

19. Lee K-s, Ferguson RM, Corpuz M, Gartner LM. Maternal age and incidence of low birth weight at term. *Am J Obstet Gynecol* 1988; 158:84-9.
20. Kiely JL, Paneth N, Susser M. An assessment of the effects of maternal age and parity in different components of perinatal mortality. *Am J Epidemiol* 1986; 123:444-54.
21. Naeye RL. Maternal age, obstetric complications, and the outcome of pregnancy. *Obstet Gynecol* 1983; 61:210-6.
22. Hardy JB, Mellits ED. Relationship of low birth weight to maternal characteristics of age, parity, education and body size. In: Reed DM, Stanley FJ, eds. *The epidemiology of prematurity: proceedings of the epidemiology workshop held at NICHD/NIH, Bethesda, Md., November 8-9, 1976*. Baltimore: Urban & Schwarzenberg, 1977:105-17.
23. Kramer MS. Determinants of low birth weight: methodological assessment and meta-analysis. *Bull WHO* 1987; 65:663-737.
24. Berkowitz GS. Clinical and obstetric risk factors for preterm delivery. *N Engl J Med* (NY) 1985; 52:239-47.
25. Harlap S, Shiono PH, Ramcharan S. A life table of spontaneous abortions and the effects of age, parity, and other variables. In: Porter IH, Hook EB, eds. *Human embryonic and fetal death*. New York: Academic Press, 1980:145-58.
26. Hook EB. Rates of chromosome abnormalities at different maternal ages. *Obstet Gynecol* 1981; 58:282-5.
27. Bulmer MG. The effect of parental age, parity and duration of marriage on the twinning rate. *Ann Hum Genet* 1959; 23:454-8.

MARKERS OF MULTIPLE HEMATOPOIETIC-CELL LINEAGES IN MULTIPLE MYELOMA

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Abstract Multiple myeloma is considered a cancer of mature plasma cells. Recent studies, however, suggest the possible involvement of early B cells and the expression of myelomonocytic antigens by myeloma cells. Using flow cytometry, we searched for evidence of the expression of genes specific for different hematopoietic lineages by tumor cells in bone marrow aspirates from 27 patients with aneuploid multiple myeloma. In addition to features characteristic of myeloma cells, we found evidence of the frequent expression by myeloma tumor cells of the pre-B-cell antigen CALLA (common acute lympho-

cytic leukemia antigen) (in specimens from 58 percent of patients) and of megakaryocytic (88 percent), myelomonocytic (65 percent), and erythroid (39 percent) surface markers. The proportion of tumor cells expressing the different markers varied among patients, from 2 to 100 percent of recognizable tumor cells.

We conclude that cells of multiple lineages are involved in myeloma — a finding that is consistent with the hypothesis that there is a common primary neoplastic lesion for all hematologic cancers. (*N Engl J Med* 1990;322:664-8.)

DIFFERENTIATION of hematopoietic progenitor cells into lineages is accompanied by the activation of genes that regulate the expression of surface antigens specific to each cell line and each stage of differentiation. These antigens, known as surface markers, are used in the diagnosis and classification of hematologic neoplasias because they indicate the lineage and stage of differentiation of the tumor cells.¹

Multiple myeloma is a B-cell cancer, typically associated with mature plasma-cell morphology and function. There is, however, increasing evidence of the involvement of early B lymphocytes in myeloma.²⁻⁸ Among the evidence for the presence of neoplastic pre-B cells in myeloma is the expression of the pre-B-cell antigen CALLA (common acute lymphocytic leukemia antigen), often without cytoplasmic immunoglobulins, in the bone marrow of about 50 percent of patients with myeloma,²⁻⁴ the presence of pre-B cells in peripheral blood,⁵ and the differentiation of CALLA-bearing lymphocytes to monoclonal plasma cells with concordant isotypes.⁶ More recent studies have demonstrated the presence of myeloid antigens on myeloma cells and found them to be associated with a poor prognosis.⁹ The presence of tumor cells expressing myeloid antigens in this B-cell neoplasia suggests the involvement of other hematopoietic

cell lines and supports the hypothesis that there is a common primary neoplastic lesion in all hematologic cancers. To determine how frequently and to what extent different hematopoietic lineages are involved in plasma-cell myeloma, we measured the expression of lineage-associated genes by tumor cells in bone marrow aspirates from patients with myeloma. We used dual-parameter flow cytometry to identify the expression of megakaryocytic, myelomonocytic, and erythroid markers by aneuploid myeloma cells.

METHODS

Patients

We studied 27 patients with a diagnosis of DNA-aneuploid myeloma. All the patients gave informed consent, and the study was approved by the human studies committee of the University of Arkansas institutional review board. Twelve patients were studied at the time of diagnosis, before the initiation of treatment, and six at relapse. Seven patients with disease that was unresponsive to therapy were studied after the end of treatment, and two who had responded to treatment were studied between courses of chemotherapy. Bone marrow aspirates treated with heparin were subjected to Ficoll-Hypaque density separation (specific gravity, 1.077 g per cubic centimeter). Light-density mononuclear cells collected from the plasma-Ficoll-Hypaque interface were examined for ploidy and monoclonal cytoplasmic immunoglobulin by DNA-cytoplasmic immunoglobulin Row cytometry as previously described.¹⁰ Because there were insufficient quantities of cells to perform all the cell assays on specimens from all patients, the order of laboratory studies was randomly assigned so as to minimize inadvertent bias.

Reagents

The expression of myeloid, megakaryocytic, erythroid, and lymphoid antigens by myeloma tumor cells was probed with monoclonal antibodies. Table 1 lists the antibodies used, the antigen and

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Table 1. Probes for the Expression of Lineage-Associated Genes.

ANTIBODY	ANTIGEN	LINEAGE	SOURCE*	ASSAY
J-5	CD10	Lymphoid, myeloid	Coulter	Direct
8-1	GPIIb/IIIa	Megakaryocytic	Coulter	Direct
1b1	CD11b	Myelomonocytic, T-cell subset	Coulter	Direct
ly9	CD33	Myeloid	Coulter	Indirect
PCA-1	26-kd Antigen	Plasma cells	Coulter	Indirect
b39	Glycophorin A	Erythroid	Griffin	Indirect

*Coulter denotes Coulter Immunology, Hialeah, Fla.; the antibody to glycophorin A was a gift from Dr. J. Griffin of the Dana-Farber Cancer Institute, Boston

lineage they recognized, their source, and whether direct or indirect immunofluorescence assays were employed. All the monoclonal antibodies were used under the conditions recommended by the suppliers. Isotype controls were obtained from the same sources and used under identical conditions.

Flow Cytometry

All studies were carried out with use of dual-parameter flow cytometry. Initially, flow cytometry was used to determine the phenotype of aneuploid cells. Aliquots containing 0.5 to 1.0×10^6 mononuclear cells were reacted with fluorescein isothiocyanate-conjugated monoclonal antibodies, washed, and fixed in 70 percent ice-cold ethanol for 1 to 14 hours. The cells were then washed, treated with ribonuclease, counterstained with propidium iodide, and analyzed on a FACScan flow cytometer (Becton Dickinson) as previously described. When fluorescein isothiocyanate-conjugated antibody was not available, a second antibody, fluorescein isothiocyanate-

conjugated rabbit antimouse immunoglobulin (Dako), was used in an indirect assay. To identify coexpression of surface antigens and cytoplasmic light chain, cells were reacted with monoclonal antibodies and fixed as described above, then reacted with phycoerythrin-conjugated goat antihuman light-chain F(ab')₂ fragments (Tago). To analyze the coexpression of nonlymphoid and plasma-cell-specific antigens, cells were reacted with the purified monoclonal antibody PCA-1 and then with phycoerythrin-conjugated goat antimouse immunoglobulin, blocked with normal mouse serum, and finally reacted with the second fluorescein isothiocyanate-conjugated monoclonal antibody. For each sample, isotype-matched and, when indicated, opposite-light-chain controls were run simultaneously. In each case, overlap of emission spectra was compensated for electronically with use of the proper control samples.

RESULTS

The results of our analysis of the expression of lymphoid, megakaryocytic, erythroid, and myelomonocytic antigens by DNA-aneuploid myeloma tumor cells are illustrated in Figure 1. In addition to reacting with the anti-CD10 antibody J-5, cells in the hyperdiploid compartment also expressed the glycoprotein IIB-IIIa complex (GPIIb/IIIa), CD11b, and CD33. The reactivity with anti-glycophorin A was questionable. The different antigens were expressed independently, as illustrated in Figure 1B, where hypodiploid myeloma cells expressing GPIIb/IIIa showed no reactivity with the anti-CD11b reagent Mol. Table 2 summarizes the results of phenotype

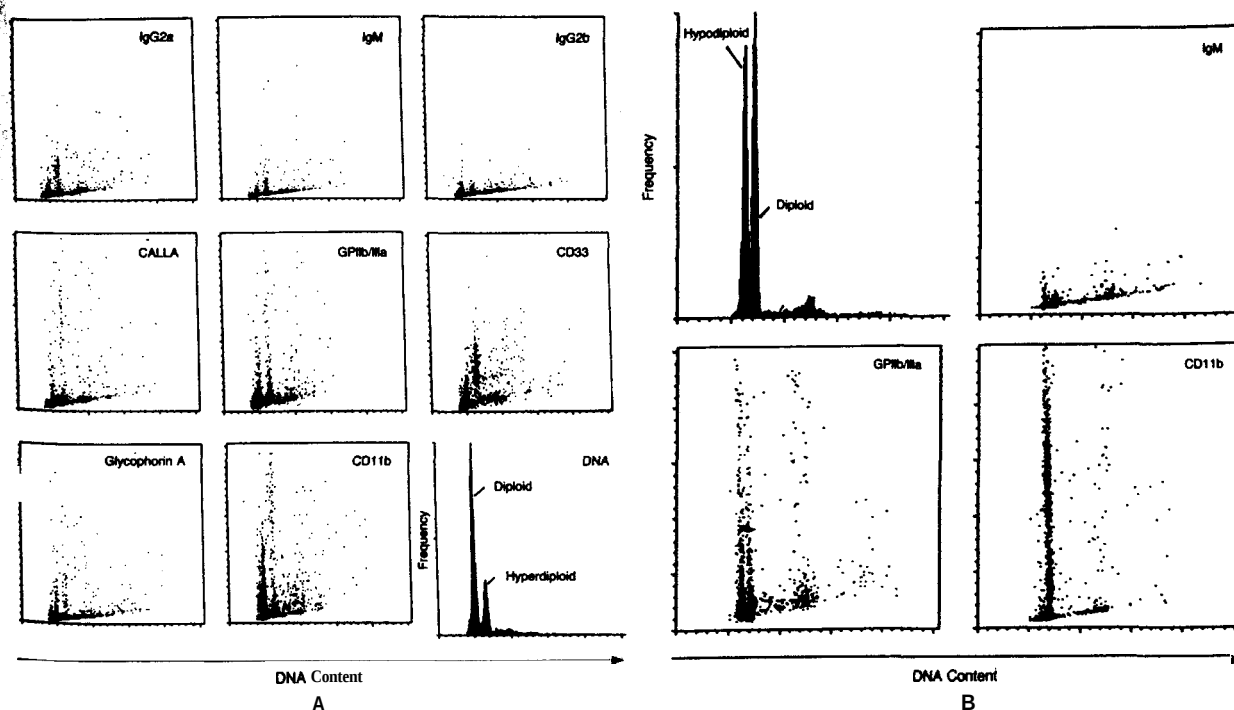


Figure 1. Multilineage Antigen Expression in Aneuploid Myeloma.

A. Panel A shows, tumor cells, identified by their hyperdiploid DNA content, reacted positively with antibodies against CALLA (11 percent Positive), GPIIb/IIIa (10 percent), CD33 (57 percent), and CD11b (13 percent). Reactivity with antibody to glycophorin A was inconclusive. The results for isotype-matched controls are shown in the top row. The abscissa indicates the degree of red fluorescence of propidium iodide as an indicator of DNA content. The ordinate indicates the frequency of green fluorescence of fluorescein isothiocyanate as an indicator of the phenotype. Hypodiploid tumor cells (Panel B), although they expressed the megakaryocytic antigen GPIIb/IIIa, did not react with the anti-CD11b antibody Mol.

analyses of cells from all 27 patients. Expression of CD10 was recorded in samples from 58 percent, GPIIb/IIIa in 88 percent, CD11b in 65 percent, and glycophorin A in 39 percent of the patients studied. The proportion of myeloma tumor cells expressing the different markers varied from patient to patient; CALLA was detected in a mean ($\pm$ SD) of 15 ± 10 percent (median, 9 percent; range, 5 to 31 percent), GPIIb/IIIa in 53 ± 37 percent (median, 32 percent; range, 7 to 100 percent), CD11b in 38 ± 34 percent (median, 20 percent; range, 5 to 100 percent), and glycophorin A in 27 ± 36 percent (median, 7 percent; range, 2 to 98 percent). In addition to these antigens, CD33 was expressed by tumor cells from all four patients examined.

Since in many cases only a fraction of the aneuploid cells reacted with the various antibodies, it was important to identify the coexpression of characteristically myeloma-associated and nonlymphoid markers. Figure 2 shows the results of such a study in a patient with marked marrow plasmacytosis (88 per-

Table 2. Expression of Lineage-Associated Genes by Myeloma Tumor Cells.

PATIENT CATEGORY	ANTIGEN			
	CD10	GPIIb/IIIa	CD11b	GLYCOPHORIN A
	no. expressing antigen/no. studied			
Studied at diagnosis	1/12	11/12	7/12	3/10
Studied at relapse	2/4	3/5	2/5	3/5
Resistant*	3/6	7/7	6/7	2/6
Other†	2/2	2/2	2/2	1/2
Total	14/24	23/26	17/26	9/23

*Patients with disease that was unresponsive to treatment, studied after treatment ended.

†Patients who responded to treatment who were studied between courses of chemotherapy.

cent of plasma cells) whose hyperdiploid tumor cells expressed both PCA-1 and GPIIb/IIIa. Although PCA-1 was expressed by 83 percent and GPIIb/IIIa by 20 percent of the cells (Fig. 2A), both antigens were present in 16 percent of all cells (Fig. 2B). Similarly, Figure 3 shows the coexpression of cytoplasmic kappa light chain and megakaryocytic, myelomonocytic, and erythroid antigens in bone marrow cells from a patient with substantial marrow plasmacytosis (90 percent of cells) and a high proportion of cells (88 percent) containing cytoplasmic immunoglobulin-kappa. Cytoplasmic immunoglobulin-kappa was coexpressed with GPIIb/IIIa, glycophorin A, and CD11b by 10, 22, and 16 percent of cells, respectively.

DISCUSSION

To evaluate the possible involvement of several hematologic cell lineages in this B-cell neoplasia, we analyzed the expression by myeloma tumor cells of genes that are specific for hematopoietic cell lines and for particular stages of differentiation. The expression of such markers has been a useful tool in the classification of hematologic cancers. The close association between the unexpected expression of lineage and differentiation markers and the clinical features of some

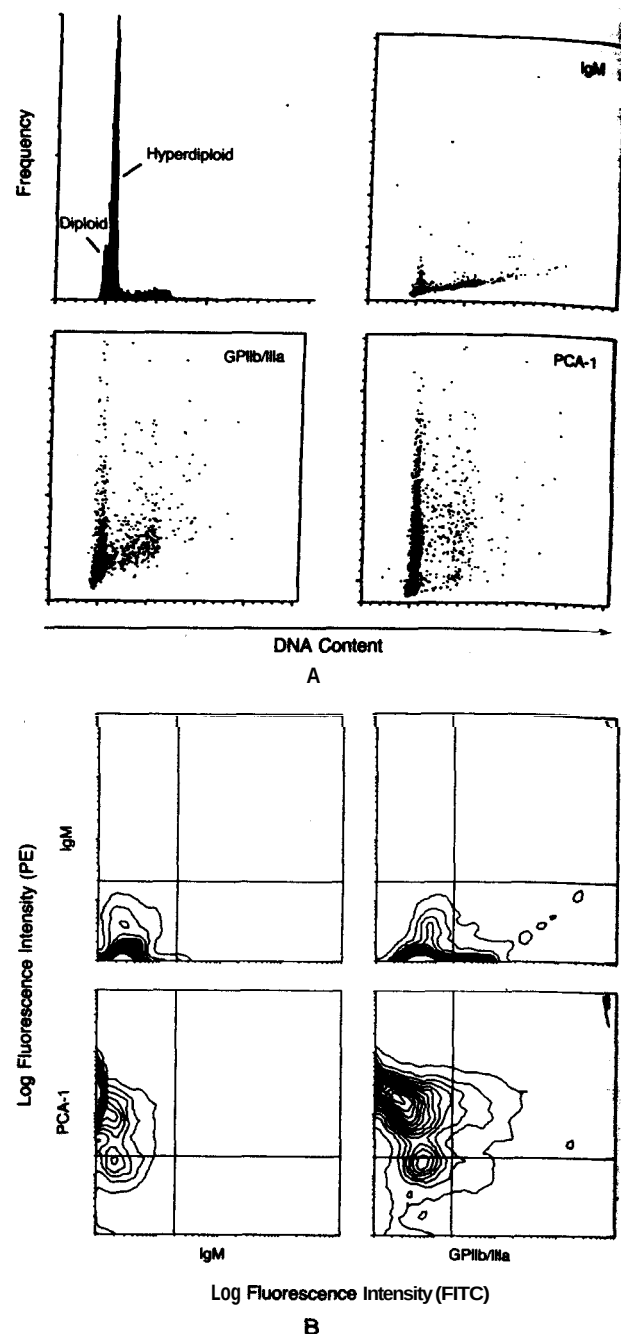


Figure 2. Coexpression of Plasma-Cell Antigen (PCA-1) and Megakaryocytic Antigen (GPIIb/IIIa) in a Patient with Myeloma. In the correlated DNA-phenotype analysis (Panel A), hyperdiploid cells expressed GPIIb/IIIa and PCA-1. Panel B shows contour plots representing the density distribution of cells analyzed for the expression of PCA-1 and GPIIb/IIIa. The lower right-hand section represents cells that were reacted with antibodies to both antigens. Within the lower right-hand section, PCA-1-positive, GPIIb/IIIa-negative cells are represented in the upper left quadrant, PCA-1-negative, GPIIb/IIIa-positive cells in the lower right quadrant, and PCA-1-positive, GPIIb/IIIa-positive cells in the upper right quadrant. The lower left quadrant shows cells negative for both PCA-1 and GPIIb/IIIa. The upper left-hand section shows cells reacted with the isotype-matched control immunoglobulins for both antibodies. The upper right-hand and lower left-hand sections show the results for individual controls. Both antibodies were IgM. PE denotes phycoerythrin, and FITC fluorescein isothiocyanate.

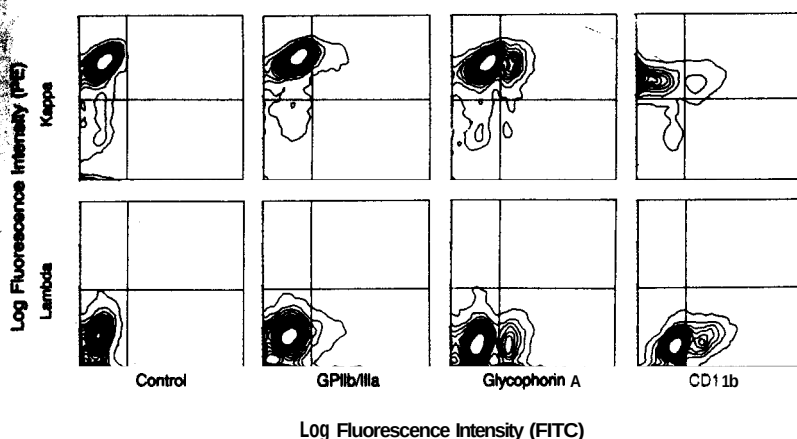


Figure 3. Density Distribution of Cells from a Patient with a Kappa-Expressing Diploid Myeloma Analyzed for the Expression of Monomorphic Cytoplasmic Immunoglobulin and Myelomonocytic, Megakaryocytic, and Erythroid Antigens.

The top and bottom rows represent cells reacted with anti-kappa and anti-lambda light chains, respectively. Within each panel, the upper left quadrant contains cells that reacted with the ordinate antibody, the lower right quadrant cells that reacted with the abscissa antibody, and the upper right quadrant cells that reacted with both antibodies and hence coexpress the two antigens. Cells that reacted with neither antibody are shown in the lower left quadrant. PE denotes phycoerythrin, and FITC fluorescein isothiocyanate.

hematologic tumors indicates that at least in some instances, the expression of these genes may have a role in the evolution of disease and should not be dismissed as an aberration, coincidental to the neoplasia or reflecting a loss of regulatory control associated with malignant transformation. The expression of pre-B-cell and myeloid antigens by myeloma cells has prognostic implications,^{2,4,9} and the expression of CALLA led to the understanding of pre-B-cell involvement in a disease typically seen as a tumor of mature B cells.^{2,3,6} The expression of megakaryocytic and erythroid genes, in addition to lymphoid and myeloid genes, by myeloma cells is now being reported by investigators using DNA ploidy, cytoplasmic immunoglobulin content, and the presence of a plasma-cell-specific antigen as three independent markers of myeloma tumor cells.

Although it was not the purpose of this study to elucidate the prognostic implications of our findings, the presence of the different markers appears to be unrelated to the disease stage. The observed frequency of "lineage infidelity" reflected by the expression of multilineage-associated genes by myeloma cells in this study (39 to 81 percent) suggests that this phenomenon is common. The absence of multilineage involvement in some patients probably reflects the low density of antigen presentation — below the level that can be detected by the available methods — on the surface of myeloma plasma cells. This phenomenon is best exemplified by the My9 antibody; no reactivity was detected when the fluorescein isothiocyanate-conjugated reagent was used, but myeloma cells expressing CD33 were readily detected in all four bone marrow samples studied with the more sensitive indirect method. Differences in the sensitivity of the methods may also account for the varying

proportions of tumor cells found to express the different antigens.

In a similar study of patients with myeloid leukemia, the association of lineage-specific antigens with specific cytogenetic anomalies was used to demonstrate the neoplastic involvement of morphologically normal lineages, suggesting that a multipotent hematopoietic progenitor cell was involved in the disease." In contrast, the coexpression of typical myeloma-cell features and nonlymphoid antigens might be interpreted as ruling out the presence of megakaryocytes and myeloid and erythroid cells in the DNA-aneuploid cell compartment; this suggestion is compatible with the apparently normal hematopoiesis observed in patients with myeloma. Rather, it suggests that the primary neoplastic lesion in plasma-cell myeloma occurs in an early hematopoietic progenitor ca-

pable of differentiation into the various lineages. This hypothesis is supported by a previous report of rearrangements of T-cell receptor gamma-chain genes and invariant chain expression in myeloma.¹²⁻¹⁴ It is still possible that, as observed in the study of myeloid leukemia," different lineages may bear yet unidentified genetic abnormalities associated with myeloma. Taken together, all the studies reporting lineage and differentiation infidelity and multilineage involvement in hematologic cancers could imply the existence of a common neoplastic progenitor for all hematologic tumors, with further events determining the phenotypic presentation of the disease.

The involvement of multiple lineages provides clues for mapping the sequence of the transforming events in terms of the development of the hematopoietic system. The high incidence of multilineage involvement in myeloma as well as other hematologic cancers could validate the sequential model of lineage commitment during hematopoietic development¹⁵ by suggesting that the neoplastic cells express genes representing the "stations" they have traversed during differentiation and lineage commitment. The coexpression of various lineage-associated genes by tumor cells could suggest that cells with less stringent differentiation, which are likely to be present in small numbers and for short periods during normal hematopoietic development, are immortalized by the neoplastic transformation. Alternatively, cells bearing neoplastic lesions could have lost the linkage between lineage commitment and the expression of surface molecules that determine microenvironmental interactions (e.g., homing), resulting in the many manifestations of each hematologic cancer.

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REFERENCES

1. Foon KA, **Todd RF** III. Immunologic classification of leukemia and lymphoma. *Blood* 1986; 68:1-31.
2. Epstein J, Barlogie B, Katzmann F, Alexanian R. Phenotypic heterogeneity in aneuploid multiple myeloma indicates pre-B cell involvement. *Blood* 1988; 71:861-5.
3. **Grogan TM**, **Durie BG**, Lomen C, et al. Delineation of a novel pre-B cell compartment in plasma cell myeloma: immunochemical, immunophenotypic, genotypic, cytologic, cell culture, and kinetic features. *Blood* 1987; 70:932-42.
4. **Durie GM**, **Grogan TM**. CALLA-positive myeloma: an aggressive subtype with poor survival. *Blood* 1985; 66:229-32.
5. Pilarski LM, Mant MJ, Ruether BA. Pre-B cells in peripheral blood of multiple myeloma patients. *Blood* 1985; 66:416-22.
6. Caligaris-Cappio F, Bergui L, Tesio L, et al. Identification of malignant plasma cell precursors in the *bone marrow* of multiple myeloma. *J Clin Invest* 1985; 76:1243-51.
7. Ruiz-Arguelles GJ, Katzmann JA, Greipp PR, Gonchoroff NJ, Garton **JP**, Kyle RA. Multiple myeloma: circulating lymphocytes that express plasma cell antigens. *Blood* 1984; 64:352-6.
8. Mellstedt H, **Hammarstrom S**, Holm G. Monoclonal lymphocyte population in human plasma cell myeloma. *Clin Exp Immunol* 1974; 17:371-84.
9. **Grogan TM**, **Durie BG**, Spier CM, Richter L, Vela E. Myelomonocytic antigen positive multiple myeloma. *Blood* 1989; 73:763-9.
10. Barlogie B, Alexanian R, Pershouse M, Smallwood L, Smith L. Cytoplasmic immunoglobulin content in multiple myeloma. *J Clin Invest* 1985; 76:765-9.
11. Keinänen M, Griffin JD, Bloomfield CD, Machnicki J, de la Chapelle A. Clonal chromosomal abnormalities showing multiple cell-lineage involvement in acute myeloid leukemia. *N Engl J Med* 1988; 318:1153-8.
12. Lee M, Selvanayagam P, Alexanian R, Stass S, Barlogie B. Rearrangement (REARR) of T-cell receptor (TcR) γ chain gene in multiple myeloma (MM). *Proc Am Assoc Cancer Res* 1987; 28:178. abstract.
13. Nami F, Selvanayagam P, Barlogie B, Saunders GF. HLA-associated invariant chain gene expression in human B cell neoplasia. *Leukemia* 1987; 1:504-7.
14. Barlogie B, Epstein J, Selvanayagam P, Alexanian R. Plasma cell myeloma — new biological insights and advances in therapy. *Blood* 1989; 73: 865-79.
15. Brown G, Bunce CM, Howie AJ, Lord JM. Stochastic or ordered lineage commitment during hemopoiesis? *Leukemia* 1987; 1:150-3.

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