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ORIGINAL PAPER

# Quantification of Dendritic Cells and Osteoclasts in the Bone Marrow of Patients with Monoclonal Gammopathy

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**Abstract** The purpose of this study was to find histological clues for reliable differentiation between monoclonal gammopathy of undetermined significance (MGUS) and myeloma when clinical parameters are controversial. Differential appearance of dendritic cells and osteoclasts, two cell types developing from the monocytic lineage upon distinct cytokine activation profile, might be a useful approach. Bone and bone-marrow biopsies performed in 105 patients were studied using histomorphometry after identification of osteoclasts (by histochemical identification of tartrate resistant acid phosphatase) and dendritic cells (by immunohistochemical detection of the S-100 protein). Patients were classified by the World Health Organization criteria but histopathological criteria were more adapted to identify MGUS (53 cases), myeloma (46), B-cell lymphoma (six) since six myeloma were not correctly classified. Histomorphometry was compared to 15 control cases. The number of marrow dendritic cell was significantly increased with B-cell lymphoma >MGUS >myeloma > controls. Dendritic cell were often mixed with lymphoma cells. Myeloma had increased bone resorption with a high osteoclast number and moderate increase in dendritic cells. B-cell lymphomas had a considerable increase in dendritic cell but presented mononucleated osteoclasts. These findings can help in the classification of MGUS in the early stages of the disease and could help to propose preventive treatments.

**Keywords** B-cell lymphoma · Bone histomorphometry · Bone resorption · Dendritic cells · Monoclonal gammopathy · Myeloma · Osteoclast

**Abbreviations**

| | |
|---|---|
| BL | B lymphoma |
| BV/TV | trabecular bone volume |
| CFU-M | colony forming unit (monocytic) |
| DC | dendritic cell |
| ES/BS | Eroded surfaces |
| IL-6 | interleukin 6 |
| M-CSF | mononuclear phagocyte colony-stimulating factor |
| MGUS | monoclonal gammopathy of undetermined significance |
| MM | multiple myeloma |
| NOc/B.Ar | number of osteoclasts (bone referent) |

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| | |
|---|---|
| NOc/T.Ar | number of osteoclasts (tissue referent) |
| Oc | osteoclast |
| OPG | osteoprotegerin |
| OS/BS | osteoid surfaces |
| OV/BV | osteoid volume |
| RANK | receptor activator of nuclear factor-κ B |
| RANKL | receptor activator of nuclear factor-κ B ligand |
| TRAcP | tartrate-resistant acid phosphatase |

## Introduction

A monoclonal gammopathy of undetermined significance (MGUS) represents the common manifestation of multiple benign or malignant disorders; it can represent an early stage of malignant disorders like B-cell lymphoma or multiple myeloma [1]. The frequency of MGUS increases with age and is characterized by the proliferation of a malignant clone without any obvious sign of myeloma or lymphoma. Despite innovative cytogenetic studies, mechanisms influencing the transformation of a MGUS into a B-cell malignancy are still not well known. Lytic bone lesions are a common feature of patients with advanced myeloma, in contrast to what is observed in other B-cell malignancies. Bone involvement is related to an excessive bone resorption through increased osteoclast number encountered in the close vicinity of metaplastic cells [2] and myeloma cells [3] together with a decreased osteoblast activity [4, 5]. Osteoclastogenesis entails two main factors, the receptor activator of nuclear factor-κ B ligand (RANKL) and its soluble antagonist osteoprotegerin. RANKL stimulates both osteoclastogenesis from colony forming unit (monocytic; CFU-M) precursors and also increases osteoclast activity. [5, 6] Dendritic cells are specialized cells having a leading part in different development stages of B lymphocytes [7]. They are involved in the proliferation of B-cells during antigenic presentation, activate the translocation of genes of the heavy immunoglobulin chains and favor the survival of plasmoblasts which present this translocation [8, 9]. Dendritic cells originate from the same stem cell than osteoclasts (CFU-M) and under particular culture conditions [mononuclear phagocyte colony-stimulating factor, interleukin 6 (IL-6)], immature dendritic cells can differentiate into osteoclasts. Because both osteoclasts and dendritic cells originate from a common origin upon different cytokine profiles, it would be of importance to investigate if changes in cell numbers vary in myeloma and B-cell lymphomas in these altered cytokine network. To our knowledge, only a few studies have tried to assess the pathophysiological links between these two cells types during B-cell monoclonal proliferations [10–15]. The present study aimed to identify and quantify bone marrow dendritic cell and osteoclasts in patients with a MGUS, after precise histochemical identification of these two cell populations. The final goal of the study was to see if the systematic osteoclast and dendritic cell count in the bone marrow microenvironment could present a diagnostic importance.

## Material and Methods

### Patients

One hundred and five patients from hematological and rheumatologic units were enrolled in this study; all of them had a monoclonal spike. Because numerous previous reports have focused on the importance of bone changes in hematological disorders (including myeloma [16], lymphoma [17], and myeloid metaplasia with myelofibrosis [18, 19]), our center includes a conjoint histological analysis of the marrow and bone compartment in these diseases. Biological parameters such as paraproteinemia, calcemia, degree of anemia, degree of renal insufficiency, C-reactive protein, β2 microglobulin, number of bone marrow plasma cells, and occurrence of urine light chains were also measured. In practice, myelomas which were included had no or few osteolytic lesions whereas some patients with MGUS had biological abnormalities such as the presence of a Bence Jones protein or a reduction of polyclonal immunoglobulins. Data of these patients were compared to results obtained from 15 control cases from patients with bone fragility without MGUS.

### Classification of Patients

Patients were classified according to the World Health Organization (WHO) classification of hematopoietic diseases) taking into account clinical and biochemical parameters [27] which considers:

- Major criteria, such as a plasmocytosis more than 30% (bone marrow aspiration), a plasmocytic tumor nodule in the biopsy, an immunoglobulin more than 35 g/L, the presence of Bence Jones protein.
- Minor criteria, such as bone marrow plasmocytosis between 10% and 30% in the bone marrow aspiration, monoclonal globulin spike less than 35 g/L, osteolytic lesions, decreased of polyclonal immunoglobulins.

The diagnosis of myeloma requires a minimum of two major criteria or one major criterion and one minor criterion, or three minor criteria always including bone marrow plasmocytosis between 10% and 30% and monoclonal globulin spike less than 35 g/L.

Because discrepancies have been reported between clinical and histopathological classifications [27], patients were then classified according to histopathological changes

observed in the bone marrow biopsy. The existence of a neoplastic population in the bone marrow was used to re-allocate patients and the subsequent histomorphometric abnormalities noted in the bone biopsy were considered. Myeloma patients were diagnosed upon an abundant interstitial or nodular plasmocytosis which tended to compress medullar adipocytes. In addition, myeloma patients presented a marked increased in bone remodeling in 100% of cases. This was frequently associated with a decreased osteoblast activity leading to an imbalance remodeling with decoupling. B-cell lymphomas presented a characteristic lymphoid or lymphoplasmocytic medullar infiltration. Neoplastic cells consisted of small lymphoid cells, which were evenly distributed in interstitial or nodular locations (paratrabecular). An increase in bone remodeling was rarely noticed but images of microresorption were evidenced. The remaining patients were considered to present a MGUS in the presence of a limited interstitial plasmocytosis and a normal or a mild increased in bone remodeling.

### Bone Biopsy with Histochemical Identification of Osteoclast and Histomorphometry

Quantitative histology of bone changes was performed as previously described in undecalcified transiliac bone biopsy specimens. Bone biopsies were performed under local anesthesia from a standardized point 2 cm below the iliac crest and 2 cm behind the anterosuperior iliac spine. The transiliac bone cores were 7 mm in diameter (Commeca-Commed, Beaucouzé, Angers, France). The specimens were immediately fixed in an ethanol-based fixative [20] and kept for 24 h at 4°C. They were then dehydrated over a period of 36 hours in 100% acetone at 4°C.

Biopsy specimens were embedded in methylmethacrylate. The infiltration of bone cores and the embedding process were performed in a cold environment to preserve bone-enzyme activities. Sections 7 μm in thickness were cut dry using a microtome with carbide tungsten knives (Microtome Leica Polycut S). Staining for routine histomorphometry was performed with a modification of the Goldner's trichrome [21] in six nonserial sections according to classical histomorphometric procedures (one section every 100 μm) [22]. Six additional sections were stained for the osteoclastic tartrate-resistant acid phosphatase (TRAcP) and were counterstained with phosphomolybdic aniline blue [23]. Measurements were made on a semiautomatic system developed in the laboratory as previously described [24]. Classical histomorphometric parameters were considered for diagnosis, including cortical thickness, trabecular bone volume (BV/TV, expressed in percentage), trabecular characteristics, osteoid parameters (osteoid volume and surfaces, expressed in percentage) according to the American Society for Bone and Mineral Research recommendations [22]. Special attention was paid with parameters of bone resorption:

- Eroded surfaces (ES/BS, expressed in %) represents the amount of trabecular surfaces occupied by scalloped areas reflecting the osteoclast activity.
- Number of Osteoclast in cancellous bone area (NOc/T.Ar, in ¢/mm$^3$) was measured in TRAcP stained sections. The results were compared with age- and sex-matched normal values.
- Number of osteoclast in the trabecular bone area (NOc/B.Ar, in ¢/mm$^3$) was derived as NOc/B.Ar = NOc/T.Ar x BV/TV.

### Bone Marrow Biopsy with Immunohistochemical Identification of Dendritic Cells

Marrow biopsies were performed during the same time than bone biopsy with a Jamshidi's needle in the immediate vicinity of the bone biopsy. Cores, 2 mm in diameter, were immediately fixed in phosphate-buffered formalin, pH 7.4, and kept for 24 h at ambient temperature. Decalcification was made using a ready-to-use decalcifying fluid (based on formic acid) according to the manufacturer's recommendations (Surgipath®, Labonord, Templemars, France). Marrow biopsies were then dehydrated, embedded in paraffin and sectioned at 4 μm. Antigen retrieval was performed by water bath heating in a citrate buffer (pH 6). Immunochemistry was performed using Envision® procedure with the Dako autostainer (Dako, Glostrup, Denmark). The Anti S-100 protein antibody was used to detect dendritic cells. It is a rabbit anti-cow polyclonal antibody (Dako, Z311, batch 129) directed against the α and β sub-units of the protein. It was used at a dilution of 1:800. S-100 protein is a calcium binding protein present in mature and immature dendritic cell, adipocytes and Schwann cells. S-100 has been used in several studies as a reliable method to quantify dendritic cell by immunohistochemistry [25, 26].

Control positive tissues were obtained using reactive tonsil tissue. Sections were counterstained with hematoxylin (Dako, dilution of 1:2). The number of dendritic cells was measured in 20 consecutive fields at a magnification of ×400.

### Statistical Analysis

Statistical analysis was performed using the Statview® release 10.0 (SAS Institute Inc., Cary, NC, USA). Results are expressed as mean and standard error of the mean (SEM) because the number of patients in each group of patients markedly differ. The Student's $t$ test was used to compare each group. Differences between groups were considered significant when $p<0.05$. Because the number of patients

**Table 1** Clinical results in 105 patients with a MGUS

| Parameters | Control | MGUS | Myelomas | MGUS | Myelomas | Lymphomas |
|---|---|---|---|---|---|---|
| | | WHO classification | | Histopathological classification | | |
| Number of cases | 15 | 53 | 52 | 53 | 46 | 6 |
| Mean age (years) and range | 57 (30–75) | 65 (37–92) | 68 (45–90) | 64 (53–77) | 65 (45–90) | 66 (37–92) |
| Sex ratio (F/M) | 5/15 | 25/53 | 30/52 | 25/53 | 26/46 | 4/6 |
| Monoclonal protein | | | | | | |
| IgG | – | 44 | 45 | 44 | 41 | 4 |
| IgM | – | 6 | 4 | 6 | 2 | 2 |
| IgA | – | 3 | 3 | 3 | 3 | - |
| Light chain κ | – | 37 | 35 | 37 | 30 | 5 |
| Light chain λ | – | 16 | 17 | 17 | 15 | 1 |
| Bence Jones proteinuria | | | | 10 | 5 | 3 |

was low in the B-cell lymphoma group, only raw values are provided and no statistical attempt was computed.

## Results

### Clinical Results

When the clinical criteria were used to classify the patients, 52 individuals were classified as MGUS and 53 as myeloma. Clinical results appear in Table 1.

When patients were stratified according to histopathological criteria, six patients had a B-cell lymphoma: two of them were lymphocytic lymphomas, made of a uniform prolymphocyte population which displayed an interstitial medullar infiltration; two were classified as Waldenström diseases and presented multiple nodules in the bone biopsy; one case was classified as follicular cell lymphoma with dense paratrabecular nodules composed of small cleaved cells.

MGUS, myelomas and B-cell lymphomas did not differ statically according to biological criteria (amount of monoclonal protein, calcemia, anemia, renal insufficiency, C-reactive protein, β2 microglobulin). All patients presented a decrease of polyclonal immunoglobulins.

Three patients had osteolytic lesions (one Waldenström, one follicular lymphoma and one myeloma).

### Histomorphometric Results

Bone volume, cortical thickness, cortical porosity, osteoid parameters, mineralization rate, mineralization lag time were non informative and did not differ among the different groups (Table 2). On the other hand, resorption parameters exhibited significant changes between the different groups.

Osteoclasts were accurately identified by the TRAcP staining as brown cells at the surface of light blue counterstained mineralized bone (Fig. 1). No other bone marrow cell exhibited TRAcP staining as reported previously [23, 24]. In control subjects, eroded surfaces were close to normal values reported in the French population (5.4±1.2%) [28]. Resorption parameters were significantly enhanced in myeloma. ES/BS was also significantly increased in the other groups when compared to controls (Fig. 2A). The osteoclast number NOc/B.Ar was higher in

**Table 2** Histomorphometric parameters in patients classified according to histopathologic criteria

| Parameters | Abbreviation | Control | MGUS | Myelomas | B-cell lymphomas |
|---|---|---|---|---|---|
| Number of cases | | 15 | 55 | 44 | 6 |
| Cortical thickness | Ct.Th (μm) | 730±309 | 822±222 | 778±302 | 614±263 |
| Bone volume | BV/TV (%) | 15.4±5.5 | 18.5±5.3 | 18.6±8.5 | 16.7±5.6 |
| Osteoid volume | OV/BV (%) | 2.3±1.4 | 1.8±1.6 | 3.6±3 | 3.2±3.4 |
| Osteoid surfaces | OS/BS (%) | 13.0±7.4 | 8.4±6.8 | 12.7±12.4 | 12.2±7.6 |
| Eroded surfaces | ES/BS (%) | 5.4±1.2 | 8.5±4.9[a] | 10.4±4.9[a] | 7.4±1.8 |
| Number of osteoclasts (tissue referent) | NOc/T.Ar (¢/mm$^2$) | 0.92±0.3 | 1.0±0.2 | 2.4±0.3[a] | 0.85±0.2 |
| Number of osteoclasts (bone referent) | NOc/B.Ar (¢/mm$^2$) | 7.6±3.4 | 12.4±1.3[a] | 23.3±2.3[a] | 10.2±2.3 |
| Number of dendritic cells (tissue referent) | NDC/T.Ar (¢/mm$^2$) | 0.6±0.1 | 8.5±1.2[a] | 5.1±0.9[a] | 28.5±30 |

Data are expressed as mean ± SEM.
[a] Significantly different from controls (see graph for values)

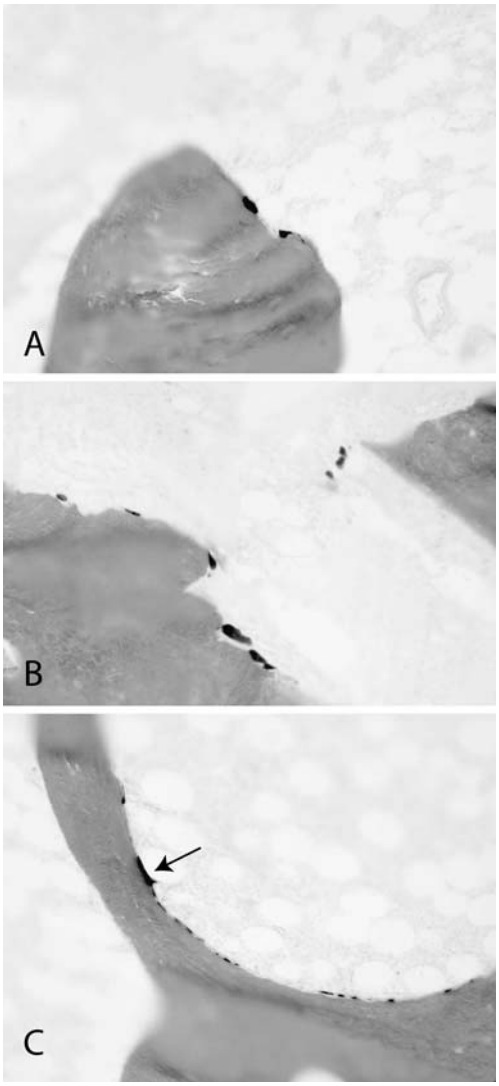


**Fig. 1** TRAcP staining in undecalcified bone biopsies **A** Control patient, two osteoclasts in an eroded lacuna; **B** In myeloma, Osteoclasts are numerous and situated in deeply eroded lacunae; **C** In B-cell lymphoma, the osteoclast population is composed of normal-sized cells (*arrow*) together with numerous mononucleated cells responsible for microresorption. Magnification ×200

myelomas compared to B-cell lymphomas and MGUS ($p<$ 0.0001; Fig. 2B). In B-cell lymphoma, patients exhibited a variable component of TRAcP+ cells that appeared mononuclear. These cells were often disposed in minute erosion lacunae, an indication of bone microresorption.

## Quantification of Dendritic Cells

With the S-100 protein antibody, dendritic cells appeared as mononuclear cells with a cytoplasmic staining often associated with a nuclear localization (Fig. 3). When using the WHO clinical classification, there was no statistical difference between the average number of bone marrow dendritic cells in myeloma and MGUS ($p=0.74$). When patients were classified according to the histopathological lesions, the average number of dendritic cells was significantly higher in MGUS than in myelomas ($p=0.042$). The average number of bone marrow dendritic cells seemed considerably higher in B-cell lymphomas than in myelomas

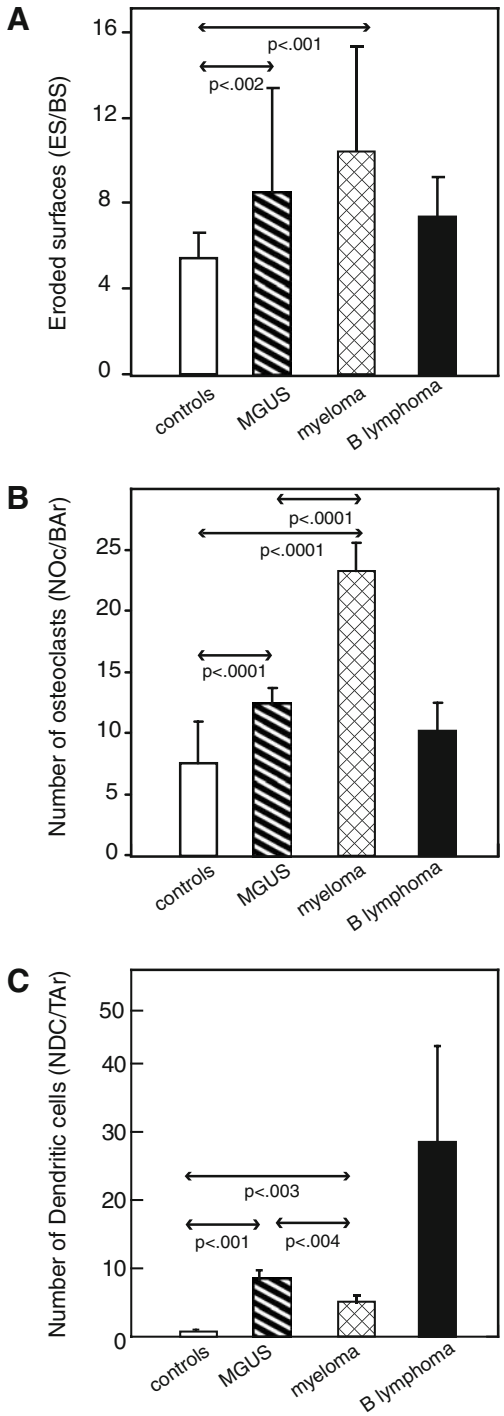


**Fig. 2** Histomorphometric analysis in the different groups of patients **A** the eroded surfaces (expressed in %); **B** the number of osteoclasts N.Oc/B.Ar (expressed in ¢/mm$^2$); **C** the number of dendritic cells (expressed in ¢/mm$^2$)

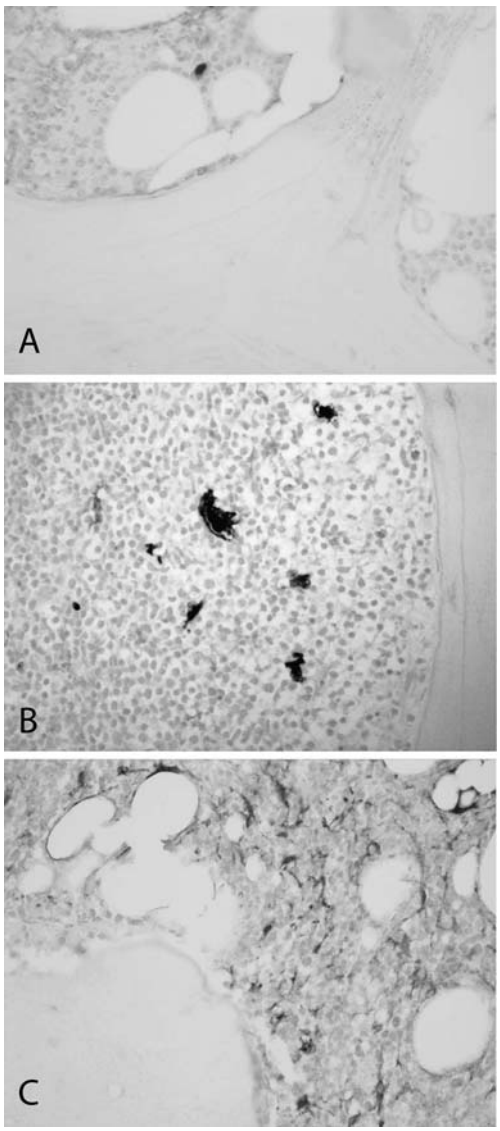


**Fig. 3** Immunohistochemistry with S-100 protein antibody. **A** Control patient, dendritic cell are rarely encountered in the marrow spaces; **B** In myeloma, dendritic cell appears dispersed with no preferential localization with regard to the tumor plasma cells; **C** dendritic cell are numerous and closely mixed with lymphoma cells. Magnification ×400

and in MGUS and control cases (Fig. 4). Large groups of dendritic cell were found mixed with lymphoma cells in four cases of B-cell lymphoma having a tumor infiltration in the bone marrow biopsy (Fig. 2C).

## Discussion

It is presently very difficult to differentiate MGUS from smoldering myeloma or lymphoma in clinicopathological practice. Biological parameters were not discriminating to differentiate myeloma from MGUS in the present series. It is probably because MGUS patients were rather atypical with abnormalities of some biological parameters (presence of Bence Jones protein, reduction of polyclonal immunoglobulins). Furthermore, the majority of myeloma patients did not had an overt myeloma in most cases, but presented only a few osteolytic lesions (or normal radiographs), a condition that probably reflect a low tumor mass.

We have previously found that bone histomorphometry could be a very interesting tool in the early diagnosis of B-cell malignancies [16]. In this study, we have used new histomorphometric parameters together with the histochemical identification of osteoclasts and dendritic cells in the bone marrow environment. Bone volume was not significantly altered because patients presented smoldering myeloma in which osteoclastogenesis did not have yet time to have deleterious consequences at this stage of the disease. Eroded surfaces are precociously increased in myeloma and thus represent a good criterion of malignancy in individuals with MGUS [3]. Some authors have clearly shown that MGUS patients with increased eroded surfaces at diagnosis had a higher propensity to develop overt myeloma or B-cell lymphoma several months or even years later [16]. In the present study, there was a significant increase in eroded surfaces between the control group and the different groups of patients with a monoclonal spike. Patients with myeloma had the highest level of bone resorption. The NOc/B.Ar was a better discriminator than NOc/T.Ar (cancellous

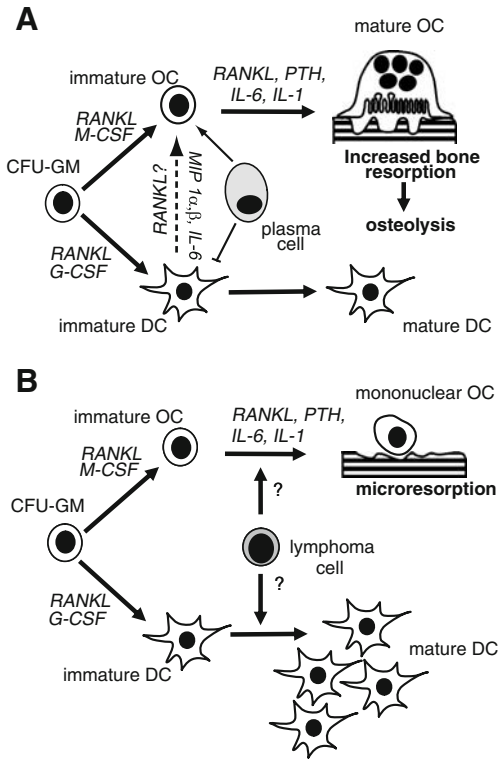


**Fig. 4** Schematic illustration of osteoclastogenesis in **A** myeloma, leading to mature multinuclear osteoclasts associated with lytic bone lesions and **B** in B-cell lymphoma, leading to mononuclear osteoclasts associated with microresorption

volume referent) for assessing osteoclastic number at the bone level. It reflects the osteoclast number in function of bone mass present in the biopsy whereas NOc/T.Ar reflects the osteoclastic activity in function of the whole tissue surface (i.e., bone and marrow cavities). MGUS are a group of disorders associated with monoclonal proliferation of B-cells. Among them, myeloma is a plasma cell malignancy characterized by the high capacity to induce osteolytic lesions. Osteolysis results from an increased resorption related to the recruitment of highly active osteoclasts associated with a decreased osteoblastic activity [5, 29]. The International Myeloma Working Group has recently reviewed the criteria for diagnosis and classification, with the aim of producing simple definitions based upon routinely available investigations [27]. However when using this classification in this series, this led to a misdiagnosis of six cases of B-cell lymphoma which were initially classified as myeloma. Differences were previously reported by our group when comparing the osteoclastogenesis in myeloma and B-cell lymphoma [17, 30, 31]. In myeloma, the cytokine network arising between plasma cells and bone cell progenitors (IL-6, MIP-1α/CCL-3...), induces the genesis of mature multinucleated osteoclasts that actively resorb bone (Fig. 4). Osteolytic lesions are observed on radiographs in about 95% of patients at the end stage of the disease. In B-cell lymphoma, the cytokine network is less well characterized, and different cytokines are advocated [32]. A mixed population of multi and mononuclear osteoclasts is observed, mononuclear TRAcP+ cells being only capable of microresorption. This explains why osteolytic lesions are usually rare in B-cell lymphoma (8–10% of cases) and occur only when the amount of these cells is dramatically increased [33].

Several reports have used immunohistochemistry directed against the S-100 protein to detect dendritic cells [34–39]. S-100 protein is expressed in nearly all types of dendritic cell. Antibody directed against the S-100 protein permitted a useful labeling of dendritic cell in the marrow cavities, similar than in the positive controls. It has been shown that the absolute number of circulating precursors of dendritic cell is significantly lower in patients with overt myeloma than in healthy subjects [15]. After maturation, peripheral blood dendritic cells from myeloma patients have been found to express significantly less HLA molecules and an impaired induction of T-cell proliferation when compared with healthy subjects [15]. Dendritic cell from myeloma patients are considered as immature or functionally defective [7, 15], partially because of IL-6 mediated inhibition of development. The number of dendritic cells in the peripheral blood of myeloma patients is reported to be sensibly normal throughout the course of the disease [14]. Commitment of the CFU-M precursor into either osteoclast, macrophages or dendritic cell lineages is determined by ligand binding to cell-surface receptors, particularly RANK for osteoclasts precursors. RANK activates the dimeric transcription factors NF-κ B and AP-1. In addition, c-Fos/AP-1 plays a positive role in osteoclasts but a negative role for macrophages and dendritic cells [12]. The differences in dendritic cell number, between myeloma and B-cell lymphoma, could be explained by different cytokines networks encountered in these two pathological conditions. IL-6 which is mainly secreted in myeloma inhibits growth and function of dendritic cell *in vitro* [40]. It was also proposed that other molecules as TGF-$\beta_1$ or IL-10 could favor a functional defect in the dendritic cell of patients with myeloma [14]. The cytokine network, leading to dendritic cell differentiation, is poorly understood [41].

The number of dendritic cells and their location in samples of B-cell lymphoma patients seems to be particular. Dendritic cells were more numerous and closely mixed with lymphoma cells; in myeloma they appeared dispersed with no preferential localization with regard to the tumor plasma cells. Interestingly, the number of dendritic cells in MGUS patients was increased at intermediate levels. In controls, dendritic cells were rarely observed and randomly distributed through the marrow spaces. The expression of a particular isotype of S-100 protein (S-100A2) has been recently shown by immunohistochemistry in 36% of cases among a group of 45 lymphomas [26]. However, the histological classification of B-cell lymphomas was not known in this work, no cytological description of the labeled cells was presented, and no mention was made if labeled cells were tumor or stromal cells. In the present study, the S-100 labeled cells could not be confounded with lymphoma cells.

In summary, myeloma patients had an increased bone resorption associated with increased osteoclast number; they also presented a moderate but significant increase in the number of dendritic cell when compared to controls. On the contrary, B-cell lymphomas had a considerable increase in the number of dendritic cell although the mechanisms of dendritic cell genesis and maturation are less well known. MGUS had intermediate levels of dendritic cells and osteoclasts, and this could represent an interesting diagnosis tool in the survey of these patients.

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