026	Vα29-Jα45	C	A	A			K	G	F												G	L	T	F
23	06-038	Vα12.1-Jα34	C	V	V			K									S	Y	N	T	D	K	L	I	F

A L I V G (P)	neutral side chain		D E	acidic side chain
S T	aliphatic side chain		N Q	amide side chain
K R H	basic side chain		C M	sulphur-containing side chain
F Y W	cyclic side chain			

Table 5. Amino acid sequences of CDR3 motifs of in-frame *TCRB* rearranged alleles in the CD8$^+$/TCRαβ$^+$ T-cell subset isolated from healthy donors.

Clone no.	Rearrangement	V					N-(D)-N										J							
34	Vβ2-Jβ2.1	C	S	A			V	K	E											E	Q	F	F	
7	Vβ2-Jβ2.7	C	S	A			H	P	T	G	T	G	A						Y	E	Q	Y	F	
30	Vβ2-Jβ2.1	C	S	A			T	P	G	Q								Y	N	E	Q	F	F	
32	Vβ2-Jβ2.1	C	S	A			G	R	E	T	D										Q	Y	F	
40	Vβ2-Jβ2.1	C	S	A			R	P	G	Q	L	G	G							E	Q	Y	F	
10	Vβ6.4-Jβ1.2	C	A	S	S		L	R	M	D	E									G	Y	T	F	
28	Vβ6.4-Jβ2.1	C	A	S			L	V	P	A	G	V	S						N	E	Q	F	F	
20	Vβ10-Jβ1.1	C	A	S	S		Y	Q	D	G	G	T	L								A	F	F	
8	Vβ12.3-Jβ2.7	C	A	S	S	E	S	S	N	S	P	I	G	Y	Q	A			Y	E	Q	Y	F	
29	Vβ12.1-Jβ2.3	C	A	S			A	G	C	D									D	T	Q	Y	F	
35	Vβ12.1-Jβ2.1	C	A				S	R	A	G	G								N	E	Q	F	F	
13	Vβ12.1-Jβ2.7	C	A	S	S		Y	D	Q	G	S								S	Y	E	Q	Y	F
24	Vβ12.3-Jβ2.1	C	A	S			G	Y	G	G	E	Q	W							N	E	Q	F	F
1	Vβ13.3-Jβ1.3	C	A	S	S		E	G	E	G								S	G	N	T	I	Y	F
2	Vβ13.5-Jβ1.5	C	A	S	S		Q	G										S	N	Q	P	Q	H	F
9	Vβ13.1-Jβ1.5	C	A	S			S	L	Q	P	P	G	T	G	G	S	E			Q	P	Q	H	F
12	Vβ13.2-Jβ1.6	C	A	S			T	G	T	G	D	D								S	P	L	H	F
16	Vβ13.3-Jβ1.1	C	A	S	S		A	E	E	G									N	T	E	A	F	F
21	Vβ13.1-Jβ1.2	C	A	S	S		T	P	R	Q	G	T								Y	G	Y	T	F
22	Vβ13.3-Jβ2.7	C	A	S	S		F	R	Q	D	P	P									E	Q	Y	F
23	Vβ13.3-Jβ2.7	C	A	S	S		E	E	G	G	T	G	S							Y	E	Q	Y	F
26	Vβ13.5-Jβ2.3	C	A	S	S		P	G											T	D	T	Q	Y	F
31	Vβ13.5-Jβ2.3	C	A	S			H	R	D	A									T	D	T	Q	Y	F
33	Vβ13.5-Jβ2.3	C	A	S	S		G	G										S	T	D	T	Q	Y	F
37	Vβ13.1-Jβ2.4	C	A	S			P	D	R	G										N	I	Q	Y	F
39	Vβ13.2-Jβ2.3	C	A	S			C	L	G	H									T	D	T	Q	Y	F

Table 5. Amino acid sequences of CDR3 motifs of in-frame *TCRB* rearranged alleles in the CD8$^+$/TCRαβ$^+$ T-cell subset isolated from healthy donors (continued).

Clone No.	Rearrangement	V						N-(D)-N								J					
43	Vβ13.2-Jβ2.3	C	A	S	S			V	S	A	G							D	T	Q	Y	F
44	Vβ13.3-Jβ2.7	C	A	S	S			E	N	R	D	R	A					Y	E	Q	Y	F
5	Vβ14-Jβ1.2	C	A	S				N	T	L	K	L	D					Y	G	Y	T	F
11	Vβ14-Jβ2.7	C	A	S	S	L		G	Q	V	A							Y	E	Q	Y	F
15	Vβ14-Jβ1.1	C	A	S				R	S	G	V						N	T	E	A	F	F
17	Vβ14-Jβ2.7	C	A	S	S	L		T	G	Q	S	L	P								Y	F
19	Vβ14-Jβ2.7	C	A	S	S	L		S	G	R	R								E	Q	Y	F
38	Vβ14-Jβ2.5	C	A	S				V	P	E	S	R						E	T	Q	Y	F
41	Vβ14-Jβ2.7	C	A	S	S	L		K	P	V	T								E	Q	Y	F
43	Vβ14-Jβ1.5	C	A	S	S			P	D	P	G	G	R					Q	P	Q	H	F
6	Vβ15-Jβ1.5	C	A	T				A	Q	G	D	S					S	N	Q	P	Q	H	F
14	Vβ15-Jβ2.2	C	A	T	S	E		A	G	T	G	P	T						G	E	L	F	F
25	Vβ15-Jβ2.3	C	A	T				P	G	Q	G	E							D	T	Q	Y	F
36	Vβ15-Jβ2.3	C	A	T	S			P	T	S	S	G	R	A	P				D	T	Q	Y	F
42	Vβ15-Jβ1.2	C	A	T	S			D	P	A	D	P							Y	G	Y	T	F
18	Vβ22-Jβ2.7	C	A	S				W	S	T	G	Y	K						Y	E	Q	Y	F
4	Vβ23-Jβ2.7	C	A	S	S			L	D	G	Q	V	S	F								Y	F

A L I V G (P)	neutral side chain	D E	acidic side chain
S T	aliphatic side chain	N Q	amide side chain
K R H	basic side chain	C M	sulphur-containing side chain
F Y W	cyclic side chain		

motifs of a large series of 60 T-LGL leukemia patients recently described by Wlodarski *et al*.[19] We found one patient in our series (06-026) with a Vβ CDR3 motif (XRDLX) which was highly homologous to the LGL-associated clonotypes of three Vβ24⁺ patients in their series. This motif was not present in 43 clones sequenced from sorted CD8⁺ T-cells from pooled PB-MNCs of five healthy adult donors (Table 5). At all there was only a very low level of homology between Vβ CDR3 sequences from healthy controls and the T-LGL leukemia clonotypes. Interestingly, in the CD8⁺ clonotypes isolated from these donors the Vβ13 gene segment was over represented, but no shared Vβ CDR3 clonotypes were found.

DISCUSSION

Monoclonal CD8⁺/TCRαβ⁺ T-LGL leukemia concerns the largest subgroup of monoclonal LGL lymphoproliferative disorders. It is generally characterized by a presentation in elderly individuals (mean age 60 years), cytopenias, autoimmune features, and an indolent clinical course.[2] However, cases with an aggressive clinical course have been reported and are associated with a CD3⁺/CD8⁺/CD56⁺/CD57⁻ phenotype.[30,31]

In suspected T-LGL proliferations, clonality can easily be detected using TCR gene rearrangement analysis and immunophenotyping, including antibodies directed against the TCR-V chains.[9,23,32] Analysis of TCR expression pattern in patients with T-LGL leukemia might reveal insight into the etiopathogenesis of this disorder. Molecular analysis of the TCR repertoire can thus be a powerful tool in the study of T-cell responses to pathogens and in autoimmune diseases. A similar TCR gene rearrangement pattern of T-LGL clones between different patients would be suggestive of a common antigenic stimulus, which might underlie the pathogenesis of this disorder. In addition, it has been suggested that the T-LGL leukemia associated cytopenias are the result of highly specific recognition and killing of individual hematopoietic cell lineages by T-LGL clones.

In the present study, we identified and characterized T-cell clonotypes in a large group of T-LGL leukemia patients. We could not detect specific predominant Vβ family usage in our group of CD8⁺/TCRαβ⁺ T-LGL leukemia patients, which is largely in line with previous findings.[15,32-34] In a recent study, Vβ CDR3 sequence analysis in a large cohort of CD8⁺/TCRαβ⁺ T-LGL leukemia patients demonstrated similarity between multiple immunodominant clonotypes.[19] In this cohort, the Vβ13 gene segment was used most frequently in the disease-related clonotypes. The Vβ13 gene segment could only be demonstrated in two out of 27 T-LGL clonotypes in our study. Remarkably, a large fraction (30%) of the clonotypes of the CD8⁺ T-cell subset from healthy individuals also used the Vβ13 gene segment. None of these Vβ13 clonotypes demonstrated identical CDR3 amino acid motifs when compared to the Vβ13 motifs in our series and the patients described by Wlodarski *et al*. Remarkably, patients with Vβ24⁺ associated LGL clonotypes also presented with a common CDR3 amino acid motif (XRDLX) which showed high similarity to the immunodominant Vβ24⁺ clonotype in one of our patients. This common "XRDLX" CDR3 amino acid motif could not be readily found among the *TCRB* sequences of CD8⁺ T lymphocytes from normal healthy individuals (Table 5). Interestingly, in all four patients, the main clinical presentation was neutropenia. Whether TCR clones with the Vβ24 XRDLX motif are responsible for neutropenia by

suppressing neutrophil precursors should be addressed in future studies.

Immunophenotypical analysis of TCR-Vα expression has been explored in a minority of T-LGL leukemia cases and sequencing of *TCRA* gene rearrangements has been performed in few patients only. Although no common usage of Vα or Jα gene segments could be found, selection of the leukemic clone because of antigenic pressure was suggested in one case.[35] In our study, we performed *TCRA* gene rearrangement analysis in 22 patients and we could not detect preferential usage of Vα or Jα gene segments either. By additional Vα CDR3 sequence analysis we also could not find a common amino acid motif in the different patients.

On the basis of our findings, we did not find straightforward evidence for common (super)antigen involvement in the pathogenesis of this disease entity since no clonotypes with identical CDR3 Vβ and Vα amino acid sequences between different patients could be identified. Nevertheless, nearly identical Vβ CDR3 sequences were shared between one case in our series and three T-LGL leukemia cases reported previously.[19] This suggests that these clones may not have evolved randomly but occur in the context of an initially oligoclonal/polyclonal immune response directed against highly similar antigenic peptides. The seemingly low level of similarity between the clonotypes in our study might also be explained by the large variability in HLA genotype in our patient series. Moreover, peptide binding is affected through the physical amino acid properties and the tertiary structure of the CDR3 structure. Therefore, the linear homology comparisons in 20 amino acid code as we performed in our study, might not be the most appropriate method in order to identify common motifs.

Though no complete identical *TCRB* or *TCRA* CDR3 motifs were found in our $CD8^+$/$TCR\alpha\beta^+$ T-LGL patient series, this does not completely rule out the involvement of common antigenic stimuli in the early pathogenesis of $CD8^+$ T-LGL leukemia. Antigen recognition depends on many more factors than linear CDR3 amino acid sequences alone. However, when clonotypes in our study were cross-referenced against a previously reported clonotypic database of 60 T-LGL leukemia patients, we could identify highly homologous clonotypes in a minority of patients. In addition, clonotypes of the leukemic LGL cases were not encountered in $CD8^+$ T cells of healthy individuals. We therefore believe that our data could still point to non-random selection of T-cell clones, possibly driven by a common pathogen. Further studies taking into account the trias of HLA genotype, peptide-groove binding, and TCR specificity, are needed to define the exact role of common antigen involvement in the pathogenesis of $CD8^+$/$TCR\alpha\beta^+$ T-LGL leukemia.

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Chapter 4.6

TCRγδ$^+$ large granular lymphocyte leukemias reflect the spectrum of normal antigen-selected TCRγδ$^+$ T-cells

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ABSTRACT

T-cell large granular lymphocyte (LGL) proliferations range from reactive expansions of activated T cells to T-cell leukemias and show variable clinical presentation and disease course. The vast majority of T-LGL proliferations expresses TCRαβ. Much less is known about the characteristics and pathogenesis of TCRγδ$^+$ cases. We evaluated 44 patients with clonal TCRγδ$^+$ T-LGL proliferations with respect to clinical data, immunophenotype and TCR gene rearrangement pattern. TCRγδ$^+$ T-LGL leukemia patients had similar clinical presentations as TCRαβ$^+$ T-LGL leukemia patients. Their course was indolent and 61% of patients were symptomatic. The most common clinical manifestations were chronic cytopenias - neutropenia (48%), anemia (23%), thrombocytopenia (9%), pancytopenia (2%) -, and to a lesser extent splenomegaly (18%). Also multiple associated autoimmune (34%) and hematological (14%) disorders were found. Leukemic LGLs were predominantly positive for CD2, CD5, CD7, CD8, and CD57, whereas variable expression was seen for CD16, CD56, CD11b, and CD11c. The Vγ9/Vδ2 immunophenotype was found in 48% of cases and 43% of cases was positive for Vδ1, reflecting the TCR-spectrum of normal TCRγδ$^+$ T-cells in adult PB. Identification of the well-defined post-thymic Vδ2-Jδ1 selection determinant in all evaluable Vγ9$^+$/Vδ2$^+$ patients, is suggestive of common (super)antigen involvement in the pathogenesis of these TCRγδ$^+$ T-LGL leukemia patients.

INTRODUCTION

T-cell large granular lymphocyte (T-LGL) leukemia is a rare heterogeneous disorder that represents a distinct group of mature chronic T-cell neoplasias. Based on characteristic clinical, morphological, immunophenotypical and molecular features it was recognized as a separate clinical entity in the World Health Organization (WHO) classification.[1] Clonal LGL proliferations are assumed to be derived from normal LGL cells, which comprise 10-15% of peripheral blood (PB) MNC.[2] The diagnosis of T-LGL leukemia is based on the finding of an abnormal CD3$^+$/CD57$^+$ cell population in combination with the presence of a clonal T-cell receptor (TCR) gene rearrangement. Clonal T-LGL proliferations usually express TCRαβ and show a CD8^{++}/CD4$^-$ phenotype, although some cases correspond to CD4$^+$/CD8$^{-/+}$dim T-LGLs.[3] Other molecules expressed on cytotoxic cells, such as CD16, CD56, CD11b and CD11c are variably expressed. A minor subgroup of T-LGL leukemias expresses TCRγδ; their CD4/CD8 immunophenotype is not well defined.

T-LGL leukemia patients may present with recurrent bacterial infections owing to (severe) neutropenia, anemia, and hepatosplenomegaly, but one third of patients appear to be asymptomatic at diagnosis.[4-6] A strong association with different autoimmune diseases (in particular, rheumatoid arthritis) and hematological malignancies has been reported.[4,5,7] In all patients with unexplained cytopenias and high numbers of cytotoxic T cells the diagnosis should be suspected and PB smears should be carefully examined for LGL cells. LGLs have typical morphological characteristics such as abundant cytoplasm and coarse azurophilic granules. The diagnosis of T-LGL leukemia can however not be excluded if no increased numbers of LGLs are found by cytomorphology, because characteristic cytomorphological

features may be absent.[5,7,8] Consequently, immunophenotyping is needed for an appropriate diagnosis.

It should be noted that not all T-LGL proliferations are clinically malignant. It has been suggested that T-LGL leukemia is at the end of a spectrum which ranges from a reactive/transient indolent disorder, via chronic lymphocytosis to clinically malignant disease requiring intensive therapy.[5,9-11] Chronic reactive proliferations of both TCRαβ⁺ and TCRγδ⁺ T-LGL cells are seen in various clinical conditions.[4,12] In these cases, lymphocytosis often is <5x 10^9/L. In T-LGL leukemia the number of PB LGLs usually is >2x 10^9/L, although lower LGL counts can be compatible with the diagnosis of leukemia as well.[5,13] By definition, TCR genes are clonally rearranged in T-LGL leukemia,[14,15] whereas in benign (reactive) proliferations polyclonal TCR gene rearrangements are found. Nevertheless, the finding of clonality certainly does not imply clinical malignancy, as most patients with clonal T-LGL proliferations have an indolent clinical course not requiring therapy.

The currently available clinical and laboratory data are mainly derived from patients with TCRαβ⁺ T-LGL leukemia, as TCRγδ⁺ T-LGL lymphoproliferations are rare. True cases of TCRγδ⁺ T-LGL leukemia have only been described anecdotally.[15-22]

We therefore aimed to assess the clinical features, immunophenotypical characteristics, and TCR gene rearrangement pattern of a large series of 44 TCRγδ⁺ T-LGL leukemias in order to get more insight into the molecular pathogenesis of this disease. A major part of the cases showed Vγ9/Vδ2 expression with the typical antigen-selected characteristics as seen in antigen-selected TCRγδ⁺ T-lymphocytes of older children and adults. Consequently, antigenic stimulation might play a role in the pathogenesis of TCRγδ⁺ T-LGL leukemia.

MATERIALS AND METHODS

Patients and cell samples

We retrospectively reviewed database files from the Erasmus MC, University Medical Center (Rotterdam, The Netherlands), the Hospital Universitario (Salamanca, Spain), and the Hospital Geral Santo António (Porto, Portugal) for cases of mature persistent TCRγδ⁺ T-cell proliferations in PB and/or BM, between 1990 and 2005. Patient samples were classified according to a combination of clinical, laboratory, histological (HE sections), cytomorphological (May-Grünwald-Giemsa staining), immunophenotypical and molecular data. In case of a confirmed hematological malignancy, the sample was classified according to the WHO classification of hematological malignancies.[1] This resulted in 53 TCRγδ⁺ T-cell proliferations of which 44 fullfilled the diagnostic criteria of T-LGL leukemia.[8,13] The diagnosis of TCRγδ⁺ T-LGL leukemia was based on the finding of a persistent (>6 months) monoclonal CD3⁺/TCRγδ⁺ LGL proliferation in PB. Both cases with increased T-LGLs (>2 x10^9/L) or a discrete T-LGL expansion as detected by flow cytometry and/or cytomorphology were included. The other diagnoses concerned persistent reactive TCRγδ⁺ T-LGL proliferations (n=4), TCRγδ⁺ hepatosplenic T-cell lymphoma (HSTCL; n=4) and TCRγδ⁺ peripheral T-cell lymphoma, unspecified (n=1). No diagnostic overlap between cases with TCRγδ⁺ HSTCL and TCRγδ⁺ T-LGL leukemia occurred, as all HSTCL patients had an aggressive clinical course requiring polychemotherapy and showed typical histopathological, immunophenotypical

($CD3^+/CD4^-/CD5^-/CD8^-/CD56^{\pm}/CD57^-$) and/or cytogenetic (isochromosome 7q) features.

In all $TCR\gamma\delta^+$ T-LGL leukemia cases, the molecular and immunophenotypical analyses were performed on PB and/or BM samples. Either erythrocyte-lysed samples (using FACSTM lysing solution; BD Biosciences, San Jose, CA) or PB/BM MNC isolated by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation were used for immunophenotyping and DNA isolation. Cytomorphological May-Grünwald-Giemsa staining of PB smears was used for detection of LGLs. In 36 patients, the BM was also examined at diagnosis. In all 36 cases this was done on bone marrow smears, while in eight of these cases trephine biopsies were also examined.

As controls, neonatal cord blood (NCB) samples and PB samples from children and adults were analyzed after informed consent was given by them or their parents. All patient and control samples were obtained according to the guidelines of the Medical Ethics Committee of Erasmus MC, Rotterdam.

Immunophenotyping and analysis of Vγ and Vδ domains of expressed TCR chains

Cells were analyzed for cell membrane expression of T- and NK-cell associated antigens, including CD2, CD3, CD4, CD5, CD7, CD8, CD16, CD56, CD57, and HLA-DR antigens, and for TCRαβ (BMA031 and WT31) or TCRγδ (11F2) expression. In about half of cases expression of the adhesion molecules CD11b and CD11c was studied. Immunofluorescence stainings were performed as described[3,23] and evaluated on either FACScan or FACSCalibur (BD Biosciences) flow cytometers. Data analysis was performed using either the CellQuest or the Paint-A-Gate Pro software (BD Biosciences).

Cell samples from 38 patients were studied in more detail for Vγ and Vδ protein expression with a panel of monoclonal antibodies (MoAbs), with specificity for Vγ2/3/4 (23D12), Vγ4 (4A11), Vγ3/5 (56.3; this antibody recognizes Vγ5 domains and some Vγ3 domains), Vγ8 (R4.5), Vγ9 (IMMU360 and Ti-γA), Vδ1 (R912 and δTCS1), Vδ2 (IMMU389 and BB3), Vδ3 (P11.5B), and non-Vδ1 (IMMU515) MoAbs in combination with anti TCRαβ and CD3 reagents. MoAb 4A11 was purchased from T Cell Diagnostics (Woburn MA), TCRδ1 and δTCS1 from T Cell Sciences (Cambridge, MA); MoAbs 23D12, R4.5, IMMU360, R912, IMMU389, P11.5B, and IMMU515 were obtained from Beckman Coulter/Immunotech; the Ti-γA, BB3, and 56.3 MoAbs were kind gifts of Dr T Hercend (Villejuif, France), Dr L. Moretta (Genova, Italy), and Dr D Kabelitz (Kiel, Germany), respectively.

NCB (n=35) samples and PB samples from healthy children (eight different age groups; n=358) and adults (n=51) were analyzed to determine the following subsets: $CD3^+$ T lymphocytes and $CD3^+/TCR\gamma\delta^+$ T lymphocytes. Part of these results have been described previously.[24] We also analyzed the expression of Vγ9 (Ti-γA), Vδ1 (δTCS1), and Vδ2 (BB3) by $TCR\gamma\delta^+$ T lymphocytes in NCB samples (n=10) and PB samples from healthy children (three different age groups; n=15) and adults (n=15).

DNA isolation

High molecular weight DNA from fresh or frozen MNC was either extracted using a phenol-chloroform extraction-based protocol, followed by ethanol precipitation and resolution in TE buffer,[25] or isolated using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's protocol.

TCR gene rearrangement analysis

Amplification reactions were performed in an automated thermocycler (model ABI 9600/2700; Applied Biosystems, Foster City, CA, USA) according to the BIOMED-2 multiplex PCR protocol.[26] In order to amplify the most commonly recognized canonical *TCRG* gene rearrangement that involves the Vγ9 and Jγ1.2 gene segments, we performed an additional singleplex PCR reaction using BIOMED-2 Vγ9 and BIOMED-1 Jγ1.2 primers. All BIOMED-2 multiplex PCR kits were obtained from InVivoScribe Technologies (San Diego, CA, USA; www.invivoscribe.com). After PCR amplification of *TCRG* and *TCRD* gene rearrangements, products were subjected to both heteroduplex (HD) and GeneScan (GS) analysis for identification of monoclonal gene rearrangements.[27,28]

Sequence analysis

PCR products found to be monoclonal in HD and GS analysis were directly sequenced except for cases with more than one clonal product. In such cases, homoduplexes were excised from the polyacrylamide gel, eluted, and directly sequenced.[29] Sequencing was performed either on the ABI377 fluorescent sequencer (Applied Biosystems), using the dye terminator cycle sequencing kit and AmpliTaqFS DNA polymerase (Applied Biosystems), as previously described[30] or on an ABI 3100 Genetic Analyzer. Assignment of Vγ, Jγ, Vδ, Dδ, and Jδ gene segments and reading frames of the involved *TCRG* and *TCRD* gene rearrangements was done using the IMGT database.[31]

RESULTS

Clinical and laboratory features of TCRγδ⁺ T-LGL leukemia

Table 1 summarizes the most relevant clinical and hematological findings at diagnosis of the 44 TCRγδ⁺ T-LGL leukemia patients enrolled in this study. The median age was 63 years (range 34-88) and there was no male or female predominance. Twenty-seven patients (61%) were symptomatic at presentation. T-LGL leukemia cases frequently (48%) presented with neutropenia. Also some T-LGL leukemias presented with multiple cytopenias, including anemia in eleven (23%), thrombocytopenia in four (9%), and pancytopenia in one (2%). Fatigue and B symptoms (fever, night sweats, weight loss) were present in five patients (11%) and recurrent infections occurred in 5 cases as well (11%). Splenomegaly and hepatomegaly were found in 18% and 7% of cases, respectively.

An associated disease was found in 23 cases (52%) (Table 1). In our series, 15 patients (34%) had a co-existent autoimmune disorder. The most common autoimmune manifestation was rheumatoid arthritis (RA), which was diagnosed in seven patients (16%). Co-existence of a second hematological malignancy was demonstrated in six patients (14%).

Examination of PB smears showed an increased number of T-LGLs with abundant cytoplasm containing azurophilic granules (Figure 1A) in 25 out of 44 cases (57%), similar to normal TCRγδ⁺ T-cells (Figure 1B). In the remaining 19 cases, we found evidence for the presence of a discrete LGL expansion with low LGL counts (Table 1). Cytomorphological BM examination demonstrated LGL infiltration in 29 out of 36 cases (81%). Histopathological analysis of BM biopsies in HE sections demonstrated lymphocytic infiltration in eight evaluable patients.

Table 1. Characteristics, clinical presentation and immunophenotype of 44 patients diagnosed with TCRγδ$^+$ T-LGL leukemia.

Case no.	Sample	Age, sex	Immunophenotype	Main clinical presentations[1]	Associated disease	LGL x10^9/L	Therapy
1	93-042	37,F	CD3/5/8	Asymptomatic	Sarcoidosis	0.4	None
2	98-046	73,F	CD3/8/16$^-$/56/57	Neutropenia/anemia/ B symptoms	-	1.3	RBC transfusion
3	99-104	78,F	CD3/8/16/56/57	Asymptomatic	RA	3.5	Corticosteroids
4	92-028	58,F	CD3/16$^-$/56$^-$/57	Neutropenia/anemia/ B symptoms	AIHA	4.3	CHOP
5	94-056	78,F	CD3/8/16$^-$/56$^-$/57	Neutropenia B symptoms	RA	3.9	COP
6	94-076	37,F	CD3/8/16/56$^-$/57	Neutropenia/anemia	-	5.5	Cyclophosphamide
7	95-106	68,F	CD3/8$^-$/16/56/57	Neutropenia	RA	9.0	Unknown
8	98-078	53,M	CD3/8/16/56/57	Neutropenia	RA	0.5	Fludarabine
9	99-025	76,F	CD3/8/16$^-$/56$^-$/57	Thrombocytopenia	-	9.0	Corticosteroids, CSA, cladribine
10	99-275	75,M	CD3/8/16$^-$/56$^-$/57	Neutropenia	-	8.5	Unknown
11	00-029	71,F	CD3/8$^-$/16$^-$/56$^-$/57	Asymptomatic	RA	1.7	None
12	00-091	53, F	CD3/8/16$^-$/56/57	Asymptomatic	Thyroid carcinoma	1.8	None
13	91-010	46,M	CD3/8$^-$/16/56$^-$/57	Neutropenia	Psoriasis	2.5	None
14	02-066	81,M	CD3/8$^-$/16$^-$/56$^-$/57	Asymptomatic	Interstitial lung disease	1.4	Corticosteroids
15	02-123	59,M	CD3/8$^-$/16$^-$/56$^-$57	Asymptomatic	MGUS	0.6	None
16	03-017	77,M	CD3/8$^-$/16/56$^-$/57	Anemia	-	19.1	COP
17	04-172	69,M	CD3/8/16$^-$/56$^-$/57	Pancytopenia	-	0.5	Corticosteroids/ azathioprine
18	04-216	34,M	CD3/8/16$^-$/56$^-$/57	Thrombocytopenia/ anemia	PRCA	3.9	Fludarabine
19	04-209	40,F	CD3/8$^-$/16/56/57	Asymptomatic	-	0.4	None
20	05-168	85,F	CD3/8$^-$/16/56$^-$/57	Neutropenia/anemia	AITD	0.5	None
21	05-181	87,M	CD3/8$^-$/16$^-$/56$^-$/57$^-$	Thrombocytopenia	-	0.2	None
22	05-251	52,M	CD3/8$^-$/16/56/57	Neutropenia/ thrombocytopenia / B symptoms	RA	1.3	MTX
23	05-259	84,M	CD3/8/16/56$^-$/57	Anemia	PRCA	4.6	RBC transfusion, EPO, MTX, CSA, alemtuzumab
24	00-5736	72,F	CD3/8/16$^-$/56$^-$/57	Neutropenia/anemia	RA	2.0	None
25	00-5723	88,M	CD3/8$^-$/16$^-$/56$^-$/57	Neutropenia/anemia	PRCA	3.6	RBC transfusion
26	00-8703	64,F	CD3/8$^-$/16$^-$/56$^-$/57	Neutropenia B symptoms	-	8.3	Unknown

Table 1. Characteristics, clinical presentation and immunophenotype of 44 patients diagnosed with TCRγδ⁺ T-LGL leukemia (continued).

Case no.	Sample	Age, sex	Immunophenotype	Main clinical presentations[1]	Associated disease	LGL x10⁹/L	Therapy
27	00-9586	78,M	CD3/8⁻/16⁻/56⁻/57	Neutropenia	-	1.4	G-CSF
28	00-10012	79,F	CD3/8/16⁻/56/57	Neutropenia	-	0.9	None
29	00-10868	66,M	CD3/8/16/56/57⁻	Asymptomatic	-	8.3	None
30	00-11804	67,F	CD3/8/16⁻/56/57	Asymptomatic	B-CLL	1.7	None
31	00-12429	49,M	CD3/8/16/56⁻/57	Neutropenia/anemia/ B symptoms	AIHA	8.7	CSA, pentostatin, ATG
32	00-14296	85,F	CD3/8/16⁻/56⁻/57⁻	Asymptomatic	SMZL	3.0	Corticosteroids
33	00-15621	44,F	CD3/8⁻/16/56/57	Neutropenia	-	5.0	Corticosteroids
34	00-15881	50,M	CD3/8/16⁻/56/57	Neutropenia	-	0.3	None
35	03-1242	77,M	CD3/8/16/56/57	Asymptomatic	-	2.2	None
36	03-2757	40,F	CD3/8⁻/16/56/57⁻	Asymptomatic	-	2.0	None
37	03-4647	65,M	CD3/8/56/57	Asymptomatic	-	2.8	None
38	03-5313	55,M	CD3/8/16/57⁻	Neutropenia/anemia	AML	1.3	Unknown
39	04-2112	35,M	CD3/8/16/56/57	Asymptomatic	-	1.0	None
40	04-2234	49,M	CD3/8/16⁻/56/57	Neutropenia	Pre B-ALL	0.9	Unknown
41	04-2617	46,M	CD3/8/16/56/57	Asymptomatic	-	5.7	None
42	00-7882	56,M	CD3/8/56⁻/57⁻	Neutropenia	-	6.0	None
43	00-4598	62,M	CD3/8/16/56/57	Asymptomatic	TCRαβ⁺ T-LGL/ MGUS	5.7	None
44	04-4168	73,F	CD3/8/16/56/57	Asymptomatic	-	2.5	None

[1] Neutropenia was defined as absolute neutrophil count (ANC) less than 1.5 x10⁹ neutrophils/L
Anemia was defined as hemoglobin level <10 g/dL
Thrombocytopenia was defined as platelet count <150 x 10⁹/L
AIHA, autoimmune hemolytic anemia; AITD, autoimmune thyroiditis; AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; B-ALL, B-cell acute lymphoblastic leukemia; B-CLL, B-cell chronic lymphocytic leukemia; CSA, cyclosporine A; MGUS, monoclonal gammopathy of undetermined significance; MTX, methotrexate; PRCA, pure red cell aplasia; RA, rheumatoid arthritis; RBC, red blood cell; SMZL, splenic marginal zone lymphoma

All patients tested (n=22) were seronegative for hepatitis B surface antigen (HBs Ag) and anti-hepatitis C antibody (HCV Ab). We did not detect EBV or CMV DNA sequences in PB MNC DNA in the 30 patients tested.

Therapy and follow-up of TCRγδ⁺ T-LGL leukemia

Table 1 shows the different therapeutical strategies in the individual patients. In 21 cases the clinical course was indolent and no therapy was required, while another 18 patients were treated with one or more different approaches. The therapeutic strategey was largely aimed at improving cytopenias and included granulocyte-colony stimulating factor, erythropoietin, red blood cell transfusions, variable immunosuppressive agents, purine analogs, alemtuzumab,

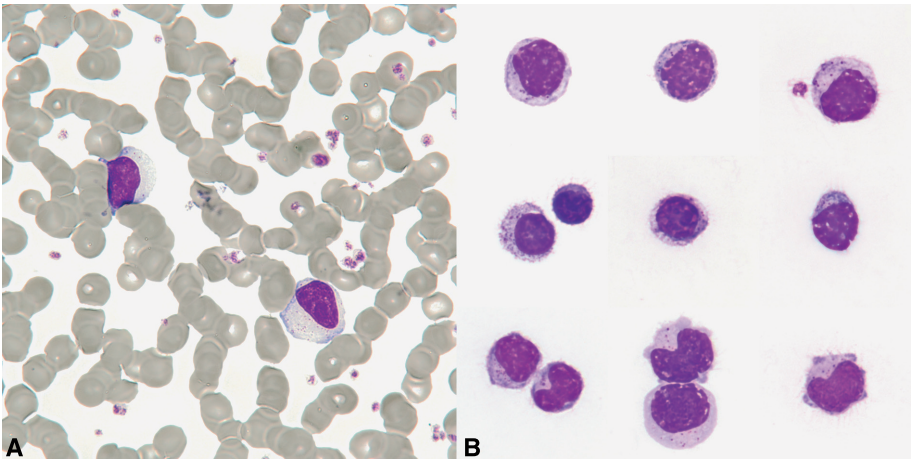

Figure 1. Morphology of TCRγδ$^+$ T-cells.
A. Large granular lymphocytes in PB of a patient with TCRγδ$^+$ T-LGL leukemia. Cells are typically large, with abundant cytoplasm containing azurophilic granules. **B**. Sorted CD3$^+$/TCRγδ$^+$ T-cells of a healthy individual. Most cells (~70%) are large granular lymphocytes. Original magnifications x 63.

and polychemotherapy with alkylating agents. From the remaining five patients no therapeutical data were available.

We observed no disease-related deaths in 18 patients followed for at least three years, which is consistent with an indolent clinical course. From the remaining patients no long-term clinical follow-up data were available.

Immunophenotypical characteristics of TCRγδ$^+$ T-LGL leukemia

In all cases, the LGLs co-expressed the TCRγδ and CD3 molecules on the cell surface in the absence of TCRαβ expression. These cells predominantly showed a CD4$^-$/CD8$^+$ immunophenotype (66% of cases), whereas the remaining cases were CD4$^-$/CD8$^-$ (34%). Variable expression was seen for CD16 (45%), CD56 (45%), while CD57 was expressed in 37 out of 43 patients (86%) (Table 1). Coexpression of CD56 and CD57 was seen in 43% of patients, while 41% of cases showed the CD16/CD57 immunophenotype. The vast majority of leukemic LGLs expressed CD2 (100%), CD5 (86%), CD7 (93%), and CD11c (83%). Heterogeneous expression was seen for HLA-DR (64%) and CD11b (58%) among the cases tested.

Immunophenotypical and molecular Vγ/Vδ analysis of TCRγδ$^+$ T-LGL leukemia

The TCR repertoire was studied using specific anti-TCR Vγ/Vδ domain MoAbs in 38 out of 44 patients, with a dominant TCR Vγ/Vδ combination being expressed in each case. Monoclonal TCR gene rearrangements could be found in all 44 patient samples, as detected by both PCR based HD and GS analysis. Detailed information concerning the TCR Vγ/Vδ expression and configuration of *TCRG* and *TCRD* genes of each TCRγδ$^+$ T-LGL leukemia is summarized in Table 2. Results of sequence analysis of *TCRG* and *TCRD* gene rearrangements and Vγ/Vδ protein expression was concordant in 33 cases. In case no. 24 and 44 clonal out-

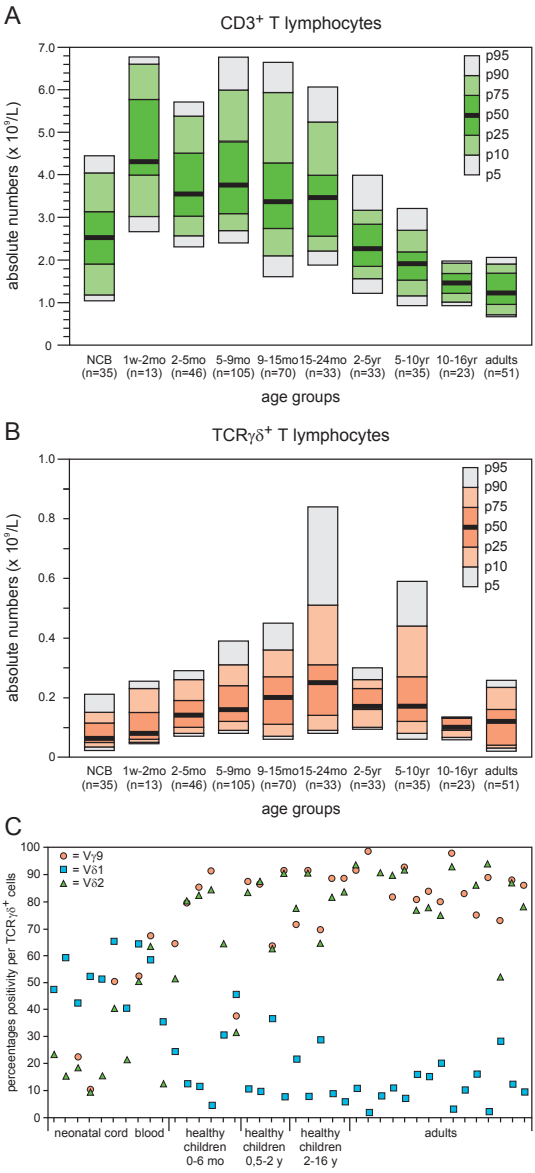

Figure 2. Development of normal TCRγδ⁺ T lymphocytes.
A. Absolute number of CD3⁺ T lymphocytes in neonatal cord blood, blood from healthy children (eight different age groups) and blood from healthy adults.[24] **B**. Absolute number of TCRγδ⁺ T lymphocytes in neonatal cord blood, blood from healthy children (eight different age groups) and blood from healthy adults. **C**. Vγ and Vδ usage by TCRγδ⁺ T lymphocytes in neonatal cord blood, blood from healthy children (three different age groups) and blood from healthy adults. The expression of Vγ9 (Ti-γA), Vδ1 (δTCS1), and Vδ2 (BB3) was assessed within the TCRγδ⁺ T-cell population, defined as CD3⁺/TCRαβ⁻.

of-frame *TCRG* gene rearrangements were found and no DNA was left for further *TCRG* and *TCRD* gene rearrangement analysis. In the remaining nine cases, no or incomplete flowcytometric analysis of Vγ/Vδ chains was performed.

Combined molecular and immunophenotypical data demonstrated the Vγ9/Vδ2 phenotype to be present in 21 out of 44 cases (48%), whereas fifteen cases (34%) were positive for Vδ1 in combination with Vγ other than Vγ9. In four patients (9%) a Vγ9/Vδ1 immunogenotype was detected. The four remaining cases demonstrated expression of Vγ9/Vδ3, Vγ2/Vδ3, Vγ5/Vδ2, and Vγ3/Vδ2. The predominant expression of Vγ9 (n=26; 59%), Vδ2 (n=23; 52%) and Vδ1 (n=19; 43%) in TCRγδ$^+$ T-LGL leukemias reflects the Vγ/Vδ usage of normal TCRγδ$^+$ T-lymphocytes in PB from healthy adult individuals.

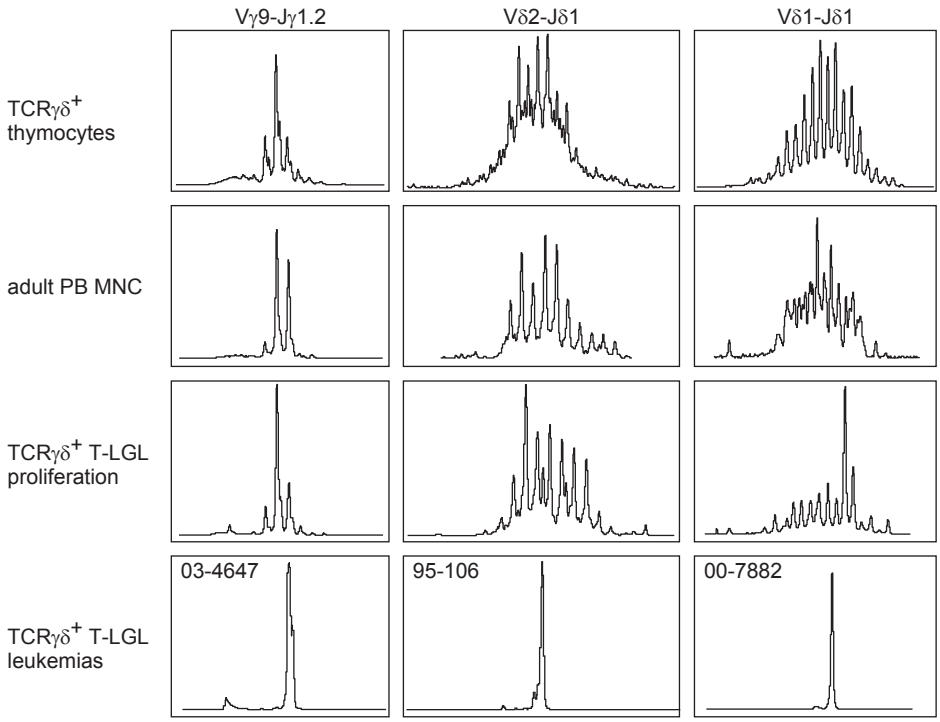

Figure 3. PCR based GeneScan analysis of Vγ9-Jγ1.2, Vδ2-Jδ1, and Vδ1-Jδ1 gene rearrangements in different cell types.

Polyclonal gene rearrangements can be identified in DNA isolated from TCRγδ$^+$ thymocytes and adult MNC. Oligoclonal gene rearrangements are found in a TCRγδ$^+$ T-LGL proliferation and clear monoclonal gene rearrangements can be identified in PB DNA from patients with TCRγδ$^+$ T-LGL leukemia (patients 03-4647, 95-106, and 00-7882).

Table 2. Vγ/Vδ protein expression and configuration of *TCRG* and *TCRD* genes in cell samples from 44 TCRγδ⁺ T-LGL leukemia patients.

Case no.	Sample no.	Sample	Vγ/Vδ expression	HD/GS PCR analysis and sequencing				Invariant T in first CDR3 codon (AA) in Vδ2-Jδ1
				TCRG rearranged alleles		*TCRD* rearranged alleles		
1	93-042	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	canonical (+)	Vδ2-Jδ1	clonal (+)	+ (Val)
2	98-046	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Ile)
3	99-104	BM	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Leu)
4	92-028	PB/BM	Vγ5/Vδ1	Vγ5-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
5	94-056	PB	Vγ5/Vδ1	Vγ5-Jγ1.1	clonal (+)	Vδ1-Jδ2	clonal (+)	NA
6	94-076	PB/BM	Vγ3/Vδ1	Vγ3-Jγ2.1	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
7	95-106	PB	Vγ5/Vδ2	Vγ5-Jγ2.3	clonal (+)	Vδ2-Jδ1	clonal (+)	–
8	98-078	PB/BM	Vγ8/Vδ1	Vγ8-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
9	99-025	PB	Vγ5/Vδ1	Vγ5-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
10	99-275	PB	Vγ5/Vδ1	Vγ5-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
11	00-029	PB	Vγ3/Vδ1	Vγ3-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
12	00-091	PB/BM	Vγ9/Vδ2	Vγ9-Jγ1.2	canonical (+)	Vδ2-Jδ1	clonal (+)	+ (Ile)
13	91-010	PB	Vγ4/Vδ1	Vγ4-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
14	02-066	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Leu)
15	02-123	PB/BM	Vγ8/Vδ1	Vγ8-Jγ1.3/2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
16	03-017	PB	Vγ8/Vδ1	Vγ8-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
17	04-172	PB/BM	NT/Vδ3	Vγ2-Jγ2.1	clonal (+)	Vδ3-Jδ1	clonal (+)	NA
18	04-216	PB/BM	Vγ4/Vδ1	Vγ4-Jγ2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
19	04-209	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Leu)
20	05-168	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Val)
21	05-181	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	canonical (+)	Vδ2-Jδ3	clonal (+)	NA[b]
22	05-251	PB	NT/Vδ2	Vγ3-Jγ2.3	clonal (+)	Vδ2-Jδ3	clonal (+)	NA[b]
23[a]	05-259	PB	Vγ8/Vδ1	Vγ8-Jγ1.3/2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
24	00-5736	PB	Vγ9/Vδ2	Vγ10-Jγ1.3/2.3	clonal (-)	NT		NT
25	00-5723	PB	Vγ9/NT	Vγ9-Jγ1.1	clonal (+)	Vδ1-Jδ2	clonal (+)	NA
26	00-8703	PB	NT	Vγ9-Jγ2.1 Vγ9-Jγ1.3/2.3	clonal (+) clonal (+)	Vδ1-Jδ1	clonal (+)	NA
27	00-9586	PB/BM	Vγ9/Vδ3	Vγ9-Jγ1.2	clonal (+)	Vδ3-Jδ1	clonal (+)	NA
28	00-10012	BM	NT	Vγ5-Jγ1.3/2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
29	00-10868	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Val)
30	00-11804	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ3	clonal (+)	NA[b]
31	00-12429	PB	NT	Vγ4-Jγ1.3/2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
32	00-14296	PB	NT	Vγ9-Jγ1.2	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
33	00-15621	PB	NT	Vγ2-Jγ1.3/2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA

Table 2. Vγ/Vδ protein expression and configuration of *TCRG* and *TCRD* genes in cell samples from 44 TCRγδ$^+$ T-LGL leukemia patients (continued).

Case no.	Sample no.	Sample	Vγ/Vδ expression	HD/GS PCR analysis and sequencing				Invariant T in first CDR3 codon (AA) in Vδ2-Jδ1
				TCRG rearranged alleles		*TCRD* rearranged alleles		
34	00-15881	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Val)
35	03-1242	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Val)
36	03-2757	PB/BM	Vγ9/Vδ2	Vγ9-Jγ1.2	canonical (+)	Vδ2-Jδ1	clonal (+)	+ (Leu)
37	03-4647	PB/BM	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Leu)
38	03-5313	BM	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Ile)
39	04-2112	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+(Leu)
40	04-2234	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ2	clonal (+)	NA[b]
41	04-2617	PB	Vγ9/Vδ2	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Leu)
42	00-7882	PB/BM	Vγ9/Vδ1	Vγ9-Jγ1.3/2.3	clonal (+)	Vδ1-Jδ1	clonal (+)	NA
43	00-4598	PB	NT	Vγ9-Jγ1.2	clonal (+)	Vδ2-Jδ1	clonal (+)	+ (Leu)
44	04-4168	PB	Vγ9/Vδ2	Vγ4-Jγ1.3/ 2.3	clonal (-)	NT		NT

(+) In-frame gene rearrangement

(-) Out-of-frame gene rearrangement

AA, amino acid; BM, bone marrow; HD, heteroduplex; GS, Genescan; PB, peripheral blood; NA, not applicable; NT, not tested

[a] This case was reported previously[50]

[b] The invariant T nucleotide was not detected in Vδ2-Jδ3 and Vδ2-Jδ2 gene rearrangements

Detailed comparison of normal TCRγδ$^+$ T-lymphocytes and TCRγδ$^+$ T-LGL leukemias

To gain further insight into the pathogenesis of TCRγδ$^+$ T-LGL leukemias we compared normal TCRγδ$^+$ T-cells and TCRγδ$^+$ T-LGL leukemias in more detail. We evaluated the absolute size of the CD3$^+$ and the CD3$^+$/TCRγδ$^+$ lymphocyte subpopulations in NCB and PB from healthy individuals of different age groups (Figure 2). The absolute number of T lymphocytes increases immediately after birth, remains relatively stable until two years of age, and subsequently decreases threefold to adult levels (Figure 2A). A fivefold increase of absolute numbers of TCRγδ$^+$ T lymphocytes occurs during the first 2 years of life, followed by a gradual decrease to adult levels (Figure 2B). In NCB, approximately half of the TCRγδ$^+$ T lymphocytes expressed Vδ1, while 30-40% expressed Vγ9 and/or Vδ2 (Figure 2C). However, during early childhood the distribution of Vγ and Vδ gene usage changes to 10-15% Vδ1, 80-85% Vδ2, and 85-90% Vγ9 in older children and adults (Figure 2c).

GeneScan patterns of the most frequently used *TCRG* (Vγ9-Jγ1.2) and *TCRD* (Vδ2-Jδ1 and Vδ1-Jδ1) gene rearrangements in both immature and mature TCRγδ$^+$ T-cells are illustrated in Figure 3. A polyclonal GS pattern can be found in normal TCRγδ$^+$ thymocytes and MNC. Oligoclonal gene rearrangements are detected in a persistent reactive TCRγδ$^+$ lymphoproliferation, while clear monoclonal peaks are present in PB samples from TCRγδ$^+$T-LGL leukemia patients.

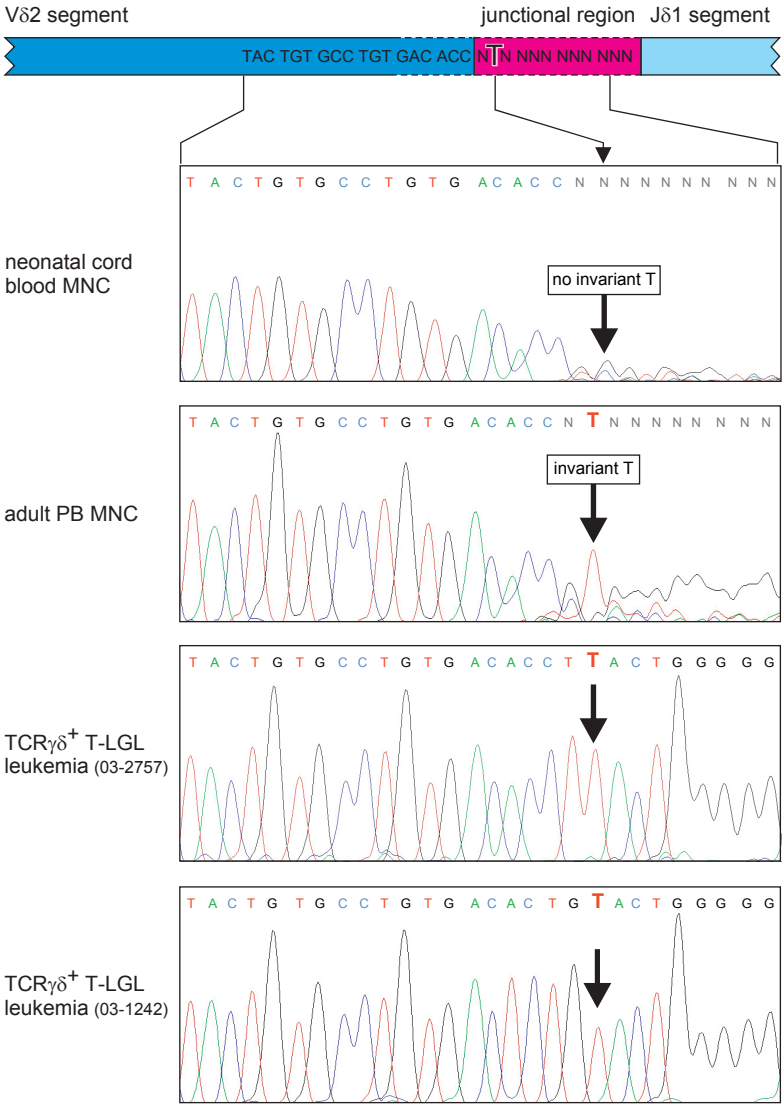

Figure 4. Invariant T nucleotide in the first codon of the Vδ2-Jδ1 junctional region.
The invariant T nucleotide is absent in Vδ2-Jδ1 junctional regions of neonatal cord blood MNC, but is present in polyclonal PB MNC of healthy adults. In all 16 analyzed Vδ2-Jδ1 junctional regions of clonal Vγ9⁺/Vδ2⁺ T-LGL leukemias, the invariant T nucleotide could be detected as well.

Out of 21 TCRγδ$^+$ T-LGL leukemia samples bearing the Vγ9/Vδ2 phenotype, 19 were further sequenced, demonstrating in-frame Vγ9-Jγ1.2 and Vδ2-Jδ1 gene rearrangements in 16 cases. Two cases showed Vδ2-Jδ3 gene rearrangements and one a Vδ2-Jδ2 gene rearrangement. The earlier reported invariant T-nucleotide at the relative second position in the first codon of the Vδ2-Jδ1 junctional regions was present in all 16 cases (100%) (Figure 4). This first codon encoded leucine (50%), valine (32%) or isoleucine (19%) (Table 3). The invariant T nucleotide is a peripheral antigen selection marker that is absent in DNA isolated from thymocytes and cord blood MNC but is present in DNA from normal adult PB MNC (Figure 4).[32] As no similar marker is known for Vδ1 junctions, further evaluation of the Vδ1 phenotype for signs of antigen selection was not possible.

Taken together our data show that TCRγδ$^+$ T-LGL leukemias are quite similar to normal PB TCRγδ$^+$ T-cells in Vγ/Vδ usage and the presence of the Vδ2 selection determinant, and differ in their CD4/CD8 pattern.

DISCUSSION

TCRγδ$^+$ T-LGL leukemias form a rare subgroup of mature T-cell malignancies, representing approximately 5% of CD3$^+$ T-LGL leukemias. We retrieved and evaluated the clinical and laboratory data of 44 cases diagnosed with TCRγδ$^+$ T-LGL leukemia and performed an extensive morphological, flowcytometric and molecular genetic analysis of the leukemic T-cell populations. By comparison with normal TCRγδ$^+$ T-cells, we attempted to gain insight into the molecular pathogenesis of TCRγδ$^+$ T-cell proliferations.

It has been suggested that the clinical and hematological features of TCRγδ$^+$ T-LGL leukemia do not differ from TCRαβ$^+$ T-LGL leukemia,[7,20] but data on large series are lacking. Here we describe the clinical presentation and laboratory parameters of an unprecedentedly large group of newly diagnosed patients with TCRγδ$^+$ T-LGL leukemia. The most prominent clinical manifestations of the disease consisted of cytopenias and to a lesser extent the presence of splenomegaly and frequent association with autoimmune diseases and other neoplasms, as previously described for TCRαβ$^+$ T-LGL leukemia.[3-6,33] Similar to TCRαβ$^+$ cases, most TCRγδ$^+$ T-LGL leukemias have an indolent clinical course and do not require management with cytotoxic drugs. As eradication of the malignant clone does not seem to be essential for disease control, effective immunomodulatory agents such as methotrexate and cyclosporin seem to be preferred over intensive cytotoxic regimens.[34-37] However, results of future international standardized treatment protocols should reveal the optimal therapeutic strategy for both TCRαβ$^+$ and TCRγδ$^+$ T-LGL leukemias.

In the majority of T-LGL leukemia cases, the leukemic LGLs express CD3, CD8 and TCRγδ. With regard to TCRγδ$^+$ cases both CD4$^-$/CD8$^+$ and CD4$^-$/CD8$^-$ phenotypes have been described.[19,21,38] In contrast to the CD4$^-$/CD8$^-$ immunophenotype of most normal TCRγδ$^+$ T-lymphocytes in PB as well as TCRγδ$^+$ thymocytes, TCRγδ$^+$ T-ALL, and TCRγδ$^+$ HSTCL, the TCRγδ$^+$ T-LGL leukemias in our study were predominantly CD8$^+$ (n=29; 66%) (Table 3), demonstrating the activated memory immunophenotype[3,39] of most leukemic TCRγδ$^+$ T-LGL proliferations. The most common immunophenotype of T-LGL leukemias (CD3$^+$/CD8$^+$/CD57$^+$)[6] was found in 25 out of 44 patients (57%) and CD57 expression was

Table 3. Vγ/Vδ expression usage and CD4/CD8 immunophenotype in TCRγδ⁺ T-LGL leukemias, PB T-lymphocytes, thymocytes, T-ALL, and HSTCL.

	TCRγδ⁺ thymocytes [49,51,52]	TCRγδ⁺ T-ALL[23]	TCRγδ⁺ PB T-lymphocytes in healthy adults[51] (this study)	TCRγδ⁺ T-LGL leukemias (this study)	TCRγδ⁺ HSTCL [53,54] (Sandberg, unpublished results)
Immunophenotype					
CD4⁻/CD8⁺	15-20%	3% (1/30)	10-15%	66% (29/44)	6% (2/32)
CD4⁻/CD8⁻	80-85%	23% (7/30)	80-85%	34% (15/44)	78% (25/32)
Vγ/Vδ usage					
Vγ9	20-25%	31% (8/26)	85-90%	57% (25/44)[a]	0% (0/4)
Vδ1	60-75%	80%(24/30)	10-20%	43% (19/44)	81% (22/27)
Vδ2	20-25%	7% (2/30)	80-85%	52% (23/44)[a]	11% (3/27)
Invariant T in Vδ2-Jδ1 junctional regions	NT	NT	70-75%	100% (16/16)	NT

HSTCL, hepatosplenic T-cell lymphoma; NT, not tested; T-ALL, T-cell acute lympoblastic leukemia
[a] The combined Vγ9⁺/Vδ2⁺ immunophenotype was detected in 48% (21/44) of patients.

observed in nearly 90% of cases. The CD3⁺/CD56⁺ phenotype with variable expression of CD16 and CD57 was found in 45% of cases in our series. In contrast to the report by Gentile et al.,[40] the clinical presentation and disease course of our patients showing a CD56⁺ phenotype was similar to that of the other patients, none of them suffering from severe illness.

It has been hypothesized that antigen driven T-cell expansions are the first phase in the pathogenesis of T-LGL leukemia.[41-43] Additional (genetic) events might then transform the abnormal polyclonal or oligoclonal expansion into a monoclonal proliferation.[44-46] The antigen nature and specificity of T-LGL clones remains unknown. Some cases of T-LGL leukemia occur concomitantly with viral infections, such as EBV, CMV, HIV, HTLV, and HCV.[4,47] However, we found no evidence for a potential relationship between active viral infections and TCRγδ⁺ T-LGL leukemia.

Owing to the extensive combinatorial repertoire of TCRαβ molecules, it is complicated to define antigen-selection via analysis of TCR molecules in TCRαβ⁺ T-LGL leukemias. This is in contrast to TCRγδ molecules, which show a restricted combinatorial repertoire and thereby allow better insight in antigen-selection of TCRγδ⁺ T-cells.

In thymus and NCB, TCRγδ⁺ T-cells mainly express Vδ1 chains.[48,49] However, during the expansion of TCRγδ⁺ T-cells during the first 2 years of life, the Vδ1 usage shifts to Vγ9/Vδ2 usage (Figure 2). Importantly, we have previously demonstrated that this thymus-independent peripheral expansion of TCRγδ⁺ T-cells is accompanied by selection for an invariant T nucleotide in the first codon of the Vδ2-Jδ1 junctional region.[32] Consequently, the expanded Vγ9⁺/Vδ2⁺ T-lymphocytes are antigen-selected. Apparently these T lymphocytes have proliferated in response to a ubiquitous antigen or micro-organism, which has not yet been identified, for example, a (super)antigen. The antigen-selected and activated state of the

T-LGLs is further supported by the expression of the activated memory T cell marker CD8 in the majority of cases. Nevertheless, at this point we cannot completely exclude the possibility that LGL proliferations result from secondary oncogenic events in randomly selected TCRγδ$^+$ T-cells. This needs to be further addressed in future studies.

In our series of adult TCRγδ$^+$ T-LGL leukemia patients, nearly half showed the Vγ9/Vδ2 phenotype. The Vγ9-Jγ1.2-Cγ1 and Vδ2-Jδ1-Cδ immunogenotype could be confirmed in 16 cases. In all 16 patients the antigen-selected invariant T nucleotide could be identified in the first codon of the Vδ2-Jδ1 junctional region. Expression of Vδ1 was found in another 19 cases. The preferential expression of the antigen-selected Vγ9/Vδ2 phenotype and the frequent usage of the Vδ1 subunit seem to reflect the spectrum of normal TCRγδ$^+$ T-cells in PB of healthy adult individuals (Figure 2C and Table 3). This contrasts with the Vγ/Vδ usage of normal TCRγδ$^+$ thymocytes, TCRγδ$^+$ T-ALL, and TCRγδ$^+$ HSTCL where Vδ1 usage predominates (Table 3). Although no clear clinical features such as autoimmune phenomena seem to correlate with certain Vγ/Vδ usage, it is interesting to note that all three T-LGL leukemia patients with pure red cell aplasia (PRCA) express the Vδ1 chain. This phenotype has been described previously in a case of TCRγδ$^+$ T-LGL leukemia and PRCA.[19]

In summary, our results show that monoclonal TCRγδ$^+$ T-LGL proliferations display similar clinical features as the more frequent TCRαβ$^+$/CD8$^+$ LGL leukemias. Leukemic TCRγδ$^+$ cells frequently display a typical cytotoxic T-cell phenotype and show a restricted usage of Vγ and Vδ families with quite a similar pattern as their normal counterparts. In addition, TCRγδ$^+$ T-LGL leukemias clearly differ in Vγ/Vδ usage and CD4/CD8 expression from TCRγδ$^+$ thymocytes and other well-recognized TCRγδ$^+$ T-cell malignancies. A strong selection determinant present in normal mature antigen-selected peripheral TCRγδ$^+$ T lymphocytes was detected in almost half of the clonal TCRγδ$^+$ T-LGL leukemias, suggesting a role for antigen stimulation in the pathogenesis of TCRγδ$^+$ T-LGL leukemia.

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Chapter 4.7

Clonal T- and NK-cell large granular lymphocyte proliferations in a single patient established by array-based comparative genomic hybridization analysis

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Clonal large granular lymphocyte (LGL) proliferations can arise from their normal T- or natural killer (NK)-cell counterparts. Both T-LGL and chronic NK-LGL leukemia/lymphocytosis are well-recognized disease entities in the World Health Organization (WHO) classification of hematopoietic malignancies. LGL leukemia is a rare and heterogeneous disorder and most cases have an indolent clinical course. Its main clinical manifestations are related to chronic neutropenia and/or anemia.[1]

Clonality assessment studies are essential to discriminate true LGL leukemia from other reactive proliferations. In T-LGL proliferations, clonality can easily be detected by PCR analysis of T-cell receptor (TCR) genes. In NK-LGL proliferations, clonality detection is difficult owing to the absence of specific clonality markers. Currently it is therefore unclear whether indolent NK-LGL leukemia, also known as chronic NK-LGL lymphocytosis or indolent NK-cell lymphoproliferative disorder, represents a reactive polyclonal process or a chronic phase of leukemia.[1] It was recently demonstrated that the NK cells in patients with molecularly (HUMARA X inactivation assay in female patients) proven monoclonal chronic NK-LGL lymphocytosis express an aberrant $CD56^{-/+\ dim}/CD11b^{-/+\ dim}$ immunophenotype.[2] This immunophenotype is clearly different from the phenotype found in aggressive NK-cell leukemia and normal or reactive/activated NK-cell proliferations, which generally show high CD56 expression and a higher reactivity for CD11b. The aberrant immunophenotype of these NK LGL cells could reflect underlying clonal genetic abnormalities.

In this study we report on the clinical, hematological, immunophenotypical, serological, and molecular features of a rare case diagnosed with T-LGL leukemia and a coexistent chronic NK-LGL leukemia.

A 73-year-old Caucasian man was admitted in to the outpatient clinic in May 2005 with anemia and atypical blood lymphocytes. He had a 3-month history of fatigue, febrile episodes and painful ulcers on his tongue. He had suffered from severe mouth infections and a perianal abscess. Inspection of the oral cavity revealed an aphthous ulcer on the tongue with edema. Further physical examination was unremarkable, without evidence of lymphadenopathy, hepatosplenomegaly, or skin lesions. Ultrasonography of the abdomen demonstrated a moderate splenomegaly.

Hematological examination revealed a white blood cell count of 8.3 x 10^9/L, with 92% lymphocytes, 5% neutrophils, 1% eosinophils, and 2% monocytes. The hemoglobin (Hb) level was 9 g/dL and the platelet count 338 x 10^9/L. Lactate dehydrogenase levels (645 U/L) were elevated, whereas liver enzymes (aspartate amino transferase/alanine amino transferase) were within normal range. Extensive serologic workout for hepatitis A, B, and C virus, and HTLV-I and II showed no evidence for acute viral infection or reactivation, whereas examination of anti-cytomegalovirus (CMV), anti-Epstein-Barr virus (EBV) and anti-Parvo B19 virus antibodies demonstrated positive immunoglobulin G seroreactivity. Blood smears revealed 70% LGL cells with typical large nuclei and abundant cytoplasm containing fine azurophilic granules. Bone marrow (BM) aspiration showed hypercellular marrow with 56% LGLs. The BM biopsy was normocellular with lymphoid infiltration and slightly de-organized hematopoiesis. Immunohistochemical staining of the BM lymphoid infiltrate on tissue sections demonstrated diffuse and focal infiltration of $CD2^+$ lymphocytes. Only part of the infiltrate showed expression of CD3, CD5, and CD8. There was heterogeneous expression of CD57. The lymphoid cells were negative for CD4, CD30, and CD56. Staining for B-cell

markers CD10, CD20, and CD79A only showed scattered B-lymphocytes and plasma cells.

Immunophenotyping was performed on peripheral blood (PB) and revealed large populations of T cells and NK cells, respectively 53% and 33% of total leukocytes (Figure 1). The $CD3^+$ T-cell population was CD2, CD5 (dim), CD7, CD8, TCRαβ, CD57, HLA-DR, CD45RA, CD45RO, CD27, CD62L (dim), CD94, and CD197 (dim) positive. Cells were negative for CD16, CD56, CD11b, CD28, and perforin. TCR-Vβ flowcytometric analysis demonstrated the presence of 90% $CD3^+$ cells reactive with a TCR-Vβ5.1 antibody (Figure 1). The $CD3^-$ NK-cell population was positive for CD2, CD7, CD16, CD57, HLA-DR (dim), CD28, CD62L, CD94, CD197, and perforin, but CD4, CD5, CD8, CD27, CD11b, CD56, CD45RA, and CD45RO negative (Figure 1). PCR based GeneScan analysis of TCR genes demonstrated clonal TCR beta (*TCRB*) gene rearrangements in a PB sample. A normal XY karyotype was found by cytogenetic analysis of PB mononuclear cells (MNCs). A diagnosis of T-LGL leukemia and chronic NK-LGL lymphocytosis was made according to WHO classification criteria.

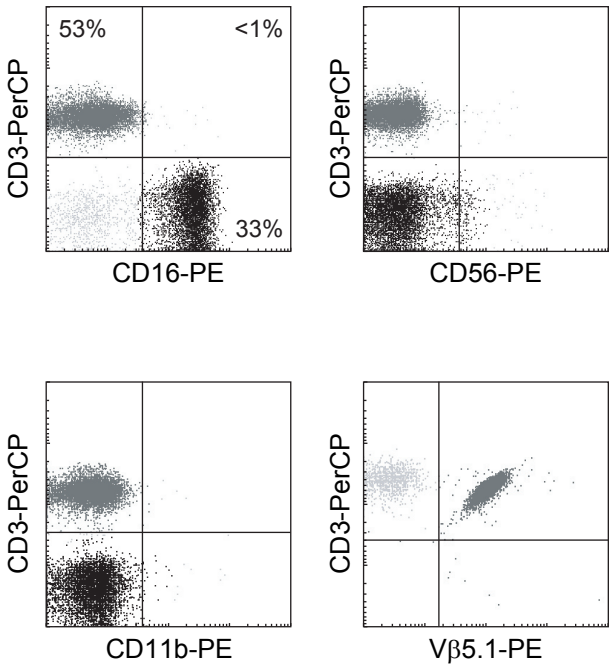

Figure 1. Immunophenotype of blood MNCs.
Immunophenotype of the T-LGL cells (shown in gray) present in the PB at diagnosis. Nearly all T-cells (90%) are positive for Vβ5.1. Note the aberrant $CD56^-/CD11b^-$ immunophenotype of the NK-LGL population (shown in black).

One year after initial presentation the aberrant T- and NK-LGL population could still be detected in PB by immunophenotyping. The patient was intended to be treated with low dose oral methotrexate (10 mg/m^2) once per week; owing to intolerance he received 10 mg weekly. During therapy he suffered from pneumococcal pneumonia with bacteremia from which the patient completely recovered. Finally, after 11 months of treatment with methotrexate, the blood count is improving with Hb level increasing to 12.5 g/dL and absolute neutrophil count of 2.0 x 10^9/L).

It has been hypothesized that persistent, perhaps viral, antigenic stimulation underlies the pathogenesis of T-LGL and chronic NK-LGL leukemia.[3-5] As both diseases have developed in our patient, we speculate that the same antigen is involved in the pathogenesis of both T- and chronic NK-LGL leukemia. Whether the T-LGL or NK-LGL clones in our case were Parvo B19 virus or CMV-triggered remains uncertain. On top of that, a secondary molecular event is most probably required to establish the full malignant phenotype of the chronic antigen stimulated LGL population.

To gain further insight into the etiopathogenesis of the LGL proliferations in our patient, both T-LGL and NK-LGL subsets were purified. To this end, total PB MNCs from the patient were labelled with fluorochrome-conjugated monoclonal antibodies (CD45-PerCP (2D1), CD3-APC (SK7), CD16.56-PE (B73.1 and My31) BD Biosciences), and VB5.1-FITC (LC4) (Immunotech, Marseille, France) for high speed cell sorting on a FACSDiVa cell sorter (BD Biosciences). Purity of the sorted populations was determined on the *FACS* Calibur (BD Biosciences) and shown to be >95% for both populations. Clonal *TCRB* and TCR gamma (*TCRG*) gene rearrangements could only be demonstrated in the sorted T-LGL population, and not in the NK-LGL subset. Southern blot analysis of both T-LGL and NK-LGL fractions showed the absence of clonal EBV genome. Array based comparative genomic hybridisation (CGH) analysis demonstrated only few subtle genetic alterations in the purified T- and NK-LGL fractions. Each population showed a different single clonal chromosomal abnormality, suggesting that the two cell proliferations did not have a common clonal origin. In the sorted T-LGL fraction a single copy loss of bacterial artificial chromosome (BAC) clone RP11-121A8 and a partial loss of the BAC clone RP11-273L18 (containing the *TCRGV9* and *TCRGV5* genes) located at 7p14.1 was observed. The clonal genetic alteration detected in the purified T-LGL subset most probably reflects physiological rearrangement of the *TCRG* locus. The purified NK-LGL fraction showed a gain of BAC clone RP11-440P5 (containing the *BCL11A* gene) located at 2p16.1 (Figure 2A and B). The proto-oncogene *BCL11A* is involved in multiple lymphoid malignancies and BCL11A expression has been described in a case of NK-cell lymphoma.[6] Its possible role in the pathogenesis of chronic NK-LGL leukemia is not known. As the gain of 2p16.1 was not reflected by increased *BCL11A* messenger RNA expression (data not shown), we conclude that the gene is unlikely to be involved in malignant transformation in our case. Therefore, the pathologically relevant secondary genetic events are probably more subtle and remain to be identified. In line with this, in indolent LGL leukemia, cells most frequently have a normal karyotype and well-defined clonal chromosomal aberrations have been reported in few cases only.

T-LGL leukemia in the presence of an NK-cell proliferation has been reported before, but the clonal nature of the NK-cell population could not be established.[7] The here presented case represents the first description of the coexistence of T-LGL and chronic NK-LGL

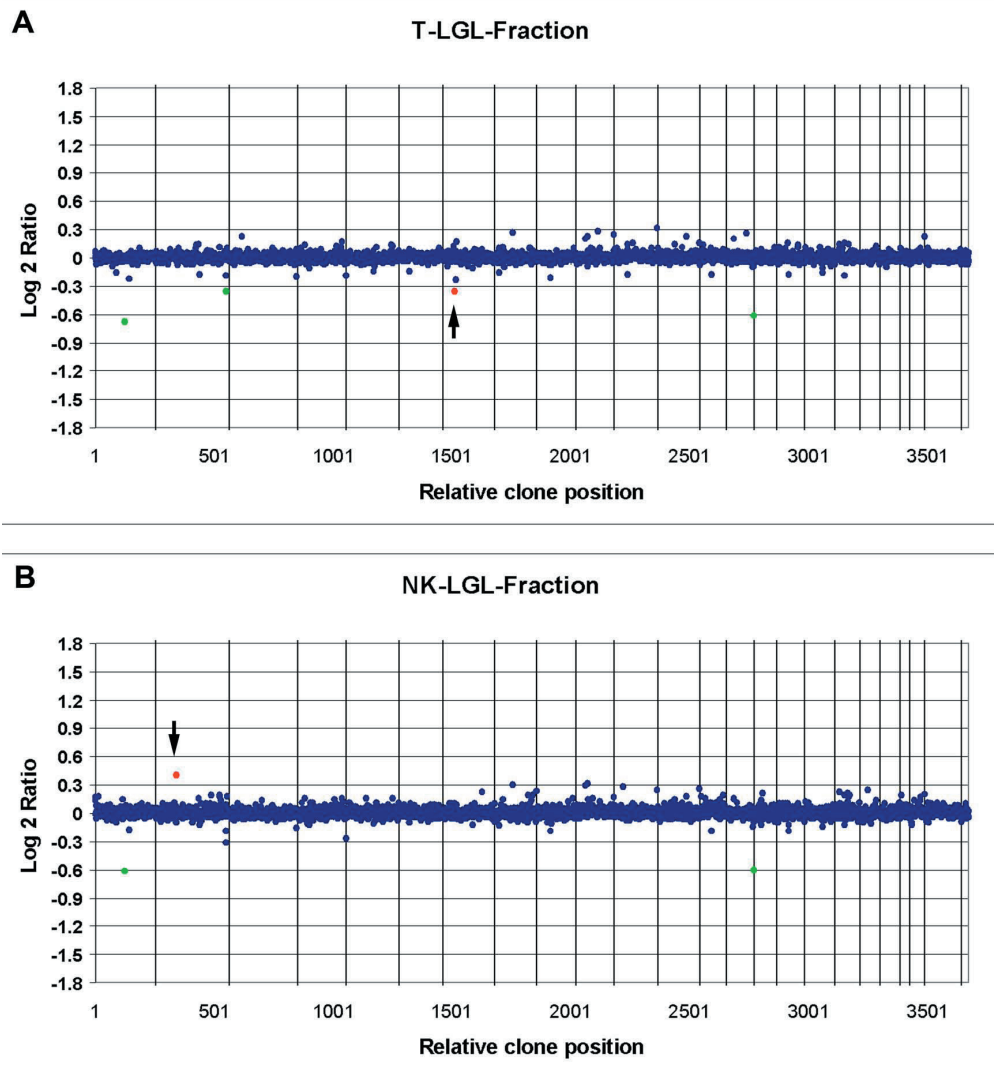

Figure 2. Array-based comparative genomic hybridization (CGH) analysis of purified leukemic T-LGL (A) and NK-LGL (B) populations.

Black dots represent clones within threshold (±0.33 log2), whereas light grey (known polymorphic region) and dark grey (altered region indicated by arrowhead) dots represent clones outside the threshold. Fabrication and validation of the array, hybridization methods, and analytic procedures have been described elsewhere in detail,[8] whereas the clone content is available in the "Cytoview" window of the Sanger Institute mapping database site, Ensembl (http://www.ensembl.org/).

populations with "clonal" immunophenotype, which was confirmed by molecular studies. Remarkably, two different clonal chromosomal aberrations were present in the T-LGL and NK-LGL proliferations. Furthermore, this case illustrates the relatively indolent clinical course of T-LGL and chronic NK-LGL leukemia and confirms that genetic instability in LGL leukemias is less common and more subtle than in other mature T-/NK-cell malignancies.

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Chapter 5

GENERAL DISCUSSION

V(D)J recombination processes underlie the generation of TCR diversity and antigen specificity during human T-cell differentiation. The studies presented in this thesis particularly focused on TCR recombination analysis in normal T cells and malignant T-cell proliferations.

The novel BIOMED-2 based PCR primers and protocols were evaluated for their additional value in diagnosing lymphoproliferations with a special focus on cutaneous lymphoproliferations. In addition, they were used for characterization of the TCR gene rearrangement status and TCR clonotypes of both normal and malignant T cells.

Analysis of gene segment usage in V(D)J rearrangements and TCR clonotypes can also give insight into the etiopathogenesis of lymphoid malignancies. To this end, complementarity determining region 3 (CDR3) sequences of *TCRD, TCRG, TCRB* and *TCRA* gene rearrangements in mature T-cell malignancies were analyzed in this thesis. These studies mainly focused on T-LGL leukemias and the possible role of antigenic stimulation in the pathogenesis of this disease entity. Additionally, secondary molecular genetic events, seemingly essential for transformation of 'benign' LGL clones into leukemic clones, were studied.

IMPLEMENTATION AND VALUE OF THE BIOMED-2 MULTIPLEX PCR PRIMERS AND PROTOCOLS IN DIAGNOSING SUSPECTED LYMPHOPROLIFERATIONS

The newly developed BIOMED-2 PCR based primers and protocols for analysis of TCR gene rearrangements are highly sensitive in detecting T-cell clonality. This strategy has now been widely validated and demonstrated to enable detection of clonal T-cell populations in a large background of polyclonal (reactive) T cells.[1-5] As conventional methods such as histomorphological analysis, immunohistochemistry and immunophenotyping can not discriminate between reactive and malignant lymphoproliferations in 5 to 15% of cases, these highly sensitive PCR based assays could definitely be of additional diagnostic value. In Chapters 2 and 3 of this thesis we demonstrate this additional value of the BIOMED-2 assays in diagnosing and staging suspected lymphoproliferations. Taking cutaneous lymphoproliferations as a paradigm for lymphoproliferations in general, we discuss the following specific applications:

1) early diagnosis in early stage lymphoma
2) dissemination of lymphomas including evaluation of the clonal relationship between multiple lymphoid malignancies in one patient
3) extra-ordinary presentation of an underlying leukemia/lymphoma
4) lineage determination.

TCR gene rearrangement analysis in early stage primary cutaneous T-cell lymphoma (CTCL)

Cutaneous T-cell proliferations are often difficult to diagnose, which is partly explained by their rarity. Discrimination between reactive (benign) cutaneous lymphoproliferations and true malignant T-cell leukemias/lymphomas developing in the skin can occasionally not be made on clinical and histomorphological features only.

A multidisciplinary diagnostic approach is required, in which the molecular clonality results are integrated.

Mycosis fungoides (MF) is the most common primary CTCL and is characterized by a long natural history. Many patients show non-specific scaly eruptions years before a diagnostic histology develops. Although the majority of patients has stable disease for decades, a minority develops tumor-stage MF and may finally evolve to systemic wide-spread disease, with an aggressive clinical course.[6] MF patients might logically benefit from early and correct diagnosis and thereby better treatment, improving their prognosis. In Chapter 3 we describe the results of T-cell clonality analysis in a large group of patients with cutaneous lymphoproliferations. We could demonstrate clonal T-cell populations in the skin in up to 65% of patients with early stage MF and overall in ~70% of CTCL patients. When early stage MF was not considered, TCR clonality was detected in 78% of all cases. These results are in line with other sensitive PCR based studies.[7] It reflects the sensitivity and relevance of these molecular diagnostic tools in clinical practice, especially in early stage MF. However, our studies also show that clonal TCR gene rearrangements can be detected in a minority of patients with reactive skin lesions. It should be emphasized that monoclonality does not necessarily imply malignancy and molecular results should therefore always be correlated with the clinical, histopathologic, cytomorphologic and phenotypic data of the individual patient. Nevertheless, since clonal dermatitis is known to progress to overt CTCL in 25% of cases,[8] clinical and histologic follow-up of the skin lesions of such patients is strongly recommended.

Extracutaneous dissemination and evaluation of clonal relationship

Primary CTCL may become progressive and thereby involve extracutaneous tissues such as lymph node (LN), peripheral blood (PB), liver, spleen etc, but rarely bone marrow (BM). Evaluation of the clonal relationship between two lymphoproliferations in a single patient is another important application of molecular TCR gene rearrangement studies. We demonstrate in Chapter 3 that the multiplex PCR based heteroduplex and GeneScan analyses are rapid and highly sensitive methods to demonstrate clonal relationship between multiple skin lesions and between skin and extracutaneous sites in patients with suspected disseminated CTCL.

Extracutaneous dissemination has important implications for treatment and prognosis. In our patients with early stage MF no clonal TCR rearrangements were identified in samples from extracutaneous sites (Chapter 3.2). However, identical clones were detected in LN, BM, and PB samples in 67% of patients with late stage MF. Especially in LN specimen and uncommon extracutaneous localizations, molecular clonality studies are of additional diagnostic value. Consistent with our findings, Assaf *et al*.[9] demonstrated that T-cell clonality analysis is an important tool in differentiating benign dermatopathic lymphadenitis from early CTCL involvement. However, molecular clonality studies point out that dominant T-cell clones detected in the PB of patients with CTCL using routine PCR techniques are rarely tumoral and are more often related to age.[10] The fact that the finding of clonality does not imply malignancy is even further stressed by the profound loss of TCR repertoire complexity in the PB of CTCL patients.[11] Therefore, we emphasize that in CTCL patients with suspected extracutaneous dissemination, samples should always be analyzed in parallel with the skin biopsy sample in order to reliably demonstrate clonal identity. Sézary syndrome is characterized by erythroderma and malignant T-cell populations in skin, PB and LN.[12] The demonstration of identical clonal

T cells in PB, skin and LN is an important diagnostic criterion allowing discrimination from benign forms of erythroderma.[13] As malignant and benign clonal T-cell expansions can occur in the blood and skin of patients with Sézary syndrome,[14] parallel analysis of the samples is recommended.

Skin relapses frequently occur in primary CTCL. Especially primary cutaneous $CD30^+$ lymphoproliferative disorders frequently relapse in the skin.[12] The clinical presentation is highly variable and skin relapses might occur many years after the initial diagnosis was made. As is demonstrated in Chapter 3.4, molecular clonality analysis can easily demonstrate identical clonal TCR gene rearrangements between multiple skin lesions. These studies therefore discriminate true relapses from benign skin lesions or secondary cutaneous malignancies.

T-cell malignancies with secondary skin involvement

Cutaneous localizations of T-cell lymphomas and T-cell leukemias occur most frequently in adult T-cell leukemia/lymphoma (ATLL; >50% of cases), anaplastic large cell lymphoma (ALCL; ~20% of cases), and T-PLL.[15] It is rarely observed in patients with immature T-cell malignancy (T-ALL).[16-18] Skin involvement may occur at presentation concomitantly with BM/LN infiltration, may be the first sign of relapse or may even precede the underlying T-cell lymphoma/leukemia by several months. Accurate diagnostics of these skin lesions is thus critical for treatment and prognosis. These skin lesions do not show common or typical dermatological characteristics. The malignant T cells are often difficult to detect in routinely stained sections.[19] A multidisciplinary diagnostic approach is required, which is clearly illustrated in a case presenting with a skin localization of an underlying T-cell leukemia (Chapter 4.2). It underlines the close collaboration, which should exist between clinicians, pathologists, cytomorphologists, immunologists, and molecular biologists in order to reach the correct diagnosis. In case of clear clonal TCR gene rearrangements in a skin lesion, an underlying malignant T-cell disorder should be considered.

Lineage determination

It is clear from various reports that Ig and TCR gene rearrangements are not necessarily restricted to B- and T-cell lineages. Crosslineage TCR gene rearrangements occur relatively frequently in immature B-cell malignancies, particularly in precursor B-ALL.[20] Also acute myeloid leukemias and mature B-cell malignancies might contain TCR gene rearrangements.[21] NK-cells in principle do not rearrange their TCR genes and therefore TCR gene rearrangement analysis could be helpful in discriminating malignant NK-cell disorders from T-cell malignancies. Although extremely rare, it is clinically very important to discriminate cutaneous T-cell lymphomas from the clinically more aggressive NK-cell malignancies localized in the skin.[22] Since cutaneous T-cell lymphomas might also express NK-cell markers, such as CD56, immunohistochemical studies could be extended with molecular gene rearrangement analysis to support lineage determination.

T CELL DEVELOPMENT AND TCR REPERTOIRE FORMATION

TCR repertoire bias established during human thymocyte development

In this thesis we also studied TCR diversity (repertoire) in human PB T lymphocytes by BIOMED-2 based PCR GeneScan analysis. The TCR repertoire is generated by TCR gene rearrangements during thymic development. This process gives rise to a unique type of functional TCRαβ or TCRγδ complex expressed by each T cell. Selection for in-frame TCR gene rearrangements results in thymocytes expressing functional TCR molecules on their membrane. The naïve T-cell repertoire in the thymus is eventually defined by positive and negative selection, ensuring that only lymphocytes with TCRs that recognize foreign peptides bound to self-MHC can reach the periphery. Although mathematical estimates of the TCR diversity are in the range of 10^{12}-10^{15} TCRα and TCRβ pairings, the necessity of self-tolerance and positive and negative selection reduce the actual repertoire of TCRαβ pairs to 2×10^{7}.[23] Our studies in Chapter 2.1 show the timing and extent of selection for in-frame TCR gene rearrangements during T-cell development.

The *TCRB* and *TCRA* loci in TCRγδ^{+} thymocytes largely remain in germline configuration and complete in-frame *TCRA* and *TCRB* rearrangements are hardly detected. On the basis of our data and the work of others, we conclude that the potential to develop into the TCRγδ lineage is the highest in the early (DN3/4 and ISP CD4^{+}) stages of thymocyte development. The TCRγδ potential rapidly decreases from the ISP CD4^{+} stage onwards as the TCRαβ potential increases. This is reflected by the observation that selection for complete in-frame *TCRB* gene rearrangements already occurs from the CD34^{+} CD38^{+} CD1a^{+} to ISP CD4^{+} stage, which is consistent with our previous findings and the demonstration of intracellular TCRβ protein.[24, 25] It is shown that *TCRA* gene rearrangements are already present in the ISP CD4^{+} stage, which strengthens our previous notion that *TCRA* recombination is initiated at the transition from CD34^{+} CD38^{+} CD1a^{+} to ISP CD4^{+} stage.[24] We are the first to show that selection for in-frame *TCRA* gene rearrangements in man takes place from the DP CD3^{-} stage into the DP CD3^{+} stage.

Interestingly, the *TCRD* and *TCRG* loci are highly rearranged in both TCRαβ^{+} and TCRγδ^{+} thymocytes, but they are out-of-frame in TCRαβ^{+} thymocytes and peripheral TCRαβ^{+} lymphocytes.

Peripheral selection of human T lymphocytes is mediated via antigenic stimulation

A first TCR repertoire bias is generated during thymic selection, but the TCR repertoire is further modulated in the periphery, during immune response. In response to antigen, specific T cells that show preferential usage of particular TCR gene segments are selected. It has been suggested that the peripheral TCR repertoire bias is the result of continued antigenic stimulation, which promotes the selective outgrowth of T-cell clonotypes that have optimal TCR structural characteristics. As an example, we show in Chapter 4.6 that the expansion of TCRγδ^{+} T-lymphocytes after birth mainly concerns T cells with invariant expression of Vγ9-Jγ1.2 and Vδ2-Jδ1 receptors, which are apparently selected for their specificity for a (super)antigen.[26] In line with this finding, we demonstrate in Chapter 2.1 that massive positive selection for in-frame Vδ2-Jδ1 and Vγ9-Jγ1.2 gene rearrangements occurs in the periphery. In-frame Vδ1-Jδ1 and VγI-Jγ1.3/2.3 gene rearrangements can be detected at high frequency

in thymic subsets, whereas Vδ2-Jδ1 and Vγ9-Jγ1.2 gene rearrangements are virtually absent. The selection and expansion of Vγ9/Vδ2$^+$ T-cells likely account for the observed decline in Vδ1-Jδ1 and VγI-Jγ1.3/2.3 gene rearrangements observed in mature TCRγδ$^+$ T cells. Although the (super)antigenic stimulus leading to extensive proliferation of Vγ9/Vδ2$^+$ T cells is currently unknown, these cells have been shown to proliferate in response to non-peptidic phosphoantigens broadly expressed by mycobacteria and other pathogens.[27, 28]

Immunosenescence: through antigenic stimulation in association with HLA genotype

Under physiological circumstances T cells are programmed to undergo rapid clonal expansion upon recognition of antigens in the context of HLA molecules through the antigen-specific binding sites of TCR molecules, leading to activation of signaling cascades.

The occurrence of persistently elevated numbers of (oligo)clonal T cells in healthy elderly adult individuals is a general feature of immunosenescence, i.e. ageing of the immune system.[29] As a consequence, an accumulation of oligoclonally expanded memory lymphocytes occurs in the elderly individual. The diversity of the antigen-recognition repertoire is therefore markedly decreased, from approximately 10^8 in young adults to 10^6 in the elderly.[30] This dramatic age-related diversity reduction is most prominent in the CD8$^+$ T-cell compartment and less frequently detected in CD4$^+$ T cells.[31, 32] However, both fractions show the potential of an impaired immune response, contributing to the partial immunodeficiency of the elderly.

The accumulation of memory T cells with age may reflect an adaptive immune response to the decline of production of naïve lymphocytes through homeostatic expansion, as well as the cumulative effect of chronic viral infections. Chronic stimulation of T cells occurs during persistent viral infections, but also in several other pathological conditions such as autoimmune diseases, hematologic disorders and following bone marrow transplantation. As 50-90% of human populations are infected with EBV and CMV, it seems likely that both CD4$^+$ and CD8$^+$ expansions are at least partly driven by these chronically infective viruses. Clonal T-cell populations found in the elderly have been demonstrated to strongly proliferate on coculture with CMV.[33, 34] The CD8$^+$ TCR bias can thus result from the preferential selection of T cells with high affinity TCRs for viruses that chronically persist in the host, especially CMV but possibly also EBV.[35-38]

It is well established that certain HLA alleles constitute predisposition to an exaggerated immune response. Interestingly, recent studies show a clear association between specific TCR Vβ clones of anti-hCMV CD4$^+$ and CD8$^+$ T cells and particular HLA haplotypes. (Rodriguez-Caballero *et al.* submitted) Expansion of common TCR Vβ clonotypes in CD4$^+$ and CD8$^+$ PB T cells from hCMV seropositive individuals underlines the potential relevance of a group of CMV epitopes which are specifically recognized by a particular TCR in the context of the optimal HLA haplotype.[39, 40]

Healthy elderly adults with such clonal T-cell expansions are usually diagnosed with T-cell clonopathy of undetermined significance (TCUS). Although this is a benign condition, the clinical presentation is highly variable. Patients with TCUS may be completely asymptomatic or may present with several hematological abnormalities.[41, 42] Patients with clonal CD3$^+$/CD57$^+$ T-cell expansions who have progressive disease with multiple cytopenias, recurrent infections and associated autoimmune features are diagnosed with T-cell large granular lymphocyte (T-LGL) leukemia, often requiring therapy.[43]

TCR REPERTOIRE OF MALIGNANT T-CELL CLONES

PCR based heteroduplex and GeneScan analyses are highly sensitive in characterizing the biased profiles (polyclonal/oligoclonal/monoclonal) of TCR gene rearrangements. This method will identify both the small clones of normal memory cells as well as the very large expanded clones as they can be detected in T-cell leukemia. In contrast, TCR V chain antibody typing will only identify the very large clones. After amplification of *TCRB, TCRA, TCRG*, and *TCRD* gene rearrangements, sequence analysis of PCR products results in precise identification of TCR clonotypes.

Sequencing of TCR gene rearrangements in patients with mature T-cell leukemias might contribute to the understanding of the etiology/pathogenesis of these disorders. The complementarity-determining regions (CDRs) of the TCR-V chains are involved in antigen binding and recognition. Especially the CDR3 region of the TCR molecules is a unique antigen-specific region. As the structure of the CDR3 region results from V(D)J recombination and junctional diversification, analysis of clonotypes might provide insight into the role of antigenic stimulation in the etiopathogenesis of mature T-cell leukemias.

In this thesis, we have identified and characterized leukemia-specific T-cell clones in a large cohort of patients diagnosed with T-PLL, Sézary syndrome, and T-LGL leukemia to search for disease related TCR clonotypes, which might reflect an antigenic stimulation signature.

TCR clonotypes in T-PLL

Translocations that juxtapose the *TCRA/D* locus with the oncogenes *TCL1* and *TCL1b* at 14q32.1 are found in ~90% of T-PLL cases. Activation of *TCL1* is thought to play a crucial role in the pathogenesis. Until now, antigenic stimulation has never been established to play a role in T-PLL development. We studied in-frame *TCRB* gene rearrangements in a large series T-PLL patients (n=27) and found random usage of Vβ gene segments without common Vβ CDR3 motifs. The Vβ-Jβ2/Vβ-Jβ1 ratio was 2.7 illustrating a bias towards Jβ2 usage which is in keeping with the mature phenotype of T-PLL cells.

TCR clonotypes in Sézary syndrome

There is some evidence for the involvement of superantigen in driving T-cell expansion in cutaneous T-cell lymphoma.[44,45] Especially in early stage MF it is assumed that malignant T cells proliferate in response to chronic stimulation by antigen present in the skin. Recently, Morgan *et al*.[46] analyzed Vβ CDR3 regions of patients diagnosed with early and late stage MF and Sézary syndrome. A strong association between Jβ1 gene segment usage and disease severity was found, which was supposed to reflect an abnormal TCR repertoire. Although no consensus amino acid CDR3 motif could be found, a role for superantigen involvement in early-stage disease was suggested. The Vβ-Jβ2/Vβ-Jβ1 ratio in our series of patients with advanced stage MF and Sézary syndrome (n=34) was 1.1 (unpublished results), which however still clearly differs from the over representation of the Jβ2 gene segment in the PB TCR repertoire of healthy adults (Vβ-Jβ2/Vβ-Jβ1 ratio of 2.6). No shared/restricted Vβ gene segment usage and CDR3 motifs could be detected in our series of patients either, indicating that there is no strong evidence for a common antigen to be involved in this disease entity.

TCR clonotypes in T-LGL leukemia

The most striking evidence that antigenic stimulation plays an etiopathogenic role was found in T-LGL leukemia. Much of this evidence was based on the finding of highly homologous TCR clonotypes between different patients.

Nearly identical clonotypes were found at high levels in $CD3^+/TCR\gamma\delta^+$ T-LGL leukemia and $CD3^+/CD4^+/TCR\alpha\beta^+$ T-LGL leukemia cases. The invariant T nucleotide in the first codon of the Vδ2-Jδ1 junctional region and the resulting neutral amino acid could be demonstrated in nearly 50% of $TCR\gamma\delta^+$ T-LGL leukemia cases by *TCRD* sequence analysis as presented in Chapter 4.6. In all patients with the Vγ9-Jγ1.2-Cγ1 and Vδ2-Jδ1-Cδ immunogenotype, the invariant T nucleotide could be identified. On the basis of our findings we therefore conclude that the leukemic LGL proliferation in a high proportion of these cases is antigen-selected. The results described in Chapter 4.4 demonstrate that the LGLs in 42% of patients with $CD4^+$ T-LGL leukemia show membrane expression of the TCR-Vβ13.1 chain. Sequence analysis of *TCRB* gene rearrangements in these patients demonstrated a common Vβ CDR3 amino acid motif which gives strong evidence for a chronic antigen-driven T-cell stimulation in $CD4^+$ T-LGL leukemia.

Consistent with previous studies,[47,48] our study presented in Chapter 4.5 confirmed the absence of identical *TCRB* or *TCRA* CDR3 motifs in patients with $CD3^+/CD8^+/TCR\alpha\beta^+$ T-LGL leukemia. However, this finding might not be too surprising given the extent of diversity of HLA-class I background observed in our patients. Nevertheless, we found a highly similar Vβ24 CDR3 motif of an immunodominant clonotype in one of our patients upon cross-reference against a large series of previously reported clonotypes, suggesting non-random clonal selection.[49] Other, yet to be identified TCR clonotypes might be present as well in the cohort. In addition, the *TCRB* clonotypes of the T-LGL leukemia cases were not encountered in the $CD8^+$ T cell repertoire of healthy adult individuals, although such comparison would probably have to be refined by checking sorted effector T-cell fractions ($CD8^+/CD27^-/CD28^-$) as well.

Antigen stimulation hypothesis and the spectrum of T-LGL proliferations

The spectrum of T-LGL proliferations ranges from activated cytotoxic T cells to T-LGL leukemia. Polyclonal/oligoclonal T-LGL proliferations represent the initial benign end of the spectrum reflecting the normal immune response. In Chapter 4.3 we hypothesize that TCUS originates from a clonal outgrowth of an initially polyclonal response and represents a pre-leukemic state.

The notion that T-LGL leukemia may represent an end stage of an antigen-mediated proliferation is supported by multiple lines of evidence. Firstly, LGL cells both morphologically and immunophenotypically resemble immune activated cells. LGL cells are large lymphocytes with abundant cytoplasm and azurophilic granules. These granules contain a number of proteins that play a role in cytolysis such as perforin and granzyme B, indicating that these cells are antigen-activated memory/effector T cells. Secondly, the classic immunophenotype of the malignant T cell in LGL is $CD3^+/CD27^-/CD28^-/CD45RO^-/CD45RA^+/CD57^+$.[50,51] These cells can either be CD8 positive (>95% of cases) or CD4 positive (<5% of cases). This phenotype is the equivalent of terminally differentiated effector memory T cells.

The immune activated character is even further stressed by constitutive expression of

high levels of Fas/FasL. Interestingly, the majority of CMV-specific $CD4^+$ and $CD8^+$ T cells are also late effector/memory cells with the same immunophenotype as the leukemic LGLs. Thirdly, flow cytometric analysis of $CD3^+/TCR\alpha\beta^+$ T-LGL proliferations shows TCR-Vβ chain-restricted expansions. In addition, they express NK-associated antigens and NK receptors (CD16, CD56, CD57, CD94), suggesting that they represent antigen-driven cytotoxic T cells. In Chapter 4 we demonstrate that heterogeneous expression of CD57, which is a marker of terminal differentiation, is a characteristic feature of all LGL leukemia entities. Finally, monoclonal T-cell expansions are more frequently found in association with pathological conditions characterized by chronic antigen stimulation such as in autoimmune diseases, bone marrow transplantation, solid organ transplantation, and chronic viral infection. In addition, (oligo)clonal LGL-like expansions accumulate with age in normal healthy elderly individuals.

We found strong evidence for antigenic stimulation in the pathogenesis of $CD4^+/TCR\alpha\beta^+$ T-LGL leukemia in which a clear association between the TCR-Vβ repertoire and the HLA genotype was found. TCR-$V\beta13.1^+$ usage was overrepresented and all these cases had a common Vβ CDR3 amino acid motif and had the HLA-DRB1*0701 haplotype. This motif was found at very low frequency in $CD4^+$ T-cell fractions of healthy adult donors. We believe on the basis of our preliminary data that CMV does play a role in the development of $CD4^+/TCR\alpha\beta^+$ T-LGL leukemia (unpublished observations). We also conclude that chronic antigenic stimulation plays a role in $TCR\gamma\delta^+$ T-LGL leukemias. This was especially true for the $V\gamma9/V\delta2^+$ cases. However, we have no indications that these $TCR\gamma\delta^+$ expansions were CMV or EBV driven, which is in line with a previous study that demonstrated that in vivo CMV-driven selection of $TCR\gamma\delta^+$ T lymphocytes only concerns the Vδ1 and Vδ3 subsets.[52]

It is currently unclear whether monoclonal T-cell populations detected in individuals suffering from relatively indolent disease are purely coincidental (for instance age-related) in nature or might play a pathogenic and prognostic role in particular cases. Most patients with clonal T-LGL expansions in PB are asymptomatic elderly individuals, and T-cell clones are mostly detected following routine blood analysis. Therefore, clonal proliferation of T-LGLs is very likely to be an under-diagnosed disorder which might however be responsible for subtle unexplained hematological abnormalities.[42]

T-LGL clones in the pathogenesis of other diseases than T-LGL leukemia

The exact role of malignant T-cell clones in the pathogenesis and clinical manifestation of T-LGL leukemia is still unknown. The most characteristic clinical features of T-LGL leukemia are lineage-specfic cytopenias, which might be explained by highly specific recognition and killing of individual hematopoietic cell lineages.[49] Our studies in Chapter 4 as well as previous reports in literature show an association with several bone marrow failure conditions, such as pure red cell aplasia (PRCA), myelodysplastic syndrome (MDS), paroxysmal nocturnal hemoglobinuria (PNH), and aplastic anemia (AA).[53-55] In addition, clonal $CD8^+/CD57^+/CD28^-$ effector T-cell expansions with LGL-like morphology have been observed in patients diagnosed with AA, MDS, and PNH (Figure 1).[56-60] The clinical overlap between bone marrow failure syndromes, clonal T-LGL proliferations and T-LGL leukemia might have consequences for therapeutical strategies. As the key pathogenic mechanism in all these disease entities seems to be T-cell mediated suppression of hematopoietic stem cells,

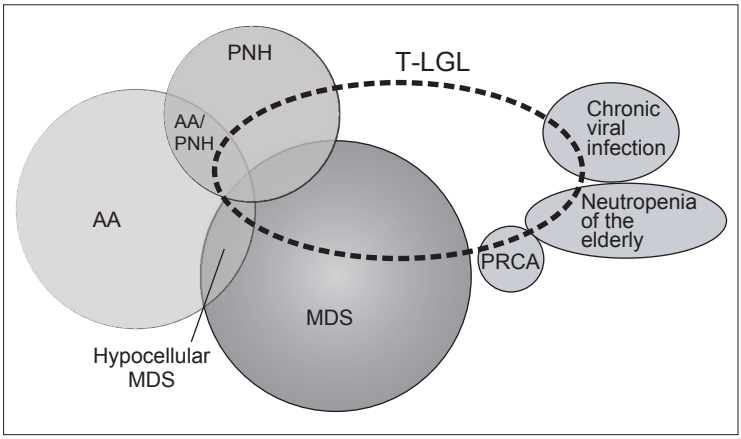

Figure 1. Venn diagram of the clinical and pathophysiologic relationships among the bone marrow failure syndromes and T-LGL proliferations.
Overlapping circles indicate clinical overlap and shared underlying pathogenic mechanisms. AA, aplastic anemia; MDS, myelodysplastic syndrome; PNH, paroxysmal nocturnal hemoglobinuria; PRCA, pure red cell aplasia; T-LGL, (Clonal) T-cell large granular lymphocyte proliferations.(Adapted from Young *et al*.[60]).

immunosuppressive therapy could be considered. It has been hypothesized that the clinical presentation spectrum is determined by the TCR specificity of the antigen-driven T-cell clone. The molecular analysis of the TCR utilization pattern and the detection of immunodominant clonotypes represents a novel approach in the study of T-cell mediated hematologic diseases. Interestingly, the (oligo)clonal $CD8^+/CD57^+$ T-LGL expansions found in the healthy elderly have been shown to suppress neutrophil development *in vitro*.[61] Sofar, we have not been able to demonstrate similar Vβ CDR3 motifs in our group of LGL leukemia patients presenting with bone marrow failure syndromes. When cross referenced against a large group of clonotypes with similar clinical presentations no identical motifs could be demonstrated either. The lack of such identical TCR specificities might be explained by the extensive diversity of the HLA background of patients.

GENETIC ABBERATIONS INVOLVED IN T-LGL LEUKEMIA

The development of LGL leukemia is likely to be a multistep process; an antigenic stimulated T-LGL proliferation might develop into TCUS and T-LGL leukemia. Whether or not a oligoclonally expanded T-cell population transforms into a leukemia is probably dependent on immunogenetic factors, the antigenic pressure, the HLA genotype and the occurrence of secondary oncogenic events.[48,62] Secondary (genetic) events seem to be essential for transformation of TCUS into clinically malignant leukemia. This is however still disputable, as the experimental data are scarce.

Because of the enormous potential to proliferate for a T cell upon antigenic stimulation, the size of the expanding T-cell population is tightly regulated.[63] Once antigen-activated T cells have gone through several cell cycles, they become highly susceptible to apoptosis,

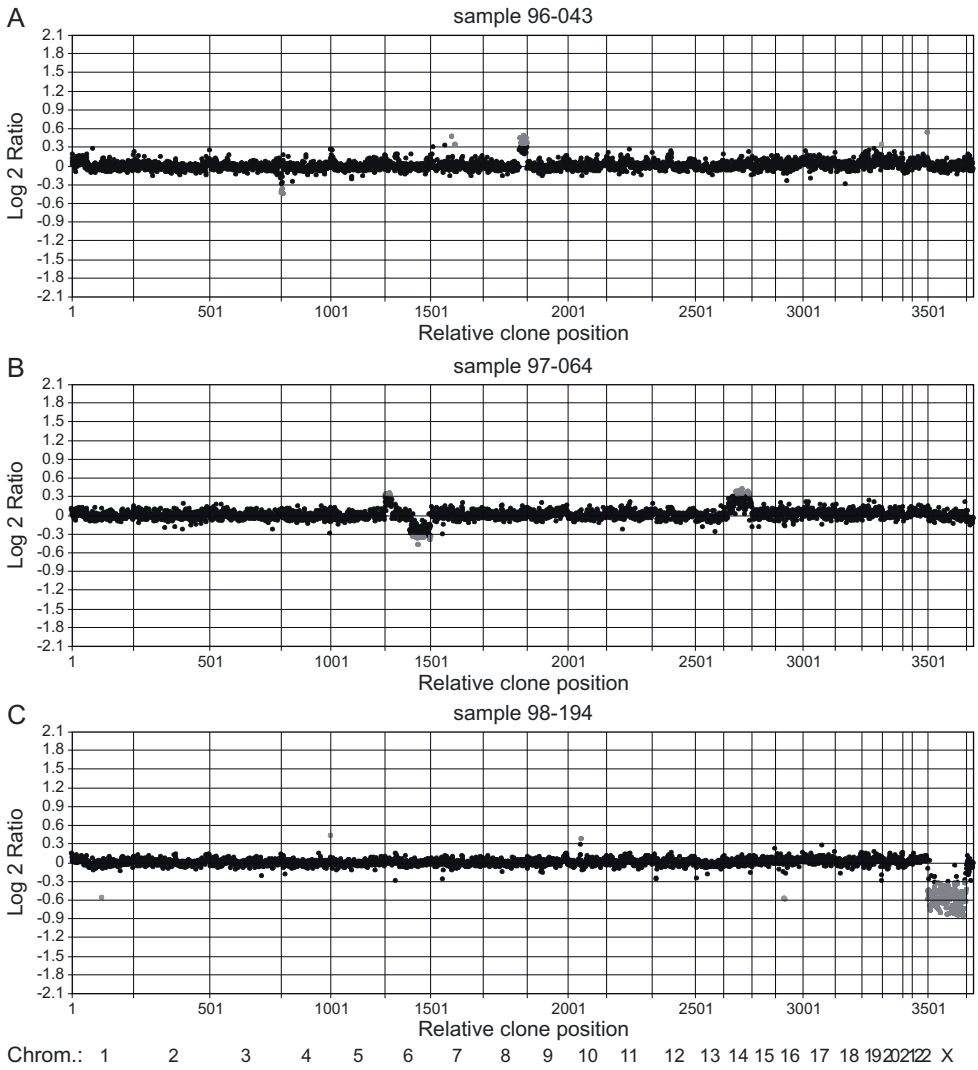

Figure 2. Array-based comparative genomic hybridization (CGH) analysis of leukemic CD8$^+$/TCR$\alpha\beta^+$ T-LGL proliferations.

Black represent clones within threshold (±0.33 log2), whereas light grey (known polymorphic region) and dark grey (altered region) dots represent clones outside the threshold. **A**. Case no. 96-043 demonstrates loss at 4p16 and gain at 8q24. This kind of gain loss pattern could be derived from an imbalanced translocation between chromosome 4 and 8. **B**. Case no. 97-064 had chromosomal gain at 6p22 and 14q13 with single copy loss at 6q16. **C**. Case no. 98-194, showing a complete loss of the X chromosome and chromosomal gain at 10q11. The clone content is available in the "Cytoview" window of the Sanger Institute mapping database site, Ensembl (http://www.ensembl.org/).

thereby eliminating excessive T cell numbers.[64] The cell surface receptor Fas (CD95) and Fas ligand are key regulators of mature T-cell apoptosis and play a central role in the process of activation-induced cell death. A central feature of LGL leukemia is a dysregulated Fas/Fas ligand apoptotic pathway leading to an antigen-driven immune response that fails to adequately terminate.[65,66] On top of that, secondary molecular events are most probably required to establish the full malignant phenotype.

TCR gene-associated translocations are frequently found in T-cell malignancies, especially in T-ALL and T-PLL. Two cases of T-LGL leukemia with genetic aberrations possibly involving the *TCRG* and *TCRA/D* loci have been described till so far.[67] We found no evidence for TCR related oncogenic events in 12 patients diagnosed with $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia, as assessed by split-signal FISH for TCR loci (Sandberg and Langerak, unpublished observations).

Microsatellite instability (MSI) has been reported to occur in a significant proportion of patients diagnosed with various hematological malignancies. Using MSI PCR analysis we show that a MSI stable phenotype is present in $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia (n=28).

Based on our results as well as the literature we conclude that there is no clear association with specific recurrent mutations and chromosomal abnormalities in T-LGL leukemia. Therefore we tried to identify relevant oncogenic events with novel molecular techniques such as array CGH analysis. We could identify clear genetic aberrations in three out of 13 cases diagnosed with $CD8^+/TCR\alpha\beta^+$ T-LGL leukemia. One had a loss of the complete X chromosome, while in the other two cases partial chromosome gains and losses were seen (Figure 2). The gain and loss patterns might indicate the presence of imbalanced translocations in those cases. These translocations might uncover the otherwise pathogenic balanced translocation. So far, no recurrent genetic aberrations could be found; nevertheless we demonstrate some aberrations that are possibly relevant for pathogenesis (Figure 2). Follow up experiments on large series of patients are useful to check for recurrent involvement of particular genomic regions by using for example split signal FISH probes. Furthermore gene expression profiling of sorted cell populations from patients with consecutive clinical stages of TCUS and malignant T-LGL leukemia might reveal genes involved in disease progression.

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ABBREVIATIONS

Ag	antigen
ALCL	anaplastic large cell lymphoma
ALL	acute lymphoblastic leukemia
BM	bone marrow
C	constant gene segment
CBCL	cutaneous B-cell lymphoma
CD	cluster of differentiation
CDR	complementarity-determining region
CGH	comparative genomic hybridization
CMV	cytomegalovirus
CTCL	cutaneous T-cell lymphoma
D	diversity gene segment
DCLWG	Dutch cutaneous lymphoma working group
DN	double negative
DP	double positive
EBV	Epstein-Barr virus
FISH	fluorescent *in situ* hybridization
HDA	heteroduplex analysis
HLA	human leukocyte antigen
Ig	immunoglobulin
IGH	immunoglobulin heavy chain gene
IGK	immunoglobulin kappa light chain gene
IGL	immunoglobulin lambda light chain gene
J	joining gene segment
LGL	large granular lymphocyte
LN	lymph node
LyP	lymphomatoid papulosis
MF	mycosis fungoides
MNC	mononuclear cell
MRD	minimal residual disease
NHL	non-Hodgkin's lymphoma
NK cell	natural killer cell
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PCR	polymerase chain reaction
RAG	recombination-activating gene
RSS	recombination signal sequence
SB	Southern blotting
SP	single positive
T-ALL	T-cell acute lymphoblastic leukemia
TCR	T-cell receptor
TCRA	T-cell receptor alpha gene

TCRB	T-cell receptor beta gene
TCRD	T-cell receptor delta gene
TCRG	T-cell receptor alpha gene
TCUS	T-cell clonopathy of undetermined significance
TdT	terminal deoxynucleotidyl transferase
T-LGL	T-cell large granular lymphocyte (leukemia)
T-PLL	T-cell prolymphocytic leukemia
UCB	umbilical cord blood
V	variable gene segment
WHO	World health Organization

SUMMARY

During T-cell development, thymocytes undergo a sequence of immunophenotypic and immunogenotypic changes resulting in the formation of mature T cells with receptors that recognize antigens with high specificity: the T-cell receptor (TCR). Recombination processes underlie the generation of the antigen-specificity of TCR molecules. The central theme of this thesis constitutes the basic aspects of V(D)J recombination of TCR genes and the diagnostic applications of the TCR in mature T-cell malignancies.

The BIOMED-2 multiplex polymerase chain reaction (PCR) primers and protocols have been developed for the detection of immunoglobulin (Ig) and TCR gene rearrangements in human lymphoid cells. Using these assays we studied TCR diversity (repertoire) formation during T-cell development in human T lymphocytes. In addition, TCR gene rearrangement analysis was performed in malignant lymphoproliferations to determine its clinical diagnostic relevance, as well as to gain insight into the pathogenesis of mature T-cell malignancies.

Analysis of the TCR alpha (*TCRA*), TCR beta (*TCRB*), TCR gamma (*TCRG*), and TCR delta (*TCRD*) loci shows the timing and extent of selection for in-frame TCR gene rearrangements during T-cell development (Chapter 2.1). Selection for complete in-frame *TCRB* gene rearrangements appears to occur from the $CD34^+$ $CD38^+$ $CD1a^+$ to immature single positive (ISP) $CD4^+$ stage. By our newly developed mutiplex PCR approach for analysis of the *TCRA* locus, we now show for the first time that selection for in-frame *TCRA* gene rearrangements in man takes place from the DP $CD3^-$ stage into the DP $CD3^+$ stage. Although the potential to develop into the TCRγδ lineage is the highest in the early (DN3/4 and ISP $CD4^+$) stages of thymocyte development, we conclude that expansion and proliferation of $TCR\gamma\delta^+$ T cells predominantly occur after birth in the periphery (Chapter 2.1 and 4.6). This mainly concerns $V\gamma9/V\delta2^+$ T cells which express invariant Vγ9-Jγ1.2 and Vδ2-Jδ1 receptor chains and are apparently selected for their specificity for a (super)antigen. These data give insight into TCR repertoire formation, selection during normal T-cell differerentiation, and the importance of antigen-induced peripheral expansion.

Mature T-cell malignancies are neoplasms characterized by an uncontrolled proliferation of post-thymic T cells. In most cases the correct diagnosis can be made with conventional diagnostics, such as histomorphology or cytomorphology supplemented with immunohistology or flowcytometric immunophenotyping. However, in 5%-15% of cases the diagnosis is more complicated and discrimination between a reactive (benign) and clonal (malignant) T-cell proliferation can only be made by sensitive molecular techniques. In Chapters 2 and 3 of this thesis, the BIOMED-2 Ig/TCR multiplex PCR assays were evaluated in a routine diagnostic setting and we demonstrated that they can accurately detect malignant lymphoid cells and can reliably replace "gold-standard" Southern blot analysis in clonality diagnostics. Based on these data we proposed a flow chart that demonstrates the most efficient and sensitive strategy in detecting T-cell clonality (Chapter 2.3).

Using cutaneous lymphoproliferations as a paradigm for lymphoproliferations in general, we demonstrate the application of TCR gene rearrangement analysis as a diagnostic tool, especially with regard to 1) early diagnosis in early stage lymphoma; 2) dissemination of lymphomas including evaluation of the clonal relationship between multiple lymphoid malignancies in one patient; 3) extra-ordinary presentation of an underlying leukemia/

lymphoma; 4) lineage determination (in selected cases only). Clonal T-cell populations were detected in the skin in up to 65% of patients with early stage mycosis fungoides (MF) and overall in ~70% of patients diagnosed with cutaneous T-cell lymphoma (CTCL) (see Chapter 3.2). When early stage MF was not considered, TCR clonality was detected in 78% of all cases. Furthermore, TCR gene rearrangement analysis could detect identical clones in lymph node, bone marrow, and/or peripheral blood samples in 67% of patients with late stage MF and identical TCR gene rearrangements could be found in skin samples of patients with underlying systemic malignant lymphoproliferations (Chapter 4.2). Altogether, a multidisciplinary diagnostic approach in which the molecular clonality results are integrated, is required for diagnosing and staging of malignant lymphoproliferative disorders.

By PCR-based TCR gene rearrangement analysis we identified and characterized leukemia-specific T-cell clones in a large cohort of patients diagnosed with mature T-cell leukemias, such as T-cell prolymphocytic leukemia (T-PLL), Sézary syndrome, and T-cell large granular lymphocyte (T-LGL) leukemia. Using this technique we searched for disease related TCR clonotypes, which might reflect an antigenic stimulation signature. Because no shared/restricted Vβ gene segment usage and CDR3 amino acid motifs could be detected in our series of patients diagnosed with T-PLL and Sézary syndrome, we conclude that there is no strong evidence for a common antigen to be involved in these disease entities (see General Discussion).

However, striking evidence was found that antigenic stimulation plays an etiopathogenic role in T-LGL leukemia. The results described in Chapter 4.4 demonstrate that the LGLs in 42% of patients with $CD4^+$ T-LGL leukemia show membrane expression of the TCR-Vβ13.1 chain. These patients display a common HLA-DRB1*0701 genotype and express identical motifs in a consistently shorter-length CDR3-TCR-Vβ sequence. This supports a chronic antigen-driven T-cell stimulation origin in $CD4^+$ T-LGL leukemia. In $CD3^+/CD8^+/TCR\alpha\beta^+$ T-LGL leukemia, no identical *TCRB* or *TCRA* CDR3 motifs in patients were found (Chapter 4.5). However, we could identify some homologous clonotypes, pointing to non-random selection of T-cell clones. Sequence analysis of *TCRG* and *TCRD* gene rearrangements in $TCR\gamma\delta^+$ T-LGL leukemias showed that nearly half of all patients share common TCR sequences, as reflected by the systematic presence of the antigen-selected invariant T nucleotide in the first codon of the Vδ2-Jδ1 junctional regions. On the basis of these results we conclude that the leukemic proliferation in a high proportion of $TCR\gamma\delta^+$ T-LGL cases is antigen-selected, most probably by a common antigen (Chapter 4.6).

Clonal T-cell populations could easily be demonstrated in seemingly healthy elderly individuals by PCR analysis and flowcytometric immunophenotyping. This condition is referred to as T-cell clonopathy of undetermined significance (TCUS). Based on our data, we propose that TCUS originates from a clonal outgrowth of an initially polyclonal response and represents a pre-leukemic state (Chapter 4.3). Immunogenetic factors, the antigenic pressure, the HLA genotype and the occurrence of secondary oncogenic events will finally determine whether or not transformation into a full malignant phenotype will occur. We show that there is no clear association with specific recurrent mutations and chromosomal abnormalities in T-LGL leukemia and conclude that the genetic alterations are more subtle. Using novel molecular techniques such as comparative genomic hybridization (CGH) array analysis we could detect aberrations that are possibly relevant for pathogenesis (see Chapter 4.7 and General Discussion).

Our data on clonality diagnostics in lymphoproliferative disorders point out that clonal TCR gene rearrangements can be identified at diagnosis in the vast majority of T-cell leukemias and lymphomas and is very helpful in discriminating reactive from malignant T-cell proliferations. Furthermore, the results of our studies on TCR repertoire in normal and malignant T-cell populations give insight into selection and expansion of normal T lymphocytes and contribute to the understanding of the etiology/pathogenesis of T-cell neoplasms. Given the highly homologous TCR clonotypes detected in T-LGL leukemia patients, antigenic stimulation seems to play an important role in initial disease development. Future studies will focus on recurrent involvement of particular genomic regions and on gene expression profiling of sorted cell populations from patients with consecutive clinical stages of TCUS and malignant T-LGL leukemia.

SAMENVATTING VOOR NIET-INGEWIJDEN

T-cellen (T-lymfocyten) zijn witte bloedcellen die betrokken zijn bij de afweer tegen ziekteverwekkers zoals virussen en bacteriën. T-cellen dragen op hun celoppervlak T-celreceptor (TCR) moleculen, waarmee ze onderdelen van de ziekteverwekker of een ander lichaamsvreemd bestanddeel (antigeen) kunnen herkennen. Gezien het feit dat er enorm veel verschillende ziekteverwekkers zijn, is het van groot belang dat er een grote diversiteit aan TCR moleculen beschikbaar is, zodat elke potentiële ziekteverwekker herkend kan worden. Iedere T-cel draagt één type (een unieke) TCR. Er bestaan twee typen TCR moleculen: TCR$\alpha\beta$ en TCR$\gamma\delta$. Omdat elk menselijk lichaam miljarden T-cellen bevat, kunnen in principe alle antigenen herkend worden met specifieke TCR moleculen. Al die miljarden verschillende TCR moleculen worden gevormd door het willekeurig aan elkaar plakken ("recombineren/herschikken") van verschillende gensegmenten die coderen voor delen van de T-celreceptoren. De aldus ontstane TCR moleculen worden uitgebreid getest: ze moeten functioneel zijn, maar ze mogen geen lichaamseigen structuren herkennen. Alleen cellen die er in slagen om de juiste TCR te produceren, mogen uitgroeien tot volwassen ('rijpe of mature') T-cellen.

Het herschikken van de "Variabele" (V) gensegmenten, de "Diversity" (D) gensegmenten en "Joining" (J) gensegmenten is een ingewikkeld moleculair proces en wordt V(D)J recombinatie genoemd. V(D)J recombinatie van TCR gensegmenten staat centraal in dit proefschrift. De TCR genherschikkingen worden gebruikt als moleculaire markers voor PCR studies. PCR genherschikkingsanalyse werd verricht in zowel normale T cellen als de kwaadaardige tegenhangers van deze cellen met speciale aandacht voor de diagnostische toepassing van deze moleculaire techniek.

Het uitrijpen tot gespecialiseerde T-cellen is eveneens een ingewikkeld proces, waarbij initiatie van TCR herschikkingen onderdeel uitmaakt van de strikte regulatie van het herschikkingsproces. Analyse van TCRα (*TCRA*), TCRβ (*TCRB*), TCRγ (*TCRG*), en TCRδ (*TCRD*) herschikkingen in normale onrijpe en rijpe T-cellen liet zien dat TCR$\alpha\beta^+$ T-cellen al zeer vroeg in hun ontwikkeling geselecteerd worden op functionaliteit en dat dit in veel mindere mate geldt voor TCR$\gamma\delta^+$ T-cellen. Selectie en expansie van TCR$\gamma\delta^+$ T-cellen bleek vooral plaats te vinden wanneer ze volledig uitgerijpt zijn en is waarschijnlijk het gevolg van chronische antigene stimulatie (zie Hoofdstukken 2.1 en 4.6).

De herschikte TCR genen zijn unieke sequenties ("DNA vingerafdruk"), waarvan wordt verondersteld dat ze verschillend zijn in elke lymfatische cel en dus ook in elke kwaadaardige woekering van T-lymfocyten (T-celleukemie en T-cel non-Hodgkin lymfoom). Rijpe T-cel maligniteiten worden gekenmerkt door ongecontroleerde deling van rijpe T-cellen. Bij de meeste patiënten kan een juiste diagnose worden gesteld op basis van klinische informatie, lichamelijk onderzoek, microscopisch onderzoek en laboratorium onderzoek. Echter in zo'n 10 tot 15 % van de gevallen leveren de gangbare diagnostische methoden geen uitsluitsel op en is het onderscheid tussen goedaardige en kwaadaardige T-cel woekeringen verre van eenvoudig. In de Hoofdstukken 2 en 3 van dit proefschrift evalueerden wij de recent ontwikkelde BIOMED-2 TCR PCR technologie en toonden wij aan dat het een erg betrouwbaar en snel hulpmiddel is in de routine-diagnostiek van lymfoproliferatieve aandoeningen. Met de uitslag van deze nieuwe 'klonaliteits'- test kan de diagnose met grote zekerheid worden gesteld en kan daarmee de 'gouden-standaard' techniek (Southern blot analyse) worden vervangen (Hoofdstuk 2.3).

De bruikbaarheid en de mogelijke toepassingen van de TCR genherschikkingsanalyse werden getest in een grote groep patiënten met lymfoproliferaties in de huid. In deze studies was specifiek aandacht voor de volgende aspecten: 1) vroege diagnose in een vroeg stadium van ziekte; 2) het aantonen van klonale verwantschap bij verdenking op uitzaaiingen naar lymfeklieren, beenmerg, bloed en/of interne organen; 3) het vaststellen van een huidlokalisatie van een onderliggende leukemie of maligne lymfoom; 4) bepalen van differentiatielijn (met name erg belangrijk voor prognose!). Het meest voorkomende type non-Hodgkin lymfoom gelokaliseerd in de huid is mycosis fungoïdes (MF). Met name in de vroege fase van de ziekte is de diagnose erg moeilijk te stellen. Met de nieuwe PCR technologie konden wij in 65% van de gevallen klonale T-cel populaties aantonen in patiënten met vroeg-stadium MF; deze is daarmee van grote aanvullende diagnostische waarde. Ook voor de andere typen cutane lymfomen gold dat de data van onze PCR analyses in 70% tot 78% van de gevallen in overeenstemming waren met de definitieve diagnose (Hoofdstuk 3.2). Bovendien bleek in het merendeel van de patiënten met een vergevorderd stadium van het cutane lymfoom of een onderliggende leukemie, klonale verwantschap aangetoond kon worden tussen de lymfoproliferaties in de huid en andere weefsels (Hoofdstukken 3.2 en 4.2). Samenvattend kan worden gesteld dat een multidisciplinaire aanpak waarin de data van de moleculaire analyses zijn geïntegreerd, vereist is voor betrouwbare diagnostiek van rijpe lymfoproliferaties.

Om meer inzicht te krijgen in de ontstaanswijze (pathogenese) van rijpe T-cel maligniteiten, bestudeerden wij in Hoofdstuk 4 leukemie-specifieke T-celklonen in een grote groep patiënten gediagnosticeerd met rijpe T-cel leukemieën, zoals T-cel prolymfatische leukemie (T-PLL), Sézary syndroom en "T-cell large granular lymphocyte" (T-LGL) leukemie. Met behulp van TCR genherschikkingsanalyse onderzochten wij of er sprake was van ziekte-specifieke TCR herschikkingen. Gemeenschappelijke TCR sequenties (motieven) tussen verschillende patiënten zijn een aanwijzing voor de betrokkenheid van aanhoudende antigene stimulatie in de ontstaanswijze van deze maligne lymfoproliferaties. Wij konden geen gemeenschappelijke motieven aantonen bij T-PLL patiënten en patiënten met het Sézary syndroom. Wij concludeerden daarom dat betrokkenheid van chronische antigene stimulatie in de pathogenese van deze aandoeningen onwaarschijnlijk is.

Analyse van TCR herschikkingen in klonale T-LGL proliferaties leverde echter wel overtuigend bewijs voor de betrokkenheid van antigene stimulatie in de ontwikkeling van deze aandoeningen.

Wij bestudeerden een grote groep patiënten met klonale $CD4^+$/$TCR\alpha\beta^+$ T-LGL proliferaties en toonden aan dat 42% van deze patiënten eenzelfde Vβ13.1 eiwitketen op het celoppervlak draagt (zie Hoofdstuk 4.4). Bij moleculaire analyse van deze patiënten bleek dat er in de *TCRB* gensequenties sterk overeenkomstige motieven aanwezig zijn en dat vrijwel alle patiënten een HLA-DRB1*0701 genotype hebben. Deze resultaten wijzen op een rol voor chronische antigene stimulatie in de pathogenese van deze ziekte-entiteit. Bij patiënten met $CD8^+$/$TCR\alpha\beta^+$ T-LGL leukemie konden daarentegen geen duidelijke gemeenschappelijke motieven worden gevonden in de TCR genen middels *TCRA* en *TCRB* genherschikkingsanalyse. De leukemie specifieke T-celklonen van onze groep patiënten werd vergeleken met een groot aantal andere T-celklonen en hierin werd toch enige homologie in TCR sequenties gevonden. Dit duidt mogelijk toch op een niet-willekeurige selectie van T-cellen, mogelijk door een antigeen (zie Hoofdstuk 4.5). Hoofdstuk 4.6 vat de resultaten samen van een studie waarin

we de sequenties van de *TCRG* en *TCRD* genen bekeken in een grote groep patiënten met TCR$\gamma\delta^+$ T-LGL leukemie. Er bleek dat bij bijna 50% van de patiënten gemeenschappelijke TCR gensequenties aanwezig waren en dat in alle *TCRD* herschikkingen in deze subgroep een selectiedeterminant aanwezig was. In een groot deel van de TCR$\gamma\delta^+$ T-LGL leukemie patiënten is de maligne proliferatie dus antigeen-geselecteerd.

Klonale T-celpopulaties konden eenvoudig worden aangetoond in het bloed van gezonde ouderen met PCR analyse en flowcytometrische immunofenotypering. De (klinische) betekenis van deze populaties is vooralsnog niet geheel duidelijk. Op basis van de resultaten van onze studies postuleren wij dat deze klonale celpopulaties mogelijk een voorstadium kunnen zijn van T-LGL leukemie (Hoofdstuk 4.3). Leukemische transformatie van deze celpopulaties is afhankelijk van immunogenetische factoren, antigene stimulatie, het HLA genotype en het optreden van secundaire chromosoomafwijkingen. Uit onze studies blijkt er geen duidelijke associatie te zijn met specifieke afwijkingen in het genoom en concluderend kan worden gesteld dat er waarschijnlijk sprake is van zeer subtiele genetische afwijkingen bij deze leukemische celproliferaties. In Hoofdstuk 4.7 is de CGH array methode beschreven. Deze methode is ontwikkeld om snel en betrouwbaar chromosoomafwijkingen te kunnen detecteren. Wij konden hiermee genetische afwijkingen aantonen die mogelijk van belang zijn in de pathogenese van klonale T-LGL proliferaties (zie Hoofdstuk 4.7 en General Discussion).

De resultaten van onze studies laten zien dat klonale TCR herschikkingen in het merendeel van de maligne T-cel proliferaties bij diagnose aantoonbaar zijn en van grote diagnostische betekenis zijn. De TCR genherschikkingsanalyse van T-celklonen draagt bij aan de immunobiologische kennis omtrent de pathogenese van mature T-celleukemieën. Omdat grote homologie in de TCR genherschikkingen van verschillende patiënten met klonale T-LGL proliferaties bestaat, lijkt chronisch antigene stimulatie een belangrijke rol te spelen in de beginfase van de ontwikkeling van T-LGL leukemie. De CGH array methode en DNA chip technologie zullen worden gebruikt om genetische afwijkingen in klonale T-cel proliferaties en T-LGL leukemieën te detecteren, die mogelijk betrokken zijn bij maligne transformatie.

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CURRICULUM VITAE

Personalia

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Opleiding

1991-1997	VWO Christelijke Scholengemeenschap Oude Hoven, Gorinchem
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juli 1998	Propedeuse
feb 2001-nov 2001	Afstudeeronderzoek Afdeling Immunologie Erasmus MC/Erasmus Universiteit Rotterdam Onderwerp: Molecular clonality analysis in cutaneous lymphoproliferations Supervisie: Prof.dr. J.J.M. van Dongen
25 nov 2001	Doctoraalexamen
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