FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 45, No. 1, 2007
pp. 5-14

Review article

Immune-mediated bone marrow failure syndromes of progenitor and stem cells: molecular analysis of cytotoxic T cell clones

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Abstract: The unique structure of the T cell receptor (TCR) enables molecular identification of individual T cell clones and provides an unique opportunity for the design of molecular diagnostic tests based on the structure of the rearranged TCR chain *e.g.*, using the TCR CDR3 region. Initially, clonal T cell malignancies, including T cell large granular lymphocyte leukemia (T-LGL), mucosis fungoides and peripheral T cell lymphoma were targets for the TCR-based analytic assays such as detection of clonality by T-γ rearrangement using γ-chain-specific PCR or Southern Blotting. Study of these disorders facilitated further analytic concepts and application of rational methods of TCR analysis to investigations of polyclonal T cell-mediated diseases. In hematology, such conditions include graft versus host disease (GvHD) and immune-mediated bone marrow failure syndromes. In aplastic anemia (AA), myelodysplastic syndrome (MDS) or paroxysmal nocturnal hemoglobinuria (PNH), cytotoxic T cell responses may be directed against certain antigens located on stem or more lineage-restricted progenitor cells in single lineage cytopenias. The nature of the antigenic targets driving polyclonal CTL responses remains unclear. Novel methods of TCR repertoire analysis, include VB flow cytometry, peptide-specific tetramer staining, in vitro stimulation assays and TCR CDR3-specific PCR. Such PCR assay can be either VB family-specific or multiplexed for all VB families. Amplified products can be characterized and quantitated to facilitate detection of the most immunodominant clonotypes. Such clonotypes may serve as markers for the global polyclonal T cell response. Identification of these clonotypes can be performed in blood and tissue biopsy material by various methods. Once immunodominant clonotypes corresponding to pathogenic CTL clones are identified they can serve as surrogate markers for the activity of the pathophysiologic process or even indicate the presence of specific antigens. The relevance of the individual clonotypes can be ascertained from clinical correlations with the activity of the disease. Quantitative clonotypic assays such as sequencing of multiple CDR3 clones or clonotypic Taqman PCR can be applied for the monitoring of the immunosuppressive therapy and prediction of relapse. Future technologies may allow for the design of clonotypic microarrays or other more clinically applicable methods of clonotypic diagnostics. Similarly, identification of immunodominant clonotypes may facilitate targeting of autoimmune or malignant clones with vaccination and induction of anti-idiotypic responses.

Key words: Marrow failure syndromes - T cell receptor - Molecular analysis

Immune-mediated bone marrow failure syndromes and cytopenias

Pathophysiology of immune mediated bone marrow failure

Idiopathic aplastic anemia (AA) can serve as a prototype of immune-mediated bone marrow failure syndromes but similar pathophysiologic mechanisms may also operate in related diseases such as paroxysmal nocturnal hemoglobinuria (PNH) or some forms of myelodysplastic syndrome (MDS). Most often these disease are characterized by global cytopenia. However, some single-lineage cytopenias such as immune-mediated pure red cell aplasia, amegakaryocytic thrombocytopenic purpura as well as certain forms of autoimmune neutropenias and white cell aplasia may be mediated by the cellular immune system. The best example of lineage-restricted cytopenias are those that accompany T cell large granular lymphocyte leukemia (T-LGL) which frequently presents with various degrees of neutropenia and less, commonly red cell aplasia.

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Various lines of laboratory evidence point towards an immune-mediated inhibition of hematopoiesis at specific differentiation stages, but the efficacy of immune-suppressive strategies targeting T cells provides the strongest argument for the involvement of T cells in the pathophysiology of AA and some other bone marrow failure syndromes. Clearly, some forms of immune cytopenias such as those seen with lupus erythematosus, and autoimmune neutropenia, are mediated by antibodies likely directed against hematopoietic progenitor or more mature cells. Theoretically, even if T cells are mostly responsible for the damage to the progenitor and stem cell compartment, one could speculate that the cellular immune response will be accompanied by a corresponding antibody response. However, serologic markers for most common types of immune-mediated bone marrow failure syndromes are missing and B cell targeting strategies such as Rituximab have not been applied to the therapy of these disorders. Similarly, attemepts of antigen identification have not yielded clinically utilizable autoantibodies.

Modes of inhibition

Effector mechanisms in hematopoietic inhibition may involve various pathways, including release of inhibitory cytokines leading to apoptosis of hematopoietic progenitor and stem cells. The specificity of such a mechanism may be difficult to reconcile with a sometimes very narrow spectrum of hematopoietic inhibition as seen single lineage cytopenias. Direct perforin/granzyme-mediated killing by cytotoxic effector cells may be another mechanism by which specific targeting of stem cells or progenitors can be explained. Clearly, the distribution of target antigens may determine the killing spectrum. Terminal CTL are likely the most efficient effector cells and so far there is little evidence that the hematopoietic inhibition in the most common forms of bone marrow failure is mediated by natural killer cells. It is possible that a CTL population lacking CD28 and expressing CD57 contains most of the pathogenic clones and can serve as a source of T cells for molecular TCR analysis.

Cellular targets

Immune elimination of stem cells due to the presence of a cross-reactive antigen or autoantigen restricted to the stem cell compartment may be responsible for AA (Fig. 1). In MDS, various possible mechanisms have been postulated to explain the occurrence of cytopenia due to inhibition of normal residual hematopoiesis. For example, the immune attack can be part of physiologic anti-tumor surveillance response to abnormal and/or dysplastic cells in the bone marrow. In such a situation, the immune attack may not be sufficiently specific and results in collateral damage with inhibition of normal hematopoiesis. Conversely, the initial immune attack may be directed against normal stem cells as in AA, resulting in selection pressure and outgrowth and escape mutant hematopoietic clones. Similar considerations apply to the evolution of glycophosphatidyl-deficient clones in PNH [55].

Target antigens and methods of their detection

Even if the damage to the hematopoietic progenitor and stem cells is mediated by the cellular immune system, the pathogenic process could elicit antibody responses. Specific target antigens have been described for Felty's syndrome associated with neutropenia [10], and AA [12,19]. Despite these reports, clinically relevant tests have not been introduced. The nature of the identified antigens does not reveal clues as to the restriction of the immune attack to the hematopoietic tissues as the described target antigens appear to be expressed in various tissues.

Clinical observations may provide some clues as to the distribution of the target antigens. It is very likely that in AA the target antigen is located on hematopoietic stem cells, while in the lineage-restricted cytopenias antigens expressed on specific progenitors are likely to be targets. This restriction may be particularly obvious in some cases of LGL leukemia associated with either neutropenia or pure red cell aplasia (Fig. 1). Similar conclusions can also be drawn with regard to the tropism of putative viral agents implicated in the pathogenesis of bone marrow failure syndromes. Even if an initial specific "inciting" antigen does exist, it is likely that the later immune response can be characterized by the responses directed against multiple antigens in concordance with the antigenic spread theory.

Molecular methods of T cell receptor analysis

A lack of knowledge of the offending antigens frustrated generations of investigators studying bone marrow failure states. Alternative strategies to investigate T cell responses in AA and other related diseases have been developed. One such method, molecular analysis of clonal T cell repertoire, is aimed at identification of immunodominant marker clones and allows for the study of the kinetics of the cellular immune response without knowledge of specific antigens [42]. The basis for this principle has been established during investigations of clonal T cell lymphoproliferations. As applied to polyclonal T cell-mediated processes, it has been hypothesized that immunodominant clones do exist and such clones can serve as markers of antigeneic processes irrespective of their recognition spectrum. Due its unique and highly specific structure, the

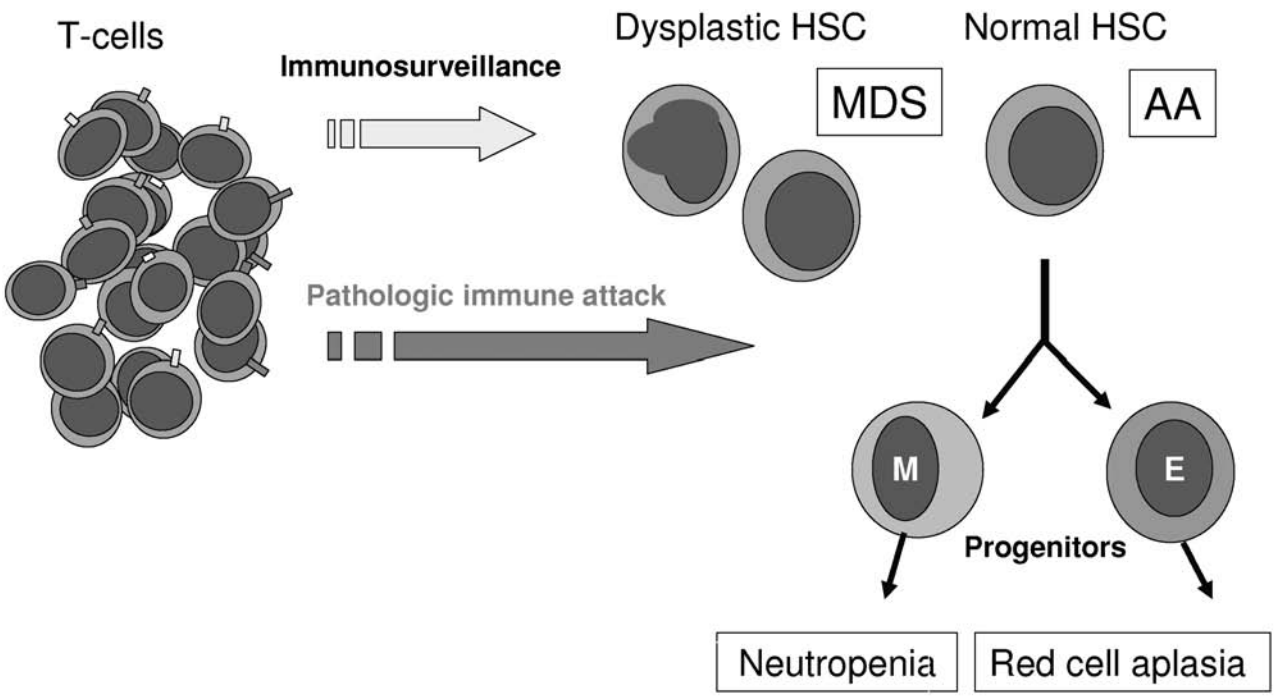

Fig. 1. Immune-mediated bone marrow failure, targets and effector cells.

rearranged portion of the variable B-chain (VB), the CDR3 region, can serve as a molecular signature of individual clones and their frequency can be easily established.

Analysis of TCR rearrangement is routinely applied to detect clonality for the diagnosis of lymphoid malignancies. However, molecular analysis of TCR can be a powerful tool in the study of T cell responses to pathogens and in autoimmune diseases [42]. The concept of oligoclonality in the context of the immune response is based on the presence of immunodominant T cell clones and has to be viewed in relation to the T cell population used for analysis. During immune reactions, a limited number of clones usually undergoes expansion triggered by foreign agents. The derailment of immune regulation allows for the expansion of T cell clones specific for "self", resulting in an autoimmune disease (Fig. 2). In hematology, LGL leukemia is suggestive of an exaggerated clonal response to a specific hematopoietic antigenic target and illustrates that an extreme expansion of a single clone can lead to a specific CTL-mediated pathology. Normally, clonal TCR rearrangement cannot be detected in unseparated blood or marrow by traditional methods. However, individual T cell clones can significantly expand, and certain T cell subpopulations (defined *e.g.*, by a specific VB chain usage or specific phenotypic markers), may demonstrate molecular oligoclonality. Expanded T cell clones may represent immunodominant responses, which can be studied by various molecular methods. In the laboratory, analysis of the individual TCR VB-chain representation, CDR3 amplification product size fragment skewing and determination of the frequency of individual clonotypic sequences were developed [42]. Such molecular analysis of TCR has been employed in the investigation of classical autoimmune diseases [5,9,15,20-22, 38,32,36] and T cell lymphoproliferative disorders[29-31].

The techniques used to study T cell clonality are based on the extraordinary biology of the TCR. The TCR is a heterodimer comprising of α (A) and β (B) chains, both encoded by rearranged V (D) J segments and a constant region (for review see [1,2,13,16,40]. This rearrangement between V, D and J regions accounts for the heterogeneity and fine specificity of antigen recognition and is mostly carried by the VB CDR3 domain. The CDR3 of the TCR VB chain is a non-germ line-encoded hypervariable region directly related to T cell recognition of peptides in the appropriate HLA context. During the recombination process, transferases add or remove nucleotides at various VB-DB or DB-JB junctions, leading to 6-8 amino acid differences between CDR3 regions within each VB chain, thus creating unique signature sequences. This process accounts for the virtually unlimited antigen-driven expansion of specific clones. As the CDR3 region appears to be responsible for the recognition of peptides presented in the context of HLA, while CDR1 and 2 regions exhibit affinity to a specific HLA type, similar CDR3 sequences that recognize a specific antigen may possibly be found in individuals with differ-

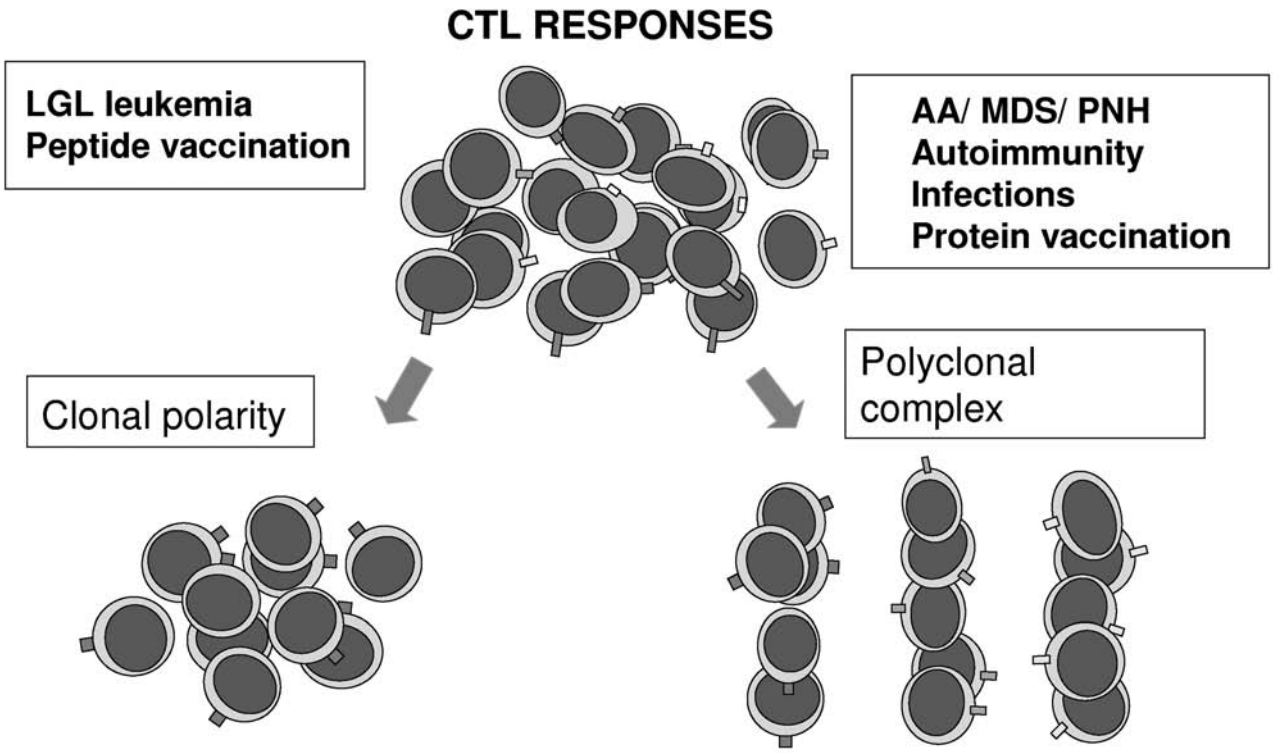

Fig. 2. Clonality of the CTL responses in bone marrow failure.

ent HLA types [1]. Despite HLA-restriction, CDR3 regions derived from different VB families may show similar sequences if the CTL clones recognize identical antigens.

Although most of the natural immune responses may be polyclonal, acutely significant oligoclonal expansions may occur. For example, in acute infection with cytomegalovirus or Epstein-Barr virus, very high frequencies of virus-specific T cells have been observed [14,28,41]. An increase in the size of the entire VB family can be detected by flow cytometry [25] or by reverse hybridization techniques [34]. LGL leukemia represents an extreme clonal expansion that results in an increase in the size of an entire VB family.

Analytic strategies

The VB chain, due to its recombinatorial and junctional diversity, is more suitable for identification of unique clonotypic markers for T cell clones. Clonal or oligoclonal T cell expansions will result in skewing of the otherwise normal Gaussian size distribution of recombined CDR3 amplification products [13,16,37, 40] that is amenable to molecular analysis by size measurement of CDR3 amplification PCR products (CDR3 spectratyping). Analysis of the VB repertoire has been called VB spectratyping. An increased frequency of individual clones can also be detected by cloning CDR3 amplification products and sequencing individual clones. Under normal circumstances, due to the great variability of the CDR3 region within each VB family, most of the cloned sequences will be unique. However, expansion of specific T cells will result in a higher probability of their amplification and cloning and consequently, many cloned CDR3 regions will be identical. The frequency of homologous CDR3 sequences corresponds to the degree of clonal overexpansion, and in LGL leukemia only identical, clonal sequences are usually obtained. The sequencing method is very labor-intense. Similar to spectratyping, it requires PCR amplification of each individual VB family, sub-cloning and sequencing of many individual clones followed by sequence analysis and calculation of the clonal frequency. Flow cytometry may be used to narrow the choice of VB families for analysis as immunodominant clonal expansions may result in relative increase in entire VB family (Fig. 3).

Recently a new multiplexed VB PCR assay has been introduced allowing for the amplification of the entire VB spectrum just in a few PCR reaction. Similar to individual VB PCR assay, the products of such PCR reaction can be sub-cloned and sequenced. This technique may be used upfront to determine most predominant clonotypes and subsequently their frequency within the given VB family can be determined using individual VB PCR. Such an approach leads to a higher level of detection of immunodominant expansions (Fig. 3) [39,51,52]. Following identification of poten-

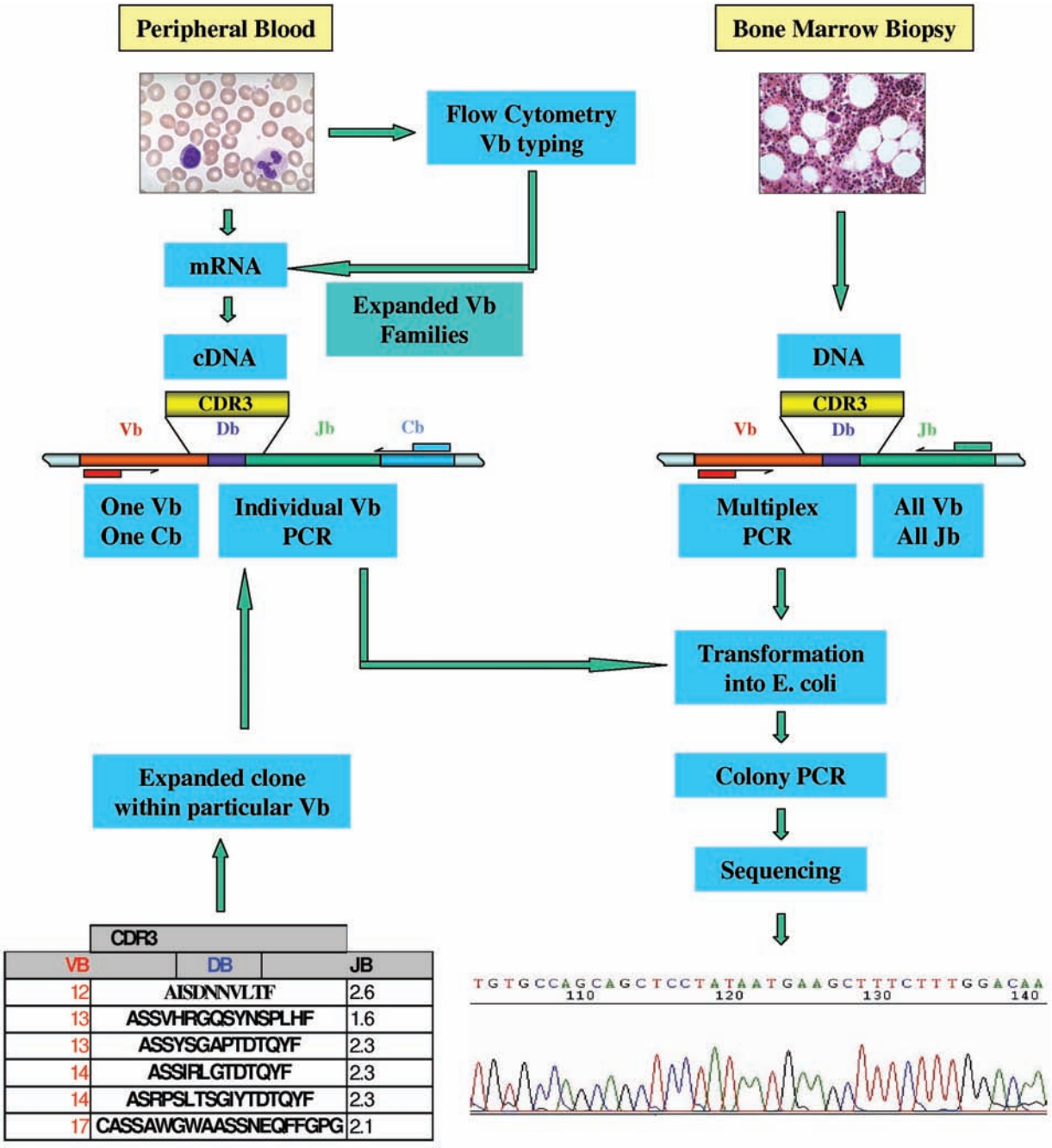

Fig. 3. Methods of molecular TCR analysis.

tially pathogenic clonotypes, clonotypic sequences can be used for the design of specific assay to determine frequency of individual clones in blood or tissues. Techniques available include clonotypic PCR and clonotypic Taqman PCR allowing for the determination of clonal frequencies (Fig. 4).

Molecular techniques can be applied not only to the detection of expanded T cell clones in blood but also in tissues affected by immune processes such as marrow and gut, liver or skin biopsies. In addition, paraffin-embedded tissues can be used as a source of nucleic acid for molecular TCR utilization analysis [3,51,52].

TCR analysis in LGL leukemia and lessons for other bone marrow failure syndromes

In many important aspects the clonal CTL expansions seen in LGL can be used as a simple model of CTL responses seen in other polyclonal CTL-mediated diseases. The investigative principles established in this disease allow for better application of molecular TCR

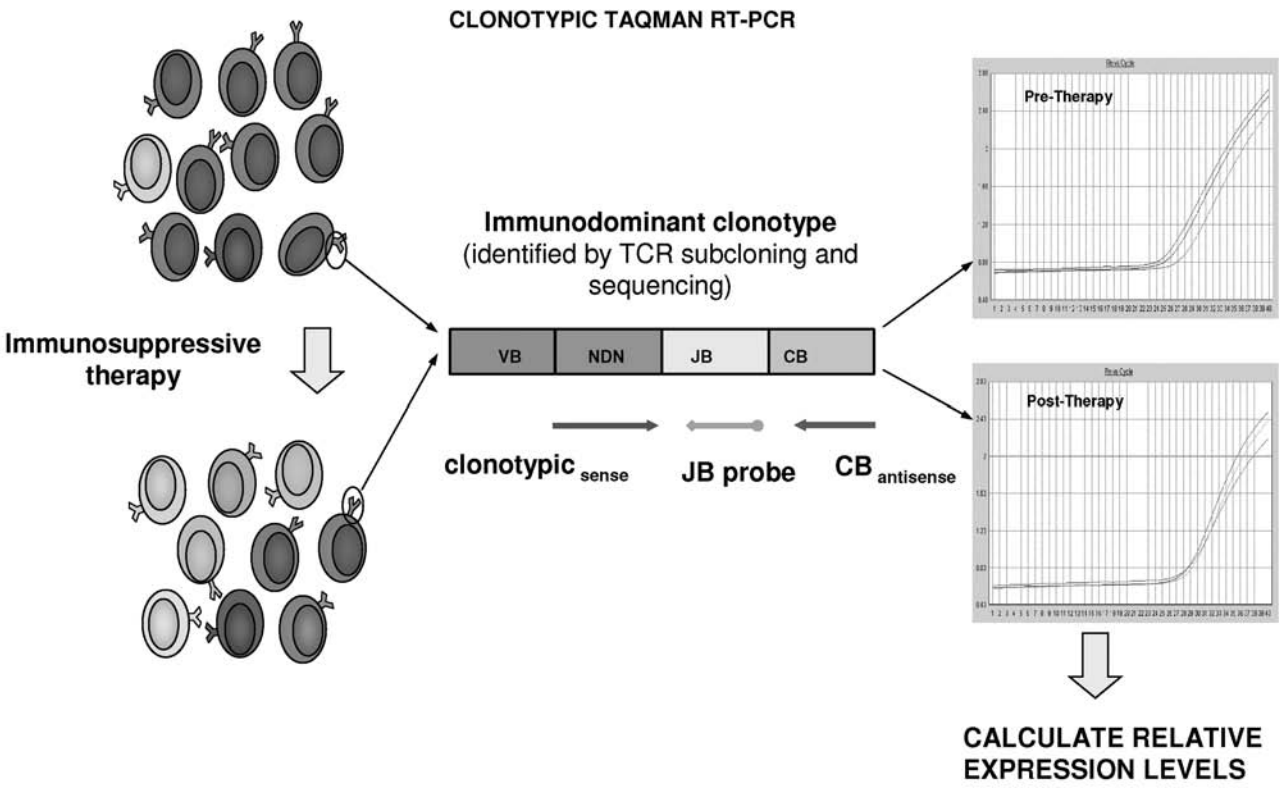

Fig. 4. Quantitation of clonotypes. Clinical application of clonotypic diagnostics.

analyses in a more complex processes. Similar to idiopathic AA, in which immune-mediated destruction of hematopoiesis has been inferred from the success of immunosuppressive therapies [52,53], LGL has been shown to respond to immunosuppression [4,7,48,49]. LGL leukemia can occur in the context of MDS and PNH [23,35,43,49,52], and in this setting it may represent an aberrant immune response to the genetically altered dysplastic cells. A pathophysiologic relationship between these immune-mediated bone marrow failure states suggests that molecular analysis of TCR repertoire can be applied to study of not only AA and PNH but also MDS. For example, MDS often evolves from AA, and hypoplastic MDS shows many clinical similarities to AA [17,50].

Flow cytometric [31] and molecular [8] VB typing have previously been applied to large cohorts of LGL patients. LGL-associated or LGL-like clonal expansions were described on molecular level in several smaller series, including patients with Felty's syndrome[6], MDS and erythroid hypoplasia [35] and patients with rheumatoid arthritis [18]. Flow cytometric detection of phenotypically abnormal CTL populations and VB CDR3 genotyping may leave a significant number of CTL proliferations unidentified. Complete molecular analysis of a large cohorts of LGL patients has been recently reported [38,52]. By refining these methods and sequencing of expanded clones a much higher detection level can be achieved.

In general, analysis of clonal utilization patterns in LGL is based on the theory that the unique and often very lineage restricted cytopenias associated with LGL leukemia may be a result of the lineage specificity of the target antigens recognized by clonal TCR (Fig. 1). Consequently it may be possible that in patients with similar presentations and matching HLA-restriction elements, identical or highly homologous clonotypes will be found. Clearly, given the estimated physiologic variability of CDR3 regions within the VB chain [2], such an event that may be rare but it would prove the nonrandom origin of clonal transformation in LGL leukemia. By studying a relative large number of patients with LGL, identical expanded clonotypes were found in 2 patients. CTL clones identified in LGL associated with neutropenia may indicate an autoimmune process modified by e.g. mutational event may result in extreme clonal polarity. Based on the diversity of the physiologic TCR repertoire and the extremely low frequency of shared clonotypes identified between healthy controls, the coincidental finding of common clonotypes is unlikely. It strongly suggests that these clones do not evolve randomly, but occur in the context of an initially polyclonal immune response directed against identical or highly similar antigenic targets. This conclusion is further supported by the

identification of sequence sharing between the two other immunodominant clonotypes identified in patients and non-expanded clonotypes found in other LGL cases. It is likely that these minor clonotypes are the remnants of an initial polyclonal response. The low frequency of these clones may be due to "dilution" by the expanding semiautonomous LGL clones. Detection of high levels of similarity between clonotypes (including both minor and immunodominant clonotypes) within a given VB repertoire of individual patients also supports our conclusions that several clones recognizing similar or identical peptides may be present prior to the expansion of immunodominant clones. The occasional presence of distinct clonotypes that are identical in their amino acid sequences but distinct in their nucleotide sequences may have resulted from selection of individual clones with identical antigenic affinity. In such cases the effective frequency of the corresponding TCR may be a sum of two independently rearranged clones.

Very analogous results to those obtained in LGL leukemia have been reported in a small cohort of intricately characterized PNH patients [47]. It is likely that clinically-manifested cases of LGL leukemia represent the extreme clonal/oligoclonal T cell lymphoproliferations that, to a lesser extent, may also be present in certain patients with otherwise unexplained cytopenias in which clonal expansion may not be detected with traditional laboratory tools. We hypothesized that high resolution T cell receptor repertoire analysis may uncover clonal cytotoxic T cell expansions that are quantitatively less pronounced than those seen in LGL but are pathophysiologicaly analogous and can serve as markers for T cell-mediated autoimmune process. We have recently studied 20 patients with unexplained neutropenia and found expanded CTL clones in a large proportion of these patients. In comparison to LGL leukemia, CTL clones found in some cases of idiopathic neutropenia were less dominant but clearly distinguishable from marginally expanded clonotypes found occasionally in healthy individuals.

Aplastic anemia, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndromes

Even in LGL leukemia not all cases will show clonality using TCR rearrangement analysis, but clearly the results of this test depend on the proportion of monoclonal cells in circulation. In classical immune-mediated bone marrow failure syndromes, polyclonality precludes positive TCR rearrangement studies. However, clonal expansions, in some cases very substantial, have been described in AA, PNH and MDS. When VB CDR3 repertoire analysis (CDR3 spectratyping) has been performed in patients with AA [27,44-46,56,57], PNH [23, 35,43,47] and MDS [11,26,35,52], some studies showed a broadly normal VB distribution pattern with overexpression of a few VB types, indicative of selective CDR3 usage. However, skewing was not limited to specific VB families, likely due to lack of selection of patients based on their HLA type. As expected in the absence of malignant lymphoproliferation, when J-regions were analyzed monoclonality was excluded [33]. These studies demonstrated that despite their complexity in AA and MDS, polyclonal CTL responses can be successfully detected and characterized.

When we have used flow cytometry to study VB family skewing as a surrogate for more stringent molecular clonal analysis, we were able to detect VB expansions within the CTL population, there was no difference in the average size or frequency of clonal expansions in AA and MDS nor was the hypocellularity (irrespective of the diagnosis) associated with more pronounced VB skewing. Genotyping of the CDR3 region demonstrated oligoclonality in around a majority of these expanded VB families. Even by this rather crude analysis a continuum of responses was observed from multiple VB families expanded in AA and MDS to extreme monoclonal expansions in typical LGL [52].

In MDS, an extensive skewing of the CDR3 region showed normalization after immunosuppressive therapy [26]. In another report, specific CTL clones were defined based on their VB and VA utilization pattern in four patients with refractory anemia. Interestingly, clinical description of the cases implies that in 3 of these RA patients an LGL clone was present, consistent with a coexisting LGL leukemia [35]. For example, while immune response in AA, MDS and PNH may be less polarized, expansions of entire VB-families and oligoclonal skewing of the TCR VB CDR3 have been observed.

Recently, we conducted a systematic analysis of the TCR repertoire in patients with MDS and AA [52]. This study focused on the development of efficient and precise tools of clonotypic diagnosis in MDS and AA rather than on the functional characterization of immunodominant clonotypes. When we applied the previously used VB CDR3 PCR of expanded VB families as well as multiplex CDR3 PCR to generate clonotypic amplicons for analysis of the frequency of amplified clonotypes. In agreement with previous reports involving smaller groups of MDS patients [26, 35,52], we found evidence for oligoclonal T cell-mediated immune responses not only in AA but also in MDS patients. The newest technologies allow for detection and characterization of immundominant clonotypes not only in freshly isolated blood and bone marrow but also in historical archived paraffin-embedded specimens. The multiplex PCR assay proved a very efficient means of identification of the most expanded clonotypes. Following multiplex PCR, VB-specific amplification using VB primers was per-

formed with primers allowing for the amplification of VB family corresponding to the redundant clone detected in the multiplex PCR. This approach allowed for quantitation of clonal expansion within VB family. Such a rational method led to identification of oligoclonally-expanded clones even when VB flow cytometry was not possible (biopsy material) or not informative. Previously this method was applied to the analysis of LGL leukemia [39,51] and now has been adopted to study MDS and AA [52].

The size of expanded clones identified in our study in MDS and AA patients was comparable, ranging from 33-100% of a given VB family. This finding has to be viewed in the context of physiologic variability of the TCR, which was previously estimated from a large number of control sequences derived from healthy individuals [52]. Interestingly, the MDS patient group included 3 patients in whom traditional flow cytometric assays detected LGL T cell clones and confirmed a diagnosis of concomitant LGL. Interestingly, using our strategy, LGL-like expansions of VB families (>30% of total CD8+ cells) were found in additional MDS patients, suggesting that LGL-like, subclinical clonal CTL responses may be more frequently encountered than can it can be extrapolated based on traditional clinical testing. Subsequent studies of T cell γ rearrangement confirmed the presence of oligoclonality in these patients. Molecular assays with TCR analysis could be useful to further refine the diagnostic criteria for some MDS patients who share overlapping features with LGL leukemia patients not detectable by routine techniques. In addition, to the sensitive detection of clonal dominance in blood, identification of oligoclonality in biopsy specimens is very instructive and would be difficult to reconcile with the argument that expanded clones are due to infection or alloimmunization.

In general, immunodominant CTL clonotypes can be detected at comparable rates in AA and MDS irrespective of the morphologic subtype. Immunodominant clones derived from blood or bone marrow can be used as markers of the activity of the disease, assuming that they are related to the pathogenic process rather than to infections or alloimmunization. The disease-specificity of the clonotypes detected in our studies was supported by the correlation of their frequency with hematologic response to immunosuppression [47,52]. Similarly, isolation of immunodominant clonotypes in bone marrow biopsy specimens as well as in blood of patients at presentation indirectly support the notion of specificity [3,51,52].

Immunodominant clonotypes can have diagnostic utility independent of their (unknown) specificity. Using quantitative sequencing methods, a correlation between the level of some but not all clonotypes and activity of AA has been demonstrated [46]. Clearly, the most stringent proof would be provided by functional analysis but such an analysis was not possible due to the lack of putative antigens needed for the assays. Of note is that should a clinical correlation between a clonotype and clinical events be established, the clonotypic sequence itself could serve a surrogate marker of the corresponding unknown antigen.

In PNH, the results of TCR VB analysis showed T cell responses involving several immunodominant clonotypes [23,24,43,47,52]. While identical sequences have not been found among PNH patients, similarities of CDR3 motifs have been observed, suggesting a possible T cell response to common inciting antigens. Sequences from the immunodominant clonotypes derived from patients allowed for the design of PCR-based assays to detect individual clonotypes. The distribution pattern of individual clonotypes established by this method indicated that imunodominant T cell clonotypic specificities were shared between the patients and also found in normal individuals, suggesting that they are either not related to the primary pathologic process or that healthy individual have CTL clones recognizing antigens involved in the autoimmune process leading to PNH. Recently, several patients with a typical PNH and significantly expanded expansions of CTL were reported [23,47]. PNH patients described in this study were subjected to intricate TCR utilization analysis, previously unknown LGL-like expansions were found in these patients suggestive of the CTL mediated immune process likely directed against hematopoietic progenitor cells.

Future potential of clonotypic diagnostics

Efficient identification or marker clonotypes may enable introduction of a novel class of diagnostic tools based on molecular methods. Such a strategy would have to be individualized due to the diverse HLA background and patient-specific antigenic recognition spectrum. Theoretically, clonotypic arrays can be designed to enable testing of large numbers disease-specific clonotypes and clonotypic utilization patterns. Such technologies may have a broad diagnostic utility. Potentially, such arrays could be HLA-independent as a large number of clonotypes could be printed that were identified in the context of many HLA-backgrounds. Finally, recognition of clonotypes and corresponding antigens may allow for the generation of predictions algorithms based on the principle of reverse genetics. Using such algorithms the sequence of antigenic peptides could be extrapolated possibly allowing for antigen identification. In specific application to immune-mediated bone marrow failure states, further refinement of the molecular analysis of TCR repertoire may lead to the identification of antigens that drive the CTL response directed against hematopoietic progenitor and stem cells.

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Received: October 4, 2006
Accepted after revision: October 23, 2006