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REVIEW

Biological features of the clone involved in primary amyloidosis (AL)

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Primary light chain-associated amyloidosis (AL) is a plasma cell dyscrasia that causes morbidity via systemic tissue deposition of monoclonal light chains in the form of fibrils (amyloid). It is the most common form of systemic amyloidosis in Western countries and is rapidly fatal. Knowledge of the pathobiology of the underlying B cell clone is of primary importance for the design and optimization of therapeutic strategies. *Leukemia* (2001) **15,** 195–202.
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Amyloid: common elements

Amyloid is an extracellular deposit mainly constituted of autologous proteins that produces a diagnostic apple-green birefringence when viewed in polarized light after Congo red staining, suggesting an organized structure. Electron microscope analysis revealed that amyloid is composed of rigid unbranched aggregated fibrils of indefinite length, 7.5 to 10 nm wide.[1] Each fibril consists of units (filaments) that are mainly composed of proteins arranged in an antiparallel, cross -pleated sheet configuration with strands perpendicular to the long axis of the filament. Exactly how fibrils are formed from filaments is unknown. A recent model involves two filaments that intertwine to form a protofibril, and two protofibrils, or alternatively three filaments directly, that intertwine to form a fibril.[2] The peculiar structural architecture of amyloid is considered responsible for its typical staining properties.[3]

Amyloid is found in a variety of hereditary and acquired pathological conditions.[4] More often systemic,[5] it can occasionally present as a single mass (amyloidoma) or confined to a specific organ, particularly the brain, upper and lower airways, lymph nodes, thyroid, Langherhan's islets or the gastrointestinal tract. Classification is based on the biochemical nature of the principal protein extracted from amyloid and approximately 20 unrelated amyloidogenic proteins are presently known.[6,7] These are usually synthesized as soluble intact precursors of the NH2-terminal fragments found in amyloid. However, cleavage is not essential for amyloidogenesis and it is unknown whether proteolysis occurs prior to, during, or after protein deposition. The most common forms of amyloidosis and their characteristics are reported in Table 1.

Amyloid fibrils are insoluble and relatively resistant to proteolytic degradation. However, amyloid deposition is not irreversible, it results from imbalance in production of the amyloid protein precursors and resorption. Clinical evidence documents that amyloid deposits frequently regress when the supply of the fibril precursor is reduced,[8] particularly in secondary AA amyloidosis,[9,10] but also in AL.[11]

Amyloid deposition rate can be extremely variable, but it is believed that a long period of time is usually needed for amyloidosis to become clinically manifested. The precise mechanisms by which amyloid deposits damage organ function are as yet undetermined. Many of the pathological effects of amyloid can be attributed to its physical presence: amyloid accumulates in the blood vessels and in the extracellular space, hindering exchanges and producing space-occupying effects and altered tissue architecture. However, clinical observations suggest that damage does not depend only on the amount of amyloid deposited.[8,12] Indeed, the relationship between the quantity of amyloid and the resulting functional disturbance is poor[11] and certainly other factors are involved. Many observations suggest that structured intermediates of amyloid fibrils may be responsible for the damaged organ function. Protofibrils, probably exposing several highly reactive sites, could bind tightly to any number of cellular targets, triggering, for example the apoptotic cascade.[13]

AL amyloidosis

Light-chain related (AL) amyloidosis is the most common form of amyloidosis in Western countries and the only one caused by a tumor. Though occasionally localized and susceptible to surgical eradication, AL amyloidosis is usually systemic, incurable and leads to organ failure and eventual death. A marrow plasma cell (PC) clone synthesizes structurally abnormal monoclonal light chains that form amyloid. Typically, numbers of clonal PC are small, proliferative activity minimal, and monoclonal protein concentration very low with lambda isotype predominance; this condition is termed 'primary amyloidosis'. In some cases, light chain-amyloidosis can complicate multiple myeloma (MM) and, occasionally, Waldenström's macroglobulinemia or B cell lymphomas, and it is sometimes difficult to distinguish between MM with light chain-amyloidosis and primary amyloidosis.[14]

Virtually any organ excluding the brain (but not brain vessels) can be targets of amyloid deposition, and in almost any combination. However, a single or a few organs dominate the clinical picture. Nephrotic syndrome and congestive heart failure (restrictive cardiomyopathy) are most frequently observed. Although AL is invariably fatal, prognosis varies widely according to the involvement of vital organs, and heart involvement is by far the most important adverse prognostic feature.[15] The current therapeutic approach is multifaceted, aimed at controlling the underlying PC dyscrasia,[8,16–19] at interfering with amyloid deposition,[20] and at providing the best supportive therapy.[8] Nevertheless, therapy is unsatisfactory with the median survival being between 2 to 3 years,[21,22]

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Table 1 The most common forms of amyloidosis and their characteristics

Type	*Fibril precursor protein*	*Clinical features*
AL	V region fragments of monoclonal light chains	Small indolent bone marrow PC clone (primary) or associated with myeloma or macroglobulinemia, (ratio of to , 3:1)
AA	SAA	Reactive amyloidosis associated with hereditary (familial mediterranean fever) or chronic inflammatory diseases, eg tubercolosis, rheumatoid arthritis, Castleman's disease
ATTR	Transthyretin	Familial (autosomal dominant mutations) or senile
A	A PP	Alzheimer's disease
A $_2$M	$_2$-microglobulin	Dialysis associated

AL, immunoglobulin light chain amyloidosis; AA, amyloid A protein amyloidosis; ATTR, transthyretin amyloidosis; A , Alzheimer's amyloidosis; A $_2$M, $_2$-microglobulin amyloidosis; SAA, serum amyloid A protein; A PP, amyloid protein precursor; V, variable region; PC, plasma cell.

and only a small subset of patients (5%) surviving for 10 years or more.[23]

Since only a few Bence–Jones proteins form amyloid, research efforts have been directed mainly towards characterizing the structural and biochemical features of amyloidogenic light chains.[24] Loss of variable (V) region domain stability because of amino acid substitutions particularly in framework,[25,26] but also in CDR regions,[27] appears to play a key role in fibril formation.[27,28] Much less attention has been focused on the study of the amyloid cell clone, though a better understanding of the tumor biology is of major importance for designing new therapeutic strategies.

The cellular composition of the amyloidogenic clone

The bone marrow is a major site of serum immunoglobulin (Ig) production, particularly IgG, and it is involved in T cell dependent antibody responses.[29–32] Plasmablasts selected for the improved antigen binding of their surface immunoglobulins leave the germinal centers and find the appropriate surface interactions with adhesion molecules and cytokine feeding in the bone marrow microenvironment, where they transform into immunoblasts and then develop into PC.[33] Marrow PC very seldom divide and probably live for long periods of time,[34] and the bone marrow milieu satisfies the requirements for their survival. Amyloidogenic PC probably have similar requirements: though clonal PC can be present in tissues and be responsible for localized forms of AL amyloidosis,[35–37] amyloidogenic PC most frequently reside in the bone marrow while amyloid deposits are systemic, caused by local deposition of circulating light chains. Monoclonal antibodies that recognize specific antigenic determinants on the tumor Ig-variable regions (private idiotopes) are particularly useful for studying the composition and antigenic phenotype of B cell clones. To this end, monoclonal anti-idiotypic antibodies were raised in mice by repeated immunization with the patients' monoclonal components.[38,39] These were used as probes to look for clonal cells in the patient's bone marrow and peripheral blood by immunofluorescence methods.[39] Results of these studies provided an initial elucidation of the clonal composition, phenotype and behavior.

The bone marrow amyloidogenic cells: lymphoid, lymphoplasmacytoid and plasma cells

Three types of amyloidogenic cells were present in the bone marrow: (1) lymphoid cells, which were slightly larger than common peripheral blood lymphocytes (47% CD45RA$^+$, 28% CD45RO$^+$, CD38, CD10, chain negative); (2) lymphoplasmacytoid cells with more abundant cytoplasm (53% CD38$^+$, CD45RA, CD45RO, CD10 negative); (3) PC that were very similar to normal PC in morphology and antigenic profile (CD38$^+$, PCA1$^+$, negative for CD56, CD10, CD20, CD45RA, CD45RO, HLA-DR). Plasma cells were mostly morphologically mature (high Ig content, large size, eccentric nuclei) and constituted a fraction (approximately 65–75%) of the PC expressing the same isotype. Cell kinetic analysis of mature PC revealed a minor, but significant, replicative activity (0.2–0.4% of cells in S-phase) in these patients, whereas normal PC do not incorporate bromodeoxyuridine (PC labeling index). Lymphoid and lymphoplasmacytoid idiotype positive cells constituted a relevant fraction (approximately 40–50%) of the amyloidogenic bone marrow clone. These results document that the amyloid clone infiltrates the bone marrow more extensively than estimated by simple morphological study.

The marrow PC labeling index was analyzed by the Mayo group[40] and failed to reveal proliferative activity in 76 of 103 (74%) amyloid patients without associated MM; PC labeling index 0 was a negative prognostic factor (median survival, 14.1 months). Since not all monotypic PC are clonal, but resting normal PC are present (labeling index = 0),[39] PC labeling index may be underestimated in AL.

The bone marrow PC percentage and intracytoplasmic light chain isotype ratio present a high degree of variability in AL amyloidosis. Infiltration is frequently minimal resembling normal bone marrow, and alteration of / ratio by immunofluorescence is of diagnostic[41,42] and prognostic value.[43] Sixty percent of patients have 10% of marrow PC[15,44] and median PC infiltration is about 7–8%.[21,43] Therefore, a 'normal' bone marrow PC count should not be used to exclude the diagnosis of AL. The degree of marrow infiltration negatively influences survival.[43,44]

The circulating amyloidogenic cells

Idiotypic monoclonal antibodies revealed a population of small lymphocytes (Figure 1) which constituted a minority (5–10%) of the total circulating B cells. By double-color immunofluorescence, Id+ cells expressed B cell associated antigens (CD19, CD20, CD22, CD45RA, HLA-DR) and surface Ig with the same isotype as the monoclonal component (post-switched B cells). Plasmacytoid (PCA1, CD38), T (CD3, CD5), monocytic (CD14), activation (CD10, CD25, CD38, CD71) and progenitor (CD34) markers were negative. These cells

Figure 1 Circulating clonal lymphocytes in AL amyloidosis. Membrane monoclonal immunoglobulins as revealed by indirect surface fluorescence using anti-idiotypic antibodies as probes. Double-staining showed positivity for the CD19, CD20 and CD22 pan-B markers.

were also negative for CD45RO, a T cell marker also present on B cells nearing the plasma cell stage. Probing with the anti-idiotypic antibodies failed to reveal circulating clonal plasma cells in the cases studied. However, alteration of the / ratio can be sometimes found in the peripheral blood PC of primary amyloidosis patients (12% of 92 cases), suggesting that a minor population (median 0.5 × 10⁶/l) of clonal mature cells can occasionally be found.[45] Cell-cycle analysis failed to detect any proliferative activity in the circulating amyloid elements, both in lymphocytes[39] and plasma cells.[45]

The involvement of peripheral blood in AL amyloidosis has also been studied by tumor-specific PCR. Using very sensitive RT-PCR procedures with primers derived from the CDR sequence of the patient's amyloid light chain, clonal elements were found in virtually all patients at diagnosis (eight of 10)[46] and in apheretic peripheral blood stem cell preparations.[46,47] Quantitative heavy chain V region-based PCR demonstrated that circulating tumor cells were rare (0.01 and 0.07% clonal cells in the two cases tested), a level of involvement similar to that found in MGUS.[48] Of course, we have no information on the nature of the cells detected by these methods, but they seem to be clinically relevant because they persist after cyclophosphamide mobilization[46] and high-dose chemotherapy (unpublished observation). As in MM, circulating elements may be therefore chemoresistant and contribute to relapse.[49,50]

The relationship between the various amyloid elements

The amyloid clone diffuses systemically and comprises cells at various stages of late B cell differentiation. The morphology and immunophenotype of the circulating amyloid lymphoid cells are compatible with post-switched B lymphocytes. Similarly to multiple myeloma,[51] a combination of IL-3 and IL-6 sustained the *in vitro* differentiation of these cells to PC that were very similar to those present in the bone marrow.[39,52] Anti-idiotypic antibodies plus complement[39] or immunotoxins to CD22,[52] a marker which is absent from PC, were capable of inhibiting the differentiation process, thus indicating that surface Ig/CD22 positive lymphocytes were responsible for the generation of the amyloid clonal PC *in vitro*. These results suggest a close relationship between the various amyloidogenic cell populations. In analogy to what is thought to occur during the normal immune humoral response, it is tempting to hypothesize that the circulating lymphoid elements are the precursors of the more differentiated bone marrow cell population: these cells should home to the bone marrow where they transform into PC, via the intermediate differentiation stages constituted by the lymphoid and lymphoplasmacytoid cells identified by the anti-idiotypic antibodies. A similar intraclonal differentiation process has been proposed for MM,[53,54] and Waldenström's macroglobulinemia,[55,56] and may therefore be common to monoclonal gammapathies. Figure 2 illustrates the proposed model of intraclonal differentiation for AL amyloidosis. However, the nature of the clonal stem cell, as well as the tumor proliferative compartment, are still elusive. Animal experimental models, such as those recently described for MM,[57,58] will perhaps help to clarify these and other issues.

Karyotypic and other genetic abnormalities in AL amyloidosis

Classic cytogenetic analysis has been hampered by the scarcity of the bone marrow PC infiltration, the fact that these cells have low proliferative activity, and the possible detection of abnormalities in non-amyloid cells especially in heavily treated patients. In a cytogenetic study, Dewald *et al*[59] detected chromosome abnormalities in five of 13 patients with AL amyloidosis. The significance of these findings was, however, confounded by the subsequent development of acute non-lymphoblastic leukemia in three of the five patients and it was therefore uncertain whether the observed anomalies were present in the amyloid clone or in other myeloid cells. Only recently, by means of a technique coupling simultaneous fluorescent staining of the monotypic cytoplasmic immunoglobulin and interphase FISH,[60] Fonseca *et al*[61] tested bone marrows from 21 patients with AL amyloidosis for numerical chromosome anomalies, using centromere-specific probes for six chromosomes frequently reported as abnormal in MM.[62] In all patients studied, nine of whom had received previous chemotherapy and one with associated MM, at least one

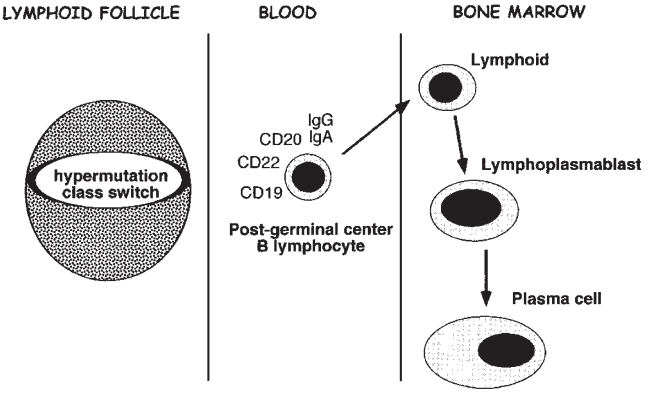

Figure 2 Hypothetical model of intraclonal differentiation in primary amyloidosis. The model is supported by *in vitro* data. The amyloid clone is constituted by post-germinal center elements: circulating mature post-switched B lymphocytes, bone marrow lymphoid, lymphoplasmacytoid cells and plasma cells. B cell antigens expressed on circulating clonal cells are reported. Neither the clonal stem cell nor the tumor proliferative compartment has been identified.

chromosomal abnormality was seen. In contrast, standard cytogenetic analysis was negative in 18 of the 20 cases (90%) in which adequate metaphases could be obtained. Multiple numerical chromosomal abnormalities were detected by FISH. Loss of chromosome 18 was particularly common in AL (72% of cases), a lesion not frequently detected in MM (6–11% of cases).[62] Coexistence of trisomy for different chromosomes, as well as trisomy and monosomy for a given chromosome, were present in several samples, thus indicating the presence of cytogenetic subclones of amyloidogenic plasma cells in a given patient, as shown in MGUS.[63] Alterations were similarly frequent in both treated and untreated patients and were therefore not therapy-related. No significant difference was observed in the prevalence of these abnormalities between AL and MGUS. Genomic instability is therefore common in AL. It will be of interest to test whether specific chromosome abnormalities have an impact on survival. In fact, although survival in AL is obviously related to the amyloid organ distribution, it is also related to therapy response, and biological variables are needed to identify those patients who are more likely to benefit from high-dose chemotherapy,[64] which is a risky therapy.[65]

Chromosomal translocations involving the Ig heavy chain locus 14q32 are common in MM and MGUS.[66,67] Several partner chromosomes are involved, but translocation at 4p16.3 is of particular interest because it leads to the apparent deregulation of two potential oncogenes, namely *FGFR3*[68,69] and *MMSET*.[70] As consequence of translocation, *IGH/MMSET* fusion transcripts are generated,[70] and these can be detected by a sensitive RT-PCR assay.[71] We recently investigated bone marrow from AL patients for the presence of *IGH/MMSET* hybrid transcripts and found that 14% of cases were positive for t(4;14)(p16.3;q32) translocation (Perfetti *et al*, manuscript in preparation). A frequency of approximately 20% was reported in MM.[71] The t(4;14) translocation appears therefore to be a recurrent genetic lesion in AL, underlying the concept that *IGH* translocations may represent early pathogenic events in plasma cell disorders.[66]

The persistence of neoplasias stands on a balance between proliferation and apoptosis of tumor cells. Many cytokines act on both these factors: they favor proliferation and prolong survival via inhibition of apoptosis. Apoptosis inhibition is therefore probably important, but this problem is only now being investigated. In a recent report, Witzig *et al*[72] developed a PC growth index that related both proliferation and apoptosis and found that there were higher proliferation and lower apoptosis rates in myeloma than in MGUS and AL; it will be of interest to test whether apoptosis in amyloid PC is lower than in normal PC, which already live for long periods of time.[34] Analysis of Bcl-2 and of other genes or proteins involved in apoptosis has not been performed in AL amyloidosis.

Angiogenesis and HHV-8

IL-6 is the major growth and survival (anti-apoptotic) factor in myeloma,[73] and probably in AL as well. Indeed, sorted amyloid PC were reported to express IL-6 receptor mRNA,[74] and a combination of IL-6 and IL-3 drove clonal circulating lymphocytes to differentiate into PC *in vitro*.[39] Amyloid PC did not express IL-6 mRNA[74] thus other cells in the bone marrow could be the source of IL-6. Kaposi's sarcoma-associated herpesvirus (KSHV), a virus found within tumor tissue in Kaposi's sarcoma, Castleman's disease, and primary effusion lymphomas, bears several genes with functional homology to cellular genes, including IL-6.[75] An association between KSHV and bone marrow dendritic cells in MM was reported.[76,77] Since viral IL-6 is able to sustain survival and proliferation of myeloma cells, albeit with much less efficiency than the human counterpart,[78] these findings led to the attractive hypothesis that KSHV was involved in MM and related PC dyscrasias through infection of marrow dendritic cells and elaboration of viral IL-6 and other cytokine products.[76] Very recently,[79] KSHV DNA sequences were found, albeit at a very low copy number, in PCR-amplified DNA from bone marrow biopsy samples and long-term bone marrow stromal cell cultures of a majority (85%) of amyloid patients. On the other hand, antibodies to KSHV were observed only in a fraction of positive cases (22%). Since the presence and role of KSHV in plasma cell dyscrasias remains controversial,[80–81] the association between KSHV and amyloidosis warrants further investigation.

KSHV is associated with increased vascularization[82] and neoangiogenesis is observed in marrow from patients with active myeloma.[83] These observations suggested the use of the anti-angiogenetic drug thalidomide to overcome chemorefractory myeloma. Indeed, thalidomide proved to be effective in a portion of myeloma patients who had relapsed after conventional therapy and autologous transplantation.[84] Thalidomide toxicity was mild and trials are ongoing in AL as well.

The amyloid V region nucleotide sequences and clonal origins – germline gene usage and organ tropism

Somatic mutations of Ig V regions are a marker of germinal center origin, and uniformity of such mutations indicates that the cell has stopped mutating and left the germinal center: ie it is a post-germinal center cell. Cells which are selected for the improved functionality of their Ig have a peculiar distribution of replacement and silent mutations in the V region: amino acid replacing substitutions are concentrated in the antigen contact regions, the CDR, whereas silent substitutions are found more frequently than expected in the framework regions, areas of structural relevance.[85,86] The study of the V region nucleotide sequences of a B cell clone can therefore provide useful information on its ontogenesis, giving essential clues to the nature of the cell of origin and on the circumstances that accompanied its development.[87,88]

The elaboration of a novel inverse-PCR strategy[89] that utilizes only primers for constant regions overcame difficulties in Ig V region sequencing. Amyloid V regions were found to be highly mutated compared to the closest germline genes in the databases or those isolated from the patients' DNA;[90] the latter were found to be identical to the germline genes present in the general population, ie mutations were all acquired and there is no apparent predisposition to develop amyloidosis at the VL germline gene level.[90] No amyloid light chain V region isolated so far is in germline configuration, but the extent of somatic changes appears to vary widely.[90] It is unclear whether deviation from the germline is greater in amyloid VL regions than in non-amyloidogenic ones. Mutations were not associated with intraclonal diversification, every clonal cell expressed VL regions sharing identical somatic mutations, ie the clone was no longer under the influence of the hypermutation process in the germinal center.[90] These findings provided evidence that the earlier amyloid cell could not possibly be a germinal or pre-germinal center cell, in agreement with the cells types identified in the immunophenotypic and functional studies employing the anti-idiotypic monoclonal antibodies (Figure 2).[39]

An important point was to test whether amyloidogenic light chains undergo the same antigen-mediated affinity selection process seen in non-pathological light chains.[85] Analysis of the nature and distribution of somatic mutations in amyloid V regions revealed significant evidence of antigen selection in a substantial proportion of cases (eight of 14).[90] These results indicate that amyloid clones developed from post-germinal center B cells selected for improved antigen binding properties, and that pathogenic light chains did not appear to result from a quality control failure. At some point in its history, the amyloid light chain was part of an antibody capable of improved functional binding to a T cell-dependent antigen.

Amyloid V region protein sequencing[91] and ELISA typing with monoclonal antibodies specific for the various VL germline gene families[92] demonstrated a strong association between the V VI family and amyloidosis: with rare exceptions, VI monoclonal light chains isolated so far are found in patients with AL. This rare subgroup of light chains seems to constitute approximately 5% of the circulating light chains,[93] but apparently up to 40% of the amyloidogenic ones.[92] A very recent genetic analysis reported, in a selected population undergoing high-dose chemotherapy, an association between the usage of the VI family and predominant or exclusive amyloid involvement of kidney (14/14 cases).[94] This work raised the possibility that the diverse organ tropism that influences the clinical picture of amyloidosis may be ruled by gene usage. We recently undertook the first analysis of V germline gene usage in a general population of AL patients[95] and found that eight of the 11 VI patients (73%) had major or exclusive kidney involvement (Perfetti *et al*, manuscript in preparation). It will be of interest to test whether gene usage may influence prognosis and, particularly, amyloid heart disease.

Therapeutic implications

Useful information can be drawn from the reported studies. Despite the fact that amyloid light chains are synthesized only by PC resident in the bone marrow, earlier clonal cells are also involved; circulating amyloid elements are present at diagnosis and in apheretic stem cell preparations, and reinfusion of clonal cells might contribute to recurrence after high-dose chemotherapy.[16] *Ex vivo* purging strategies, such as positive selection for $CD34^+$ cells[47] and/or negative selection for $CD19^+/CD20^+$ lymphocytes in stem cell preparations, may be employed. The very low proliferative activity of the amyloid elements suggests the need for strategies active on non-cycling cells, such as immunotherapy. In this respect, the cytotoxicity of the bispecific anti-CD22/anti-saporin antibodies against myeloma and amyloid circulating elements[52] convinced us to initiate a pilot study in patients with chemoresistant AL, but early development of strong human anti-mouse and anti-toxin antibody responses impeded full exploitation of the therapeutic potential of this immunotoxin (unpublished observations). Analogous immune responses were observed in three of the five myeloma patients treated with murine anti-CD19-blocked ricin.[96] The use of the anti-CD20 chimeric human–mouse antibody, Rituxan, may overcome these problems, though activity appears to be limited to the small fraction of myeloma patients with $CD20^+$ PC.[97] The immune system in monoclonal gammapathies may therefore be less altered than expected, and this offers a rationale for the development of anti-tumor vaccines. A trial of dendritic cell-based idiotype vaccination in AL has been started at the Mayo Clinic and one of the five patients has had a documented hematologic complete response.[98] In this same patient, specific T cell proliferative responses to idiotype were detectable. No toxicity was seen in any of the cases. This strategy may be particularly useful when tolerance is expected to be reduced as in the context of minimal residual disease.

In conclusion, the studies reviewed in this paper provided clues to a better understanding of the pathobiology of the amyloid clone. Much remains to be learned, but future advances in AL therapy will probably stem from progress in this field.

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