ARTICLE

# *IL21R* expressing CD14+CD16+ monocytes expand in multiple myeloma patients leading to increased osteoclasts

**Marina Bolzoni,[1] Domenica Ronchetti,[2,3] Paola Storti,[1,4] Gaetano Donofrio,[5] Valentina Marchica,[1,4] Federica Costa,[1] Luca Agnelli,[2,3] Denise Toscani,[1] Rosanna Vescovini,[1] Katia Todoerti,[6] Sabrina Bonomini,[7] Gabriella Sammarelli,[1,7] Andrea Vecchi,[8] Daniela Guasco,[1] Fabrizio Accardi,[1,7] Benedetta Dalla Palma,[1,7] Barbara Gamberi,[9] Carlo Ferrari,[8] Antonino Neri,[2,3] Franco Aversa[1,4,7] and Nicola Giuliani[1,4,7]**

[1]Myeloma Unit, Dept. of Medicine and Surgery, University of Parma; [2]Dept. of Oncology and Hemato-Oncology, University of Milan; [3]Hematology Unit, "Fondazione IRCCS Ca' Granda", Ospedale Maggiore Policlinico, Milan; [4]CoreLab, University Hospital of Parma; [5]Dept. of Medical-Veterinary Science, University of Parma; [6]Laboratory of Pre-clinical and Translational Research, IRCCS-CROB, Referral Cancer Center of Basilicata, Rionero in Vulture; [7]Hematology and BMT Center, University Hospital of Parma; [8]Infectious Disease Unit, University Hospital of Parma and [9]"Dip. Oncologico e Tecnologie Avanzate", IRCCS Arcispedale Santa Maria Nuova, Reggio Emilia, Italy

**Haematologica** 2017
Volume 102(4):773-784

## ABSTRACT

Bone marrow monocytes are primarily committed to osteoclast formation. It is, however, unknown whether potential primary alterations are specifically present in bone marrow monocytes from patients with multiple myeloma, smoldering myeloma or monoclonal gammopathy of undetermined significance. We analyzed the immunophenotypic and transcriptional profiles of bone marrow CD14+ monocytes in a cohort of patients with different types of monoclonal gammopathies to identify alterations involved in myeloma-enhanced osteoclastogenesis. The number of bone marrow CD14+CD16+ cells was higher in patients with active myeloma than in those with smoldering myeloma or monoclonal gammopathy of undetermined significance. Interestingly, sorted bone marrow CD14+CD16+ cells from myeloma patients were more pro-osteoclastogenic than CD14+CD16- cells in cultures *ex vivo*. Moreover, transcriptional analysis demonstrated that bone marrow CD14+ cells from patients with multiple myeloma (but neither monoclonal gammopathy of undetermined significance nor smoldering myeloma) significantly upregulated genes involved in osteoclast formation, including *IL21R*. *IL21R* mRNA over-expression by bone marrow CD14+ cells was independent of the presence of interleukin-21. Consistently, interleukin-21 production by T cells as well as levels of interleukin-21 in the bone marrow were not significantly different among monoclonal gammopathies. Thereafter, we showed that *IL21R* over-expression in CD14+ cells increased osteoclast formation. Consistently, interleukin-21 receptor signaling inhibition by Janex 1 suppressed osteoclast differentiation from bone marrow CD14+ cells of myeloma patients. Our results indicate that bone marrow monocytes from multiple myeloma patients show distinct features compared to those from patients with indolent monoclonal gammopathies, supporting the role of *IL21R* over-expression by bone marrow CD14+ cells in enhanced osteoclast formation.

**Correspondence:**
nicola.giuliani@unipr.it

Received: August 5, 2016.
Accepted: December 23, 2016.
Pre-published: January 5, 2017.

doi:10.3324/haematol.2016.153841

*Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/102/4/773*

## Introduction

Multiple myeloma (MM) is characterized by bone destruction, osteolytic lesions and consequently a higher risk of fractures[1] due to an increase in bone marrow (BM) osteoclast formation and osteoblast suppression.[2-4] Conversely, patients with indolent gammopathies such as smoldering MM (SMM) and monoclonal gammopathy of undetermined significance (MGUS) are characterized by the absence of lytic lesions, although they may have an increase in osteoclastic bone resorption.[5-9]

Since the close relationship between plasma cells and BM microenvironment plays a pivotal role in the pathogenesis of MM,[10] ongoing studies are focusing on the presence of potential molecular alterations in the microenvironment.[11-13] Transcriptional profile alterations have been reported in mesenchymal stromal cells and osteoblasts of MM patients correlated to osteolytic lesions and when compared to healthy donors but not MGUS patients.[13] BM monocytes play a pivotal role in bone disease,[2,4,14] angiogenesis[15] and immune system dysfunction,[16] which are hallmarks of active MM.

Enhanced bone resorption in MM patients occurs in the BM in close contact with plasma cell infiltration.[5] Contact between MM cells and BM stromal cells stimulates the production of the receptor activator of nuclear factor kappa-B ligand (RANKL), the main pro-osteoclastogenic cytokine involved in osteoclast differentiation, through its receptor RANK on the monocyte surface.[2,17] Moreover, different factors produced by MM cells can stimulate osteoclastogenesis, including interleukin (IL)-6[18] and macrophage inflammatory protein (MIP)-1α.[19,20] Recently, it has also been reported that IL-3 is increased in BM plasma from MM patients and that it induces Activin A production by BM monocytes which, in turn, stimulates osteoclastogenesis in a RANKL-independent mechanism.[21] This suggests that BM monocytes may be directly involved in enhanced osteoclastogenesis in MM.

Immunophenotypic analysis of peripheral monocytes has shown that $CD14^+CD16^+$ cells account for 5–10% of monocytes in healthy individuals but this sub-population is significantly expanded in cancer[22] and inflammatory conditions.[23,24] In psoriatic arthritis, $CD14^+CD16^+$ cells have been associated with bone erosion and identified as the main source of osteoclast progenitors.[25] More recently, it has been reported that the proportion of $CD14^+CD16^+$ cells increased in MM patients with the tumor load[26,27] and that these cells are potential markers of osteoclast progenitors.[27] However, it is not known whether there are alterations in BM monocytes in MM patients. Thus, the aim of this study was to characterize the immunophenotypic and transcriptional profiles of BM $CD14^+$ cells across a cohort of patients with different types of monoclonal gammopathies in order to identify genes that are potentially involved in enhanced osteoclastogenesis and possibly druggable as new therapeutic targets.

## Methods

### Patients

A total cohort of 50 patients with newly diagnosed active MM, 32 patients with SMM, and 20 patients with MGUS admitted to our hematology institute from 2010 until 2016 were included in the analysis of monocyte features. All of the subjects involved in the study gave their written informed consent, according to the Declaration of Helsinki. The Institutional Review Board of the University of Parma (Italy) approved all the study protocols. To define the presence of osteolytic lesions in MM patients we used X-ray skeletal survey as the first imaging procedure and alternatively a low-dose computed tomography scan or computed tomography/positron emission tomography evaluation, as recently updated by the International Myeloma Working Group.[28] The skeleton was evaluated in MM patients in the same period as the BM aspirates were taken. The presence of at least one lytic lesion on X-ray or computed tomography scan or computed tomography/positron emission tomography scan was used to define osteolytic patients. The presence of three or more lytic lesions was used to define patients with "high bone disease". MM patients with fewer than three lytic lesions or without bone lesions were considered as having "low bone disease".[29]

Not all patients' samples were suitable for all the analyses. Table 1 reports the number of patients analyzed by the different techniques used in the study.

### Immunophenotyping

Details of the immunophenotypic analyses performed are reported in the *Online Supplementary Data*.

### Isolation of primary $CD14^+$ cells and $CD14^+$ cell sorting from bone marrow samples

$CD14^+$ monocytes were isolated from BM and peripheral blood mononuclear cells by an immunomagnetic method with anti-CD14 monoclonal antibody conjugated with microbeads (Miltenyi Biotech; Bergisch-Gladbach, Germany).

With the same protocol, $CD138^+$, $CD3^+$, $CD4^+$ and $CD8^+$ cells were isolated from BM samples. The presence of potential contaminating cells in each fraction was evaluated by flow cytometry analysis, using the fluorescence-activated flow cytometer BD FACS Canto II with Diva software [Becton, Dickinson and Company (BD); Franklin Lakes, NJ, USA]. The purity of monocyte samples was >92% and an example of purity analysis is shown in *Online Supplementary Figure S1*. All the antibodies (anti-human CD14-PerCP-Cy5.5, clone MφP9; anti-human CD138 PE, clone MI15; anti-human CD3 FITC, clone SK7; anti-human CD4 FITC, clone L120; anti-human CD8 PE, clone SK1) were obtained from BD.

Primary BM mesenchymal stromal cells were obtained from the $CD14^-CD138^-$ fraction of BM mononuclear cells. Cells were incubated until confluence for 2 weeks in alpha minimum essential medium (αMEM) supplemented with glutamine, at 15% fetal bovine serum (FBS) (all these reagents purchased from Invitrogen Life Technologies; Carlsbad, CA, USA).

For $CD14^+$ cell sorting, purified BM $CD14^+$ cells were stained with PE-Cy7-conjugated anti-CD16 antibody and sorted according to the gating strategy shown in *Online Supplementary Figure S2*. The cells and gates were analyzed by FACSDiva 7 software (BD) and the cells were sorted on a FACS Aria III instrument.

**Table 1. Number of patients with the three types of monoclonal gammopathy analyzed for monocyte features by the different techniques.**

| | Immunophenotype | GEP | Real-Time PCR |
|---|---|---|---|
| MGUS | 9 | 9 | 6 |
| SMM | 15 | 15 | 11 |
| MM | 28 | 32* | 13 |

GEP: gene expression profiling; PCR: polymerase chain reaction. *9 out of 32 samples, displaying high CD138 expression, were excluded from the statistical analysis.

### Fluorescence *in situ* hybridization, microarray analysis and real-time polymerase chain reaction

These methodologies are detailed in the *Online Supplementary Methods* section.

### Monocyte treatment with cytokines

Primary CD14$^+$ cells were cultured in the presence or absence of recombinant human (rh) IL-21 (30 pg/mL) (Biovision Inc.; Milpitas, CA, USA) for 24 h and then collected for mRNA analysis.

The human monocytic cell line THP-1 was purchased from the American Type Culture Collection (Rockville, MD, USA) and was recently authenticated and tested for mycoplasma contamination. THP-1 cells were treated with rhIL-6 (20 ng/mL) (Thermo Scientific; Rockford, IL, USA) and/or rh tumor necrosis factor (TNF)-α (10 ng/mL) (OriGene; Rockville, MD, USA) for 48 h and then collected for mRNA analysis.

### Lentiviral infections

The amplified *IL21R* complementary DNA sequence was cloned into the pLenti-GIII-CMV-GFP-2A-Puro lentiviral vector (Applied Biological Materials Inc.; Richmond, BC, Canada). Recombinant lentivirus was produced by transient transfection of 293T cells.[30] Primary CD14$^+$ cells were transduced following published protocols.[31] Briefly, 8x10$^6$ CD14$^+$ cells purified from peripheral blood buffy coats of healthy donors were placed in wells in 2 mL of αMEM with 10% FBS and rh monocyte colony-stimulating factor (M-CSF; 25 ng/mL) (Peprotech, Rocky Hill, NJ, USA) in the presence of either empty or IL21R vector. As a control, CD14$^+$ cells were also seeded in the same conditions without adding the lentiviral vector. After 18 h, 2 mL of fresh αMEM with 10% FBS and rhM-CSF (25 ng/mL) were added. After 3 days, cells were collected and seeded for osteoclastogenesis assays and for *IL21R* mRNA analysis.

### *Osteoclastogenesis assays*

After cell sorting, both CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ populations were seeded at 2x10$^5$ cells/well in 96-well plates in αMEM with 10% FBS, rhM-CSF at 25 ng/mL and rhRANKL (Peprotech) at 60 ng/mL and then cultured for 28 days, replacing half the medium every 2-3 days.

In another osteoclastogenesis assay setting, 2x10$^5$ lentiviral transduced CD14$^+$ cells/well were seeded in 96-well plates in αMEM with 10% FBS, rhM-CSF 10 ng/mL, rhRANKL 50 ng/mL and the IL-21R signaling inhibitor Janex 1 (10 μM) (Cayman Chemical Company; Ann Arbor, MI, USA) and then cultured for 28 days, replacing half the medium every 2-3 days.

Finally, in the third osteoclastogenesis assay setting, MM BM mononuclear cells or purified BM CD14$^+$ cells were used (n=18). Mononuclear cells (4x10$^5$/well) or CD14$^+$ cells (2x10$^5$/well) were seeded in 96-well plates in αMEM with 10% FBS, rhM-CSF 25 ng/mL and rhRANKL 20 or 60 ng/mL in the presence or absence of rhIL-21 (30 pg/mL) and Janex 1 (10 μM), and then cultured for 28 days, replacing half the medium every 2-3 days.

In all *in vitro* osteoclastogenesis assays, each condition was performed at least in triplicate. The osteoclasts were identified at the end of the culture period as multinucleated (>3 nuclei) cells positive for tartrate-resistant acid phosphatase (TRAP) (Sigma Aldrich; Saint Louis, MO, USA) and counted by light microscopy. Osteoclast areas were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### STAT3 activity assay

The specific procedures adopted for the STAT3 activity analysis are detailed in the *Online Supplementary Methods* section.

### Bone marrow interleukin-21 levels in patients with monoclonal gammopathies

The levels of IL-21 in BM plasma were measured by enzyme-linked immunosorbent assay, as reported in the *Online Supplementary Data*.

### Statistical analysis

Quantitative variables were compared using the non-parametric Kruskal-Wallis and Mann-Whitney tests, or the parametric two-tailed Student t-test. Results are considered statistically significant at $P<0.05$. GraphPad Prism 5.0™ was used for all the statistical analyses.

## Results

### Patients with multiple myeloma have higher numbers of bone marrow CD14$^+$CD16$^+$ cells compared to patients with monoclonal gammopathy of undetermined significance

The immunophenotype of BM CD14$^+$ cells was evaluated in BM aspirates from 28 patients with active MM (median age 73 years, range 48-89; 46% female, 54% male; International Staging System stage: I=3 , II=8, III=17); 15 with SMM (median age 57 years, range 38-82; 33% female, 67% male) and nine with MGUS (median age 57 years, range 37-78; 44% female, 56% male). Forty-eight percent of the patients with active MM had evidence of osteolytic lesions. A representative example of flow cytometry analysis is reported in Figure 1A.

We found that the median percentage of CD14$^+$CD16$^+$ cells in BM samples increased significantly across the different types of monoclonal gammopathies from MGUS to active MM: MGUS: 1.9% (range, 0-3%); SMM: 3.5% (range, 1.0-7.5%); active MM: 5.25% (range, 0-20.0%) ($P$=0.0071). In particular a statistically significant difference was observed comparing MM and MGUS patients ($P$=0.0144) (Figure 1B).

There was no statistical difference in the median percentages of the BM CD14$^+$CD16$^+$ population between osteolytic MM patients (n=13) *versus* not-osteolytic ones (n=15): 4% (range, 0-20%) *versus* 7.8% (range, 0-20%), respectively (Figure 1C). Similarly, comparing MM patients with high bone disease (n=9) *versus* those with low bone disease (n=19), we did not find a statistically significant difference in the median percentages of the BM CD14$^+$CD16$^+$ population: 4.7% (range, 0-20%) *versus* 5.5% (range, 0-20%), respectively (Figure 1D).

In the same way, analyzing all myeloma patients (SMM and MM), there was no significant difference between the osteolytic patients compared to those without osteolytic lesions [osteolytic MM (n=13) *versus* not-osteolytic MM plus SMM (n=30): 4% (range, 0-20%) *versus* 4.3% (range, 0-20%), respectively].

### Bone marrow CD14$^+$CD16$^+$ cells in multiple myeloma patients are pro-osteoclastogenic in *ex vivo* cultures

To investigate whether the increased number of CD14$^+$CD16$^+$ cells observed in BM samples from MM patients could be associated with enhanced pro-osteoclastogenic activity, we sorted the BM CD14$^+$CD16$^+$ monocyte population by FACS and then tested its *ex vivo* pro-osteoclastogenic differentiation properties in comparison with the CD14$^+$CD16$^-$ cell fraction. A representative example of

flow cytometry analysis of monocyte sub-populations before and after cell sorting is reported in *Online Supplementary Figure S2*. A median of $3.5x10^5$ $CD14^+CD16^+$ cells was obtained after cell sorting from MM BM samples. Due to the limited numbers of cells, we were only able to perform osteoclastogenesis assays. Interestingly, we found that, compared to the $CD14^+CD16^-$ population, $CD14^+CD16^+$ cells generated more TRAP-positive cells with a higher number of osteoclasts showing five or more nuclei (Figure 1 E).

### A different transcriptional fingerprint characterizes bone marrow $CD14^+$ cells from patients with multiple myeloma as compared to smoldering multiple myeloma and monoclonal gammopathy of undetermined significance

We performed gene expression profiling of purified primary BM monocytes. We checked the intensity value of specific *CD14* and *CD138* probe sets (*Online Supplementary Table S1*) and discarded nine samples displaying high *CD138* expression from the analysis to further ensure that

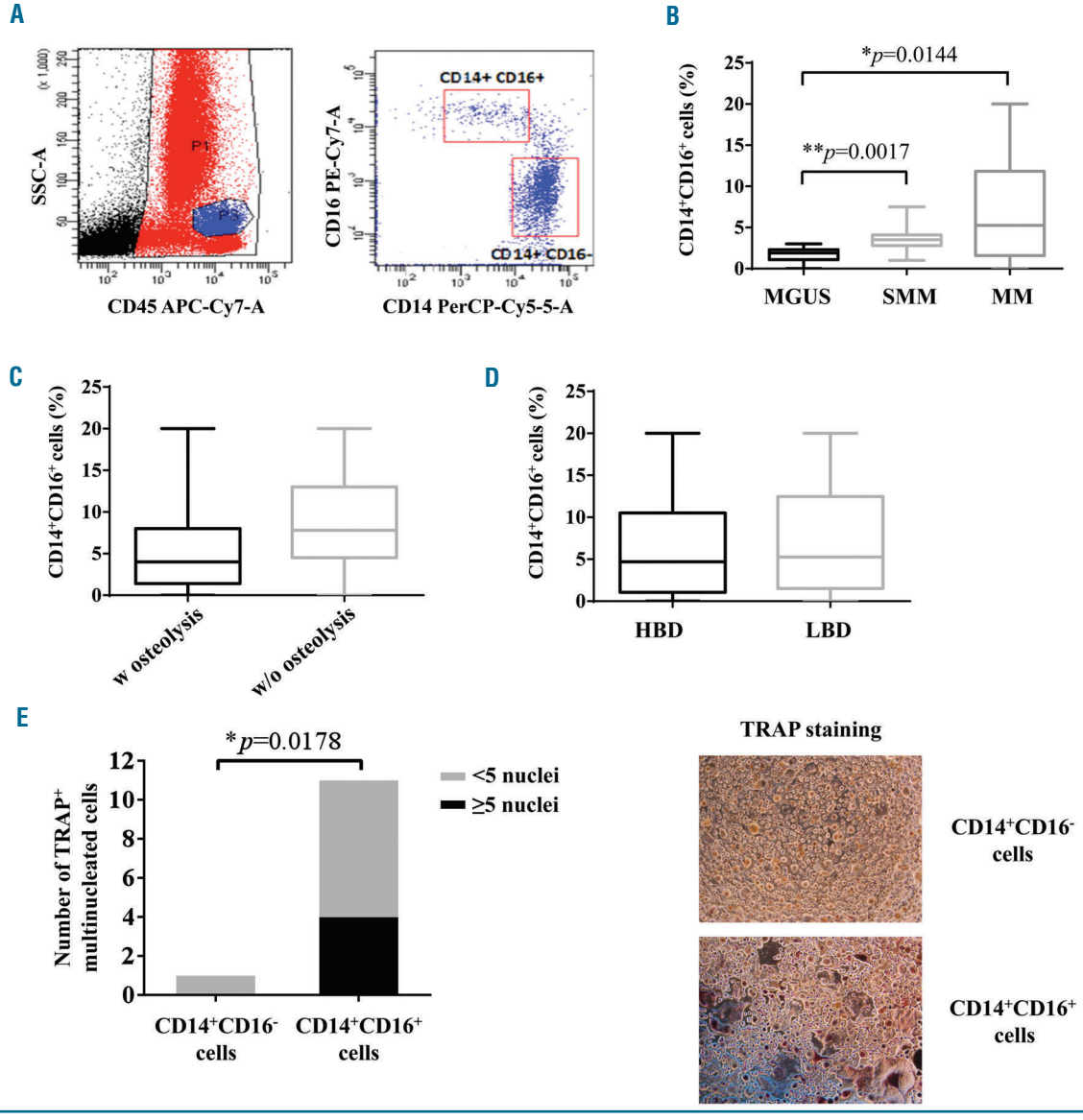


**Figure 1. Immunophenotype of bone marrow monocytes in patients with monoclonal gammopathies and their pro-osteoclastogenic *ex vivo* properties.** (A) CD14 and CD16 expression by BM monocytes in patients with monoclonal gammopathies: example of plots of flow cytometry data. (B) Box plots representing the median percentage values of $CD14^+CD16^+$ cells evaluated in BM samples obtained from patients with MGUS, SMM or MM (*P* calculated using the Mann-Whitney test). (C, D) Box plots representing the median percentage values of $CD14^+CD16^+$ cells evaluated in BM samples obtained (C) from patients with (w) or without (w/o) osteolysis and (D) from patients with high bone disease (HBD) or low bone disease (LBD). (E) Osteoclast assay. $CD14^+$ cells were purified from BM samples of patients with monoclonal gammopathies by an immunomagnetic method and then sorted into the two sub-populations $CD14^+CD16^-$ and $CD14^+CD16^+$ by a flow cell sorter as described in the Methods section. $CD14^+CD16^-$ or $CD14^+CD16^+$ cells (200,000 cells/well) were seeded in 96-well plates in αMEM with 10% FBS, rhM-CSF 25 ng/mL and rhRANKL 60 ng/mL. After 28 days of culture, osteoclasts were identified as multinucleated TRAP-positive cells and counted by light microscopy. (E) Bar graph represents the median number of osteoclasts/well of each condition, divided into osteoclasts with ≥5 nuclei or <5 nuclei (*P* calculated using the Mann-Whitney test, $CD14^+CD16^-$ *versus* $CD14^+CD16^+$ cells). On the right there is a representative image of the osteoclastogenesis assay stained by TRAP from BM sorted $CD14^+CD16^-$ and $CD14^+CD16^+$ cells (original magnification, 4x).

**Table 2.** Main clinical characteristics of the cohort of patients eligible for gene expression analysis.

| | | | | | | | MM cell genetic alterations | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Sex | Age | ISS | Osteolysis | HBD | Type | del(13q) | H | del(17p) | t(11;14) | t(4;14) | t(14;16) |
| *MGUS1* | F | 43 | | | | l | | | | | | |
| *MGUS2* | F | 52 | | | | l | | | | | | |
| *MGUS3* | M | 42 | | | | | | | | | | |
| *MGUS4* | M | 78 | | | | k | | | | | | |
| *MGUS5* | F | 54 | | | | l | | | | | | |
| *MGUS6* | M | 77 | | | | k | - | | - | - | - | - |
| *MGUS7* | M | 43 | | | | k | | | | | | |
| *MGUS8* | M | 91 | | | | l | | | | | | |
| *MGUS9* | F | 50 | | | | | | | | | | |
| *SMM1* | F | 67 | | | | l | | | | | | |
| *SMM2* | F | 72 | | | | l | - | + | - | - | + | - |
| *SMM3* | F | 58 | | | | l | | | | | | |
| *SMM4* | M | 58 | | | | k | | | | | | |
| *SMM5* | F | 64 | | | | k | | | | | | |
| *SMM6* | F | 61 | | | | l | | | | | | |
| *SMM7* | F | 64 | | | | k | - | + | - | - | - | - |
| *SMM8* | M | 56 | | | | k | | | | | | |
| *SMM9* | M | 70 | | | | k | - | - | - | | | |
| *SMM10* | F | 67 | | | | | - | | - | | | |
| *SMM11* | F | 76 | | | | k | | | | | | |
| *SMM12* | M | 83 | | | | k | + | - | - | + | - | - |
| *SMM13* | M | 47 | | | | k | | | | | | |
| *SMM14* | M | 41 | | | | k | | | | | | |
| *SMM15* | M | 65 | | | | k | + | - | - | - | + | - |
| *MM1* | M | 83 | III | + | - | l | | | | | | |
| *MM2* | M | 75 | III | | | k | | | | | | |
| *MM3* | F | 59 | II | + | - | k | + | | - | - | - | + |
| *MM4* | F | 80 | II | - | - | l | + | | + | - | - | - |
| *MM5* | M | 76 | II | - | - | l | + | | - | + | - | - |
| *MM6* | F | 79 | II | - | - | l | + | - | - | - | - | |
| *MM7* | M | 69 | I | + | + | k | | | | | | |
| *MM8* | M | 79 | II | + | + | l | | | | | | |
| *MM9* | M | 57 | I | + | - | l | + | - | + | | | |
| *MM10* | M | 81 | II | + | + | k | - | + | - | - | - | - |
| *MM11* | M | 60 | II | | | k | | | | | | |
| *MM12* | F | 78 | II | | | l | - | | - | - | - | - |
| *MM13* | M | 73 | III | + | + | k | | | | | | |
| *MM14* | M | 74 | II | + | + | l | - | | | | | |
| *MM15* | F | 73 | II | - | - | l | - | | - | + | - | - |
| *MM16* | M | 70 | I | - | - | k | - | - | - | | | |
| *MM17* | F | 84 | III | - | - | k | + | - | - | - | - | - |
| *MM18* | F | 86 | III | | | l | | | | | | |
| *MM19* | M | 73 | II | + | - | l | - | - | - | - | - | - |
| *MM20* | M | 56 | III | - | - | k | | | | | | |
| *MM21* | F | 50 | II | + | + | k | + | - | - | + | - | - |
| *MM22* | F | 75 | I | + | + | k | | | - | | | |
| *MM23* | F | 77 | III | - | - | k | - | - | | - | - | - |

MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering multiple myeloma; MM: multiple myeloma; F: female; M: male; ISS: International Staging System; HBD: high bone disease; H: hyperdploid; l: lambda k: kappa.

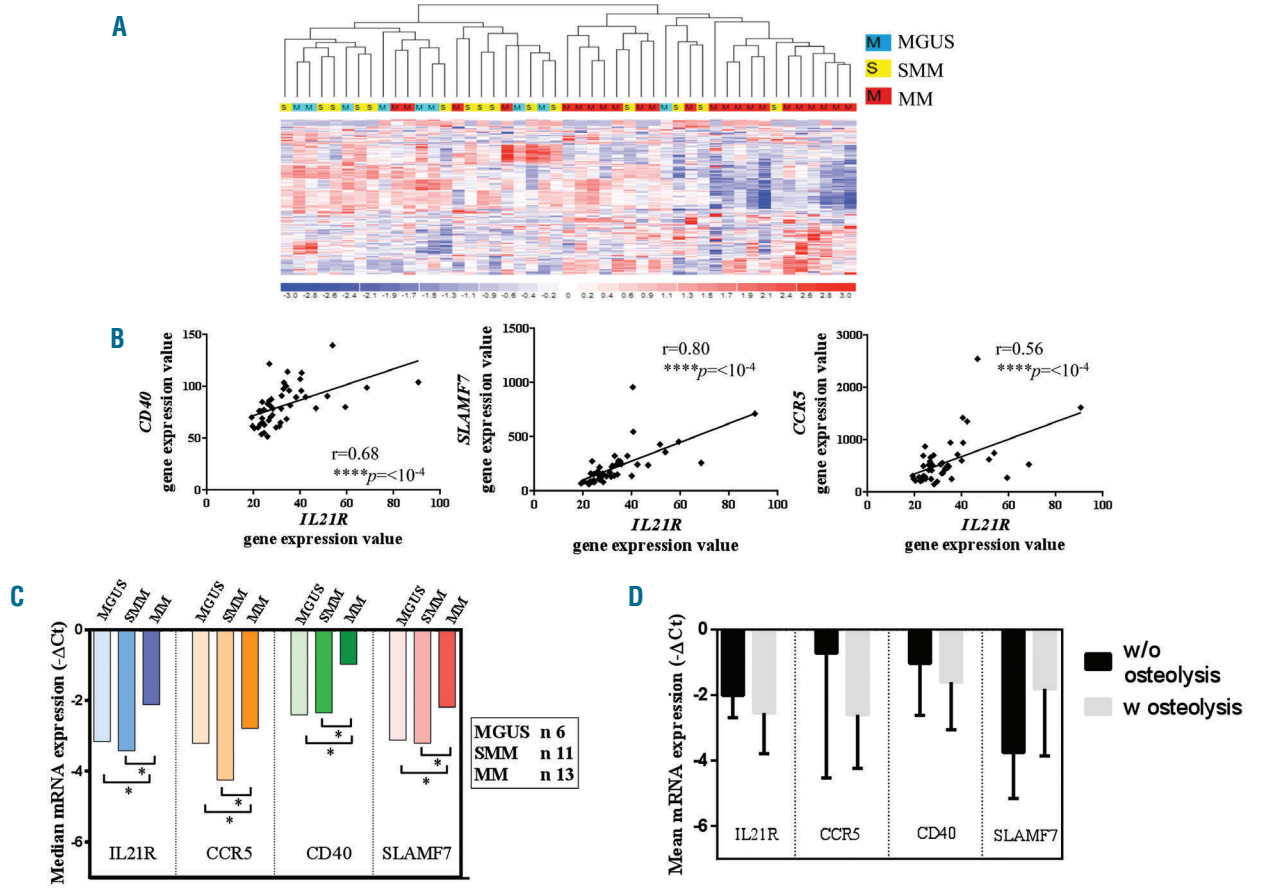


**Figure 2. Transcriptional fingerprints evaluated by gene expression profiling of purified bone marrow CD14⁺ cells from patients with different monoclonal gammopathies.** (A) Heatmap of the trancriptional profiles resulting from the unsupervised analysis of all the MM, SMM and MGUS monocyte samples. (B) Scatterplots showing the correlation between *IL21R* expression and that of *CD40*, *SLAMF7* and *CCR5* by BM monocytes as determined from gene expression analysis. The lines represent the linear regression between each couple of genes. (C) Quantitative real-time polymerase chain reaction of *IL21R*, *CCR5*, *CD40* and *SLAMF7* genes performed on BM monocytes purified from patients with monoclonal gammopathies. Values represent the median of the -ΔCt values of the reactions (*: fold change >1.5). (D) Quantitative real-time polymerase chain reaction of *IL21R*, *CCR5*, *CD40* and *SLAMF7* genes performed on BM monocytes purified from MM patients with (w) or without (w/o) osteolysis. Values represent the mean of the -ΔCt values of the reactions.

malignant plasma cells were not included in the analysis. Hence, BM monocyte samples included in the gene expression analysis were obtained from 23 MM, 15 SMM and nine MGUS patients. The main characteristics of patients eligible for gene expression analysis are reported in Table 2.

Unsupervised analysis significantly clustered together MM samples ($P$=0.0024, Fisher exact test) whereas SMM and MGUS were scattered along the dendrogram. (Figure 2A). A multiclass analysis identified 99 differentially expressed genes in CD14⁺ cells between the three classes of patients (*Online Supplementary Figure S3A*; *Online Supplementary Table S2*), whereas 78 genes (18 up- and 60 down-regulated) were differentially expressed in monocytes of MM patients as compared to SMM patients (*Online Supplementary Figure S3B*; *Online Supplementary Table S3*). The comparison of MM with samples from asymptomatic patients (SMM and MGUS) identified 254 genes differentially expressed in CD14⁺ cells; specifically, there were 62 up-regulated and 192 down-regulated genes (*Online Supplementary Figure S3C*, *Online Supplementary Table S4*). Functional annotation analysis of genes differentially expressed in symptomatic patients was performed using standard procedures with the Database for Annotation, Visualization and Integrated Discovery (DAVID) and Gene Set Enrichment Analysis (GSEA) tools (Table 3). Among the identified gene sets, it is worth mentioning those associated with the cytokine-cytokine receptor interaction pathway, Jak-STAT signaling pathway, and the interferon alpha and gamma responses. Among the differentially expressed genes, chemokines and chemokine and cytokine receptors with pro-osteoclastogenic properties such as *CCR5*, *IL21R* and *CD40*, were specifically up-regulated in CD14⁺ cells from MM patients. Importantly, monocytes in MM samples up-regulate *SLAMF7*, which is selectively expressed in plasma cells and natural killer cells in MM leading to antibody-dependent cellular cytotoxicity and direct natural killer cell activation.[32] Particularly, *IL21R* over-expression by BM CD14⁺ cells in MM was demonstrated (q-value=0), both in the multiclass analysis and when comparing MM *versus* SMM plus MGUS (*Online Supplementary Tables S2* and *S4*). Interestingly, *IL21R* gene expression in the complete database significantly correlated with the expression of *CCR5* ($P$=0.0197), CD40 ($P$<0.0001), and *SLAMF7* ($P$=0.0002) genes (Figure 2B). Moreover a further analysis between osteolytic *versus* not-osteolytic patients with active MM identified 12 genes

**Table 3.** Functional annotations* of the representative genes distinguishing BM monocytes as emerging from supervised analysis of MM *versus* MGUS plus SMM patients.

| Database for Annotation, Visualization and Integrated Discovery (DAVID ) | | |
|---|---|---|
| **Pathway Database** | **Term** | **Genes§** |
| KEGG | Cytokine-cytokine receptor interaction | *CCR5, IL21R, CSF3R, CD40* |
| | Jak-STAT signaling pathway | *IL21R, CSF3R,* |
| | Chemokine signaling pathway | *GNGT2, CCR5* |
| REACTOME | Metabolism of carbohydrates | *GOT2, GPI, PGM1, PGD, GYS1* |

| Gene Set Enrichment Analysis (GSEA) | |
|---|---|
| **Functional group category** | **Genes§** |
| INTERFERON ALPHA RESPONSE | *EPSTI1, IFI27, IFITM1, ISG20, LAP3* |
| INTERFERON GAMMA RESPONSE | *CD40, EPSTI1, IFI27, ISG20, LAP3, PIM1, SLAMF7, STAT4, VAMP5* |

*The DAVID Functional Annotation Tool v6.8 (https://david.ncifcrf.gov) and GSEA tool (http://software.broadinstitute.org/gsea) were used to classify genes into functional categories. §Genes down-regulated in MM monocytes are underlined.

(*SERPINB10*; *CDCA5*; *MYBL2*; *SELENBP1*; *TK1*; *GYPA*; *KIF18A*; *SPC25*; *HJURP*; *TAL1*; *SKA1; E2F8*) that were down-regulated in not-osteolytic MM patients. On the other hand, we did not find a significantly different gene expression signature between patients with active MM with high bone disease *versus* low bone disease.

Thereafter, in a subgroup of patients, we confirmed a significant up-regulation of *IL21R*, *CCR5*, *CD40*, and *SLAMF7* genes in CD14$^+$ cells from MM patients compared to SMM and/or MGUS patients, by real-time polymerase chain reaction (Figure 2C). Consistently with the gene expression profiling data, we did not find a significantly different expression of *IL21R*, *CCR5*, *CD40*, and *SLAMF7* genes between osteolytic *versus* not-osteolytic patients with active MM (Figure 2D).

## Bone marrow CD14$^+$ cells over-express *IL21R* mRNA in multiple myeloma patients irrespective of interleukin-21

Based on the gene expression data and previous evidence that IL-21 is a growth factor for MM cells[33,34] and that the IL-21/IL21R axis promotes osteoclastogenesis and bone destruction in pathological conditions[35,36] we further investigated the possible role of *IL21R* over-expression by CD14$^+$ cells in MM-induced osteoclastogenesis.

Firstly, by means of real-time polymerase chain reaction, we confirmed that *IL21R* mRNA levels significantly increased in BM CD14$^+$ cells across different plasma cell dyscrasias in a cohort of patients with MM, SMM and MGUS ($P$=0.036) (Figure 3A). We showed that BM CD14$^+$ cells expressed significantly higher levels of *IL21R* mRNA in MM patients than in MGUS patients ($P$=0.023, Figure 3A), and in MM patients compared to SMM plus MGUS patients ($P$=0.005, Figure 3B). The up-regulation of *IL21R* mRNA was also observed in MM CD14$^+$ cells obtained from peripheral blood (n=3, *data not shown*). The mean difference between *IL21R* expression (expressed as -ΔCt) by BM and peripheral blood monocytes purified from the same patient was 0.46±0.49 ($P$=0.64). The expression of IL-21R was also investigated at the protein level by flow cytometry: in line with the evidence that the CD14$^+$CD16$^+$ population was increased in MM patients, we found that BM CD14$^+$ cells in MM patients expressed IL-21R/CD360 and that the CD14$^+$CD16$^+$ population showed higher median fluorescence intensity (MFI) compared to CD14$^+$CD16$^-$ cells in each tested patient (mean $\Delta_{MFICD14+CD16+-MFICD14+CD16-}$ ±SD= 6.1±2.4) (Figure 3C).

Furthermore, we checked the levels of active STAT3 in BM CD14$^+$ cells, as it is well known that the signaling pathway down-stream of IL-21R leads to the activation of Jak3 and STAT3.[37,38] We found that MM CD14$^+$ cells had significantly higher levels of active STAT3 compared to MGUS cells ($P$=0.0029) and cells from asymptomatic patients (MGUS plus SMM) ($P$=0.0093, Figure 3D).

To investigate the possible mechanisms involved in *IL21R* mRNA over-expression, we treated purified BM CD14$^+$ cells obtained from MM, SMM or MGUS patients with the rhIL-21 concentration reached in the BM plasma of our cohort of patients. The addition of rhIL-21 (30 pg/mL) slightly increased *IL21R* mRNA in BM CD14$^+$ cells from MGUS and SMM patients but not from MM patients, without the difference reaching statistical significance (Figure 3E), suggesting a constitutive *IL21R* mRNA expression in MM patients irrespective of the presence of IL-21. On the other hand, using the well-established[39] monocytic cell line THP-1, we found that combined treatment with the pro-inflammatory cytokines rhIL-6 (20 ng/mL) and rhTNF-α (10 ng/mL) significantly increased *IL21R* mRNA expression compared to that of untreated controls ($P$=0.005, Figure 3F).

## Bone marrow *IL21* expression and protein levels did not significantly differ across patients with monoclonal gammopathies

Next, we evaluated *IL21* mRNA expression levels in our cohort of patients. BM mesenchymal stromal cells, CD14$^+$ and primary MM cells did not express the *IL21* gene, which was otherwise expressed by CD3$^+$ cells, in the CD4$^+$ fraction, as checked by real-time polymerase chain reaction (*Online Supplementary Table S5*). We failed to find a significant difference in *IL21* mRNA expression by CD3$^+$ cells among MM, SMM and MGUS patients, as shown in Figure 4A. Consistently, no significant difference was found in BM levels of IL-21 across the different monoclonal gammopathies, as detected by enzyme-linked immunosorbent assay (Figure 4B). The median BM IL-21 level was 32.4 pg/mL for MGUS, 24.4 pg/mL for SMM and 34.1 pg/mL for newly diagnosed MM patients.

## *IL21R* over-expression by CD14$^+$ cells is involved in osteoclastogenesis

Since *IL21R* was identified among the genes over-expressed by BM CD14$^+$ cells in MM patients, we investi-

gated its role in osteoclast differentiation. We induced *IL21R* over-expression in CD14+ cells obtained from three different healthy donors. The over-expression of *IL21R* gene was evaluated by real-time polymerase chain reaction in CD14+ cells infected with IL21R vector (CD14+ IL21R vector) and compared to that of cells infected with the empty vector (CD14+ empty vector, Figure 5A). Subsequently, we performed *in vitro* osteoclastogenesis assays. The number of osteoclasts in this set of experiments was low because the infection with lentiviral vectors strongly affects primary monocyte viability (Figure 5B,C). Interestingly, *in vitro* osteoclastogenesis assays showed that the over-expression of *IL21R* increased the number and median area of osteoclasts in the presence of RANKL and M-CSF compared to controls; consistently, the presence of Janex 1, a JAK3 inhibitor known to block IL-21R signaling, significantly reduced osteoclast formation and size in CD14+ IL21R vector cells (Figure 5B,C).

## Blocking interleukin-21 receptor signaling inhibits osteoclastogenesis

To further confirm the role of *IL21R* over-expression in osteoclastogenesis, we performed *in vitro* osteoclastogenesis assays with or without rhIL-21 in the presence or absence of Janex 1. The presence of rhIL-21 did not affect the number or area of TRAP-positive osteoclasts obtained

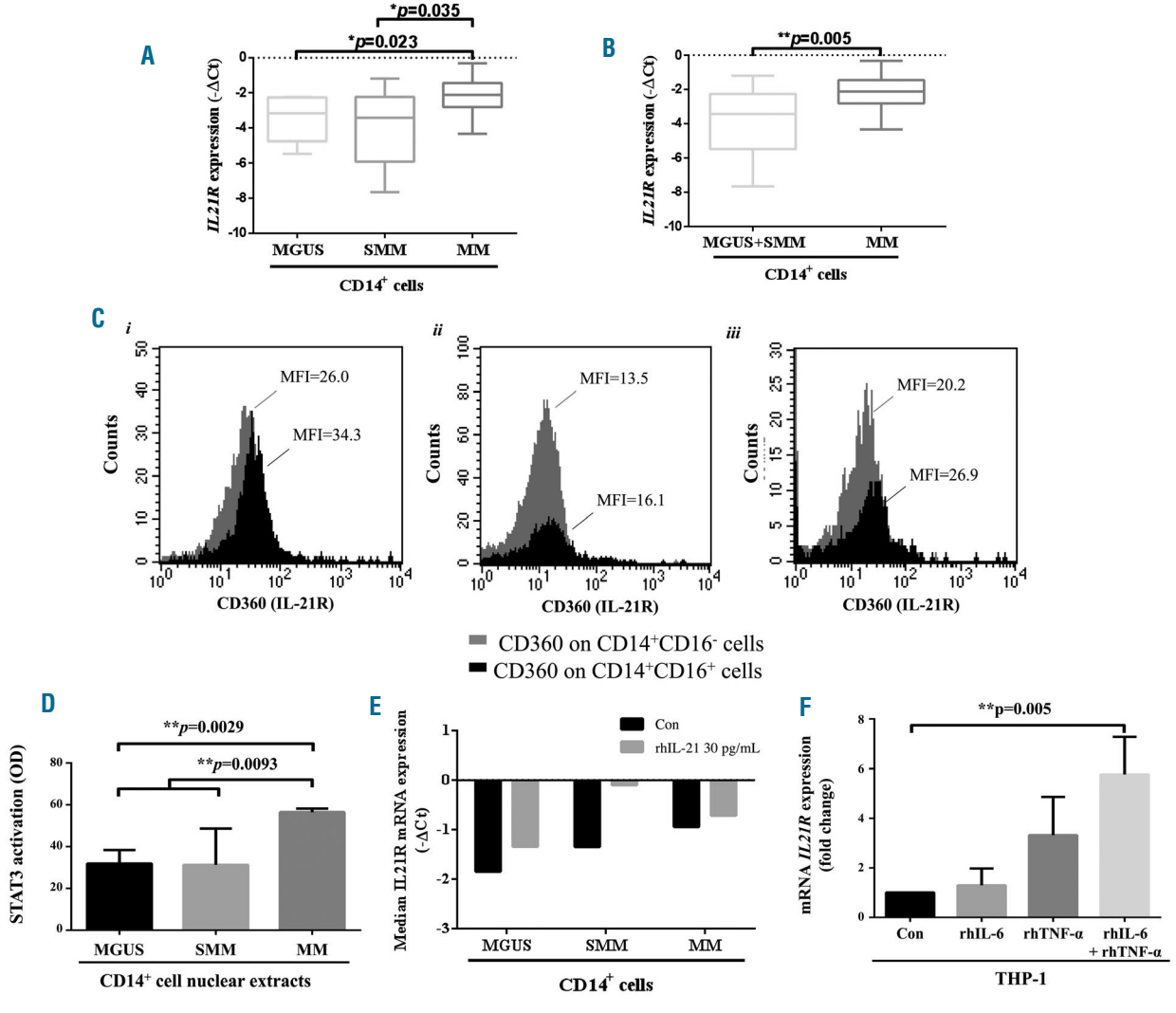


**Figure 3. IL-21R over-expression by bone marrow CD14+ cells from patients with multiple myeloma compared to those from patients with smoldering multiple myeloma or monoclonal gammopathy of undetermined significance.** (A) *IL21R* mRNA expression was evaluated by real-time polymerase chain reaction (PCR) in purified BM CD14+ obtained from patients with monoclonal gammopathies. Box plots show the median –ΔCt levels (*P* calculated by the Mann-Whitney test). (B) *IL21R* mRNA expression was evaluated by real-time PCR in purified BM CD14+ obtained from MM patients *versus* SMM plus MGUS patients. Box plots show the median –ΔCt levels (*P* calculated by the Mann-Whitney test). (C) CD360/IL-21R expression was evaluated by flow cytometry in BM CD14+CD16- and CD14+CD16+ cells. Comparison between CD360 expression by CD14+CD16- and CD14+CD16+ monocyte populations, stained with anti-CD360 or control IgG1, is shown in three representative MM patients (*i*, *ii*, and *iii*) (MFI: median fluorescence intensity). (D) Active STAT3 levels were determined by the STAT family assay kit in nuclear extracts of purified BM CD14+ cells obtained from MGUS (n=3), SMM (n=3) and MM (n=3) patients. The bar chart represents the mean±SD level of active STAT3 checked as optical density (OD) at 450 nm with a reference wavelength of 620 nm, after subtracting the blank. (E) BM CD14+ cells purified from patients with MM, SMM or MGUS were treated with or without rhIL-21 (30 pg/mL) for 24 h. *IL21R* mRNA level was evaluated by real-time PCR. The bar chart represents the median –ΔCt of *IL21R* mRNA of three replicates (Con: untreated control). (F) The monocytic cell line THP-1 was treated for 48 h with or without rhIL-6 (20 ng/mL) or TNF-α (10 ng/mL) or both cytokines. *IL21R* mRNA levels were evaluated by real-time PCR in three independent experiments (*P* calculated using the t test). The bar chart represents the mean±SD fold change of mRNA *IL21R* (Con: untreated control).

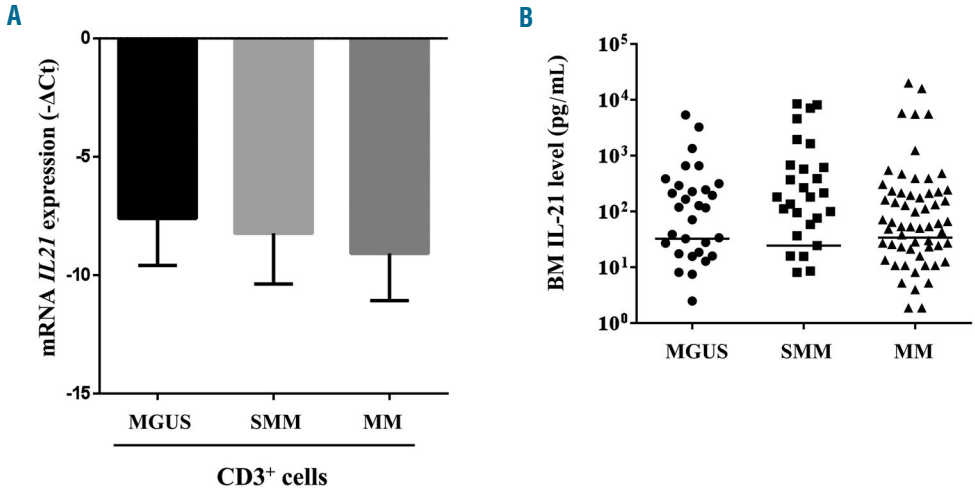


**Figure 4. *IL21* mRNA expression by the bone marrow microenvironment and levels of interleukin-21 in the bone marrow in patients with multiple myeloma, smoldering multiple myeloma or monoclonal gammopathy of undetermined significance.** (A) *IL21* mRNA expression by purified CD3$^+$ cells from MM (n=5), SMM (n=7) or MGUS (n=7) patients evaluated by real-time polymerase chain reaction. The bar chart shows the mean±SD −ΔCt of *IL21* mRNA. (B) BM IL-21 levels were evaluated by enzyme linked immunosorbent assay in a cohort of 76 newly diagnosed MM, 42 SMM and 41 MGUS patients. The scatter dot plot represents BM IL-21 levels in the cohort of patients with the lines representing median levels.

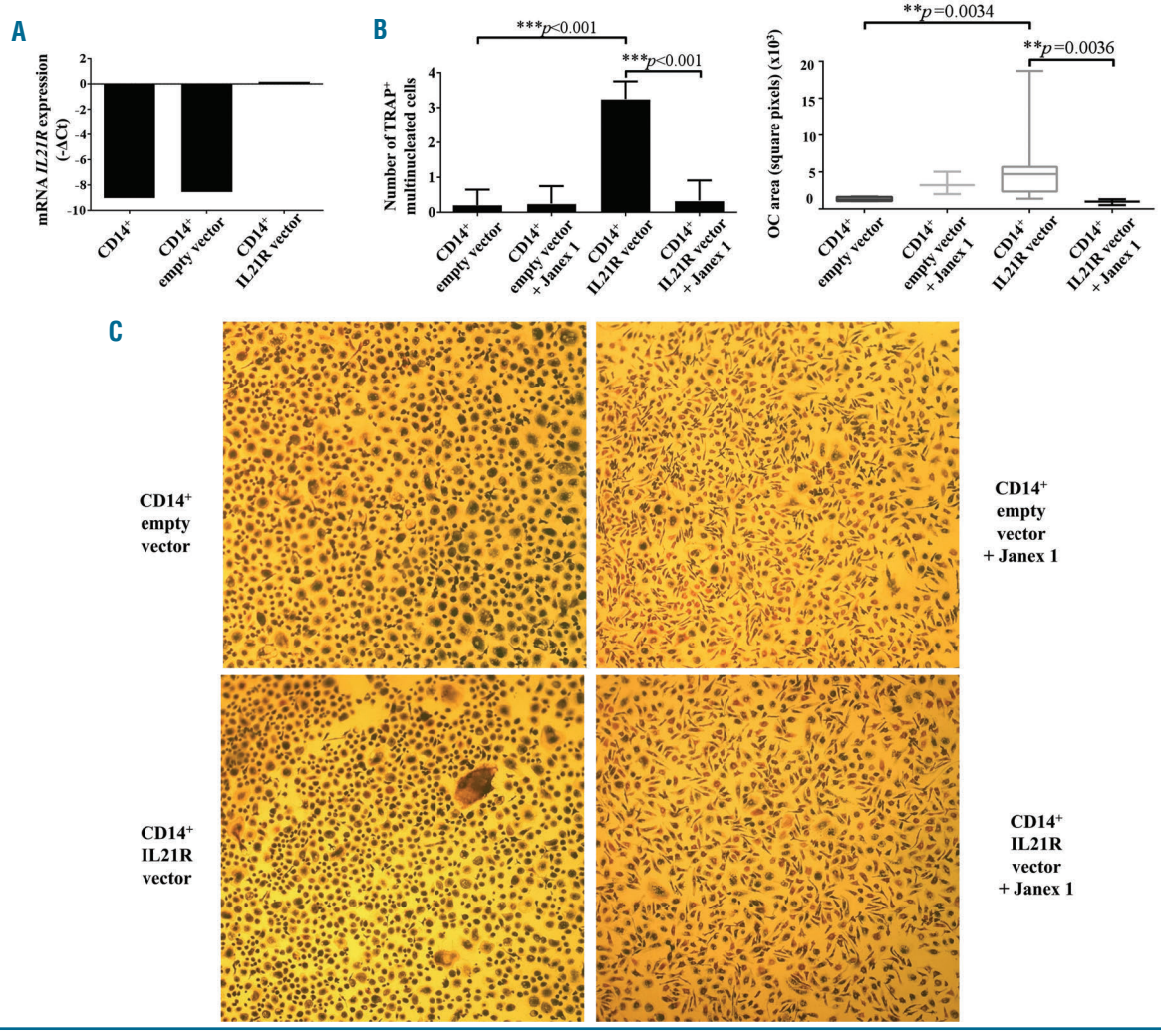


**Figure 5. *IL21R* over-expression by a lentiviral vector in monocytes increases the differentiation of osteoclasts.** (A) *IL21R* was over-expressed in peripheral blood CD14$^+$ cells obtained from three different healthy donors transduced with a specific lentiviral vector for *IL21R* (CD14$^+$ IL21R vector) as compared to those infected with the empty control vector (CD14$^+$ empty vector) or not transduced (CD14$^+$). *IL21R* mRNA levels were checked by real-time polymerase chain reaction. Bar graph represents the median −ΔCt levels of three independent experiments. CD14$^+$ transduced cells with *IL21R* or empty lentiviral vectors (200,000 cells/well) were seeded in 96-well plates in αMEM with 10% FBS, rhM-CSF 10 ng/mL and rhRANKL 50 ng/mL in the presence or absence of the IL-21R signaling inhibitor Janex 1 (10 μM) or vehicle (DMSO). After 28 days of culture, osteoclasts were identified as multinucleated (>3 nuclei) TRAP-positive cells and counted by light microscopy. (B) The bar graph shows the mean±SD number of osteoclasts for each well (*P* calculated using the t test) in three independent experiments with CD14$^+$ from three different healthy donors (left panel). The box plot represents the osteoclast area (*P* calculated by the Mann-Whitney test) in a representative experiment performed at least in triplicate (right panel). (C) Images of one representative experiment of the osteoclastogenesis assay stained by TRAP of CD14$^+$ IL21R vector and CD14$^+$ empty vector cells performed in the presence or absence of Janex 1 (original magnification, 4x).

from total mononuclear cells or CD14+ cells purified from MM BM aspirates (Figure 6A,B). No significant differences were found in osteoclast number or area between tumor and not-tumor samples treated with IL-21 (*data not shown*).

On the other hand, Janex 1 significantly suppressed osteoclastogenesis from either total BM mononuclear cells or BM CD14+ cells obtained from MM patients, both in the presence ($P$<0.001) and in the absence ($P$<0.001) of the rhIL-21, as shown in Figure 6A,B. Moreover, the presence of Janex 1 significantly reduced the median osteoclast area both in the presence ($P$=0.012) and in the absence ($P$<0.001) of rhIL-21, compared to untreated controls (Figure 6A).

## Discussion

Monoclonal gammopathies are characterized by the activation of bone resorption with a progressive increase in the number of osteoclasts from MGUS to MM.[5] Several studies have evaluated the gene expression profiles of plasma cells obtained both from patients with newly diagnosed MM or MGUS and from healthy donors to identify genes potentially related to the progression of MM.[40,41] However, while MGUS and MM can be distinguished from normal plasma cells, these two conditions cannot be easily differentiated from each other.[40,41] Transcriptional data were used to stratify MM patients with lytic lesions, identifying *DKK1* as the main over-expressed gene when focal bone lesions occur.[42] Studies analyzing BM microenvironment cells indicate that mesenchymal stromal cells and osteoblasts in MM patients have different transcriptional profiles compared to those of healthy donors and based on the occurrence of osteolytic lesions.[13] However, not all the BM biological alterations from MGUS to SMM and, finally, to active MM are clear yet.

As regards the immunophenotypic profile, we found that the median percentage of CD14+CD16+ cells in BM samples increased among the different types of monoclonal gammopathies, being significantly higher in MM than in MGUS. In our study, for the first time we sorted BM CD14+CD16+ cells and showed that they represent the osteoclastogenic fraction of CD14+ cells in MM patients, supporting the notion that inflammatory monocytes are involved in MM-induced osteoclastogenesis. In addition, CD14+CD16+ cells might contribute to the high production of inflammatory cytokines, such as TNF-α,[23] which are increased in the BM of MM patients and involved in osteoclast formation.[43,44]

Consistent with the immunophenotypic profile, the transcriptome of CD14+ cells obtained from MM patients showed up-regulation, as compared to the expression in SMM and MGUS, of genes involved in immune response, chemotaxis and osteoclastogenesis. We focused on genes

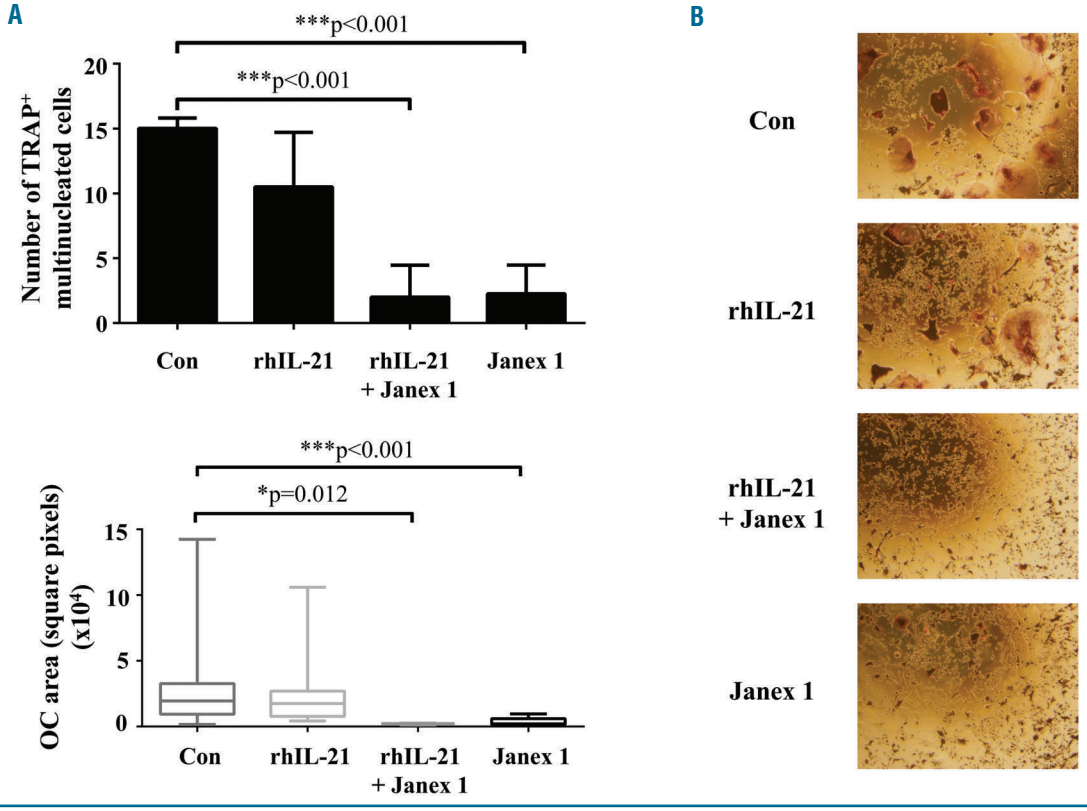


**Figure 6. Interleukin-21 receptor signaling inhibition blocks Interleukin-21 driven osteoclastogenesis.** BM mononuclear cells, obtained from MM patients, were seeded at the concentration of 4x10⁵ cells/well in 96-well plates in αMEM with 10% FBS, rhM-CSF 25 ng/mL and rhRANKL 20 ng/mL in the presence or absence of rIL-21 (30 pg/mL) and the IL-21R signaling inhibitor Janex 1 (10 μM) or vehicle (DMSO) for 28 days, replacing the medium every 3 days. At the end of the culture period osteoclasts were identified as multinucleated (>3 nuclei) TRAP-positive cells and counted by light microscopy (Con: untreated control). (A) The bar graph represents the mean±SD osteoclast number for each well (*P* calculated using the t test) (upper panel). The box plot shows the osteoclast (OC) area (*P* calculated by the Mann-Whitney test) in one representative experiment performed at least in triplicate (lower panel). (B) Representative images of osteoclasts stained with TRAP after 28 days of culture (original magnification, 4x).

potentially involved in osteoclastogenesis. The up-regulated genes included *CCR5*, whose role in bone destruction in MM has already been extensively investigated.[19,20] *IL21R* mRNA was also over-expressed by BM CD14$^+$ cells in MM but not in SMM or MGUS.

The analysis of patients with active MM according to the presence of osteolysis identified only 12 genes that were down-regulated in not-osteolytic patients. Nevertheless, we did not find a significantly different gene expression signature between the MM patients with high bone disease *versus* those with low bone disease. The lack of major differences in the immunophenotypic and transcriptional profiles of monocytes from osteolytic and not-osteolytic patients are not surprising because the main pathophysiological difference between osteolytic and non-osteolytic MM patients is the suppression of osteoblast formation rather than increased osteoclast formation and activity. Our data are supported by previous reports that all MM patients have a significant increase of bone resorption rate with unbalanced bone remodeling.[5,9] In addition, MGUS patients have a significant increase of bone resorption rate.[5,7] Consistently, in this study we did not find a large number of differentially expressed genes by monocytes across patients with the different monoclonal gammopathies.

In this study, we demonstrated the potential involvement of the IL-21/IL-21R axis in the increased osteoclastogenesis that occurs in MM patients. The ligand of IL-21R, the cytokine IL-21, is a growth factor for MM cells[33,34] and it is mainly produced by T cells.[37,45] The binding of IL-21 to its receptor leads to the activation of the Jak–STAT pathway, in particular Jak1, Jak3, STAT1, and STAT3.[37,46,47] Interestingly, a previous study showed that IL-21 up-regulation in the synovium and the serum of patients with rheumatoid arthritis is involved in osteoclastogenesis and bone destruction.[35] Nevertheless, the role of IL-21 in MM-induced osteoclast formation is largely unknown. In this study we found significant *IL21R* mRNA over-expression by CD14$^+$ cells correlated with the other osteoclastogenic genes identified such as *CCR5*, but also with *CD40* and *SLAMF7*. Interestingly, in line with the immunophenotypic profile of BM CD14$^+$ in MM patients, *IL21R* was expressed at high intensity in the CD14$^+$CD16$^+$ fraction. The up-regulation of *IL21R* in MM patients was associated with an increase of STAT3 signaling and was independent of the presence of IL-21. On the other hand, as the combination of the pro-inflammatory cytokines IL-6 and TNF-α increases *IL21R* mRNA expression in monocytes, we might suppose that these cytokines are involved in *IL21R* over-expression by BM CD14$^+$ cells. The pathophysiological role of *IL21R* over-expression by CD14$^+$ cells in the enhanced osteoclastogenesis that occurs in MM patients was further demonstrated by a lentiviral approach. These data also support the role of IL-21R signaling as a potential therapeutic target. Accordingly, it is worth remembering that the clinically approved JAK3 inhibitor tofacitinib suppresses osteoclast-mediated structural damage to arthritic joints and decreases RANKL production.[48] This study was not designed to evaluate the role of IL-21R overexpression as a potential biomarker of MM progression; however, our data suggest that IL-21R expression level could be a potential biomarker of myeloma progression. Clearly, only an appropriate prospective study evaluating IL-21R expression would be able to address this point.

In conclusion, this study supports the notion that a pro-inflammatory profile of BM CD14$^+$ cells is involved in osteoclastogenesis in MM patients, in line with a considerable amount of evidence from the literature.[19,49,50] For the first time, we highlighted *IL21R* over-expression in BM monocytes from MM patients and demonstrated its role in increased osteoclastogenesis, suggesting that IL-21R signaling could be a potential new therapeutic target for MM bone disease.

***Acknowledgments***

*This work was supported in part by a grant from the Associazione Italiana per la Ricerca sul Cancro (AIRC) IG2014 n.15531 (NG) Fondazione Italiana per la Ricerca sul Cancro fellowship [id. 18152 (MB),and id. 16462 (DT)] and AIRC IG16722 (AN). This work was also supported by a fellowship from ParmAIL (Associazione Italiana Contro le Leucemie-Linfomi e Myeloma, Parma) (VM).*

## References

1. Melton LJ 3rd, Kyle RA, Achenbach SJ, Oberg AL, Rajkumar SV. Fracture risk with multiple myeloma: a population-based study. J Bone Miner Res. 2005;20(3):487-493.
2. Giuliani N, Colla S, Rizzoli V. New insight in the mechanism of osteoclast activation and formation in multiple myeloma: focus on the receptor activator of NF-kappaB ligand (RANKL). Exp Hematol. 2004;32(8):685-691.
3. Giuliani N, Rizzoli V, Roodman GD. Multiple myeloma bone disease: pathophysiology of osteoblast inhibition. Blood. 2006;108(13):3992-3996.
4. Roodman GD. Pathogenesis of myeloma bone disease. Leukemia. 2009;23(3):435-441.
5. Bataille R, Chappard D, Basle MF. Quantifiable excess of bone resorption in monoclonal gammopathy is an early symptom of malignancy: a prospective study of 87 bone biopsies. Blood. 1996;87(11):4762-4769.
6. Kyle RA, Durie BG, Rajkumar SV, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. Leukemia. 2010;24(6):1121-1127.
7. Ng AC, Khosla S, Charatcharoenwitthaya N, et al. Bone microstructural changes revealed by high-resolution peripheral quantitative computed tomography imaging and elevated DKK1 and MIP-1alpha levels in patients with MGUS. Blood. 2011;118(25):6529-6534.
8. Agarwal A, Ghobrial IM. Monoclonal gammopathy of undetermined significance and smoldering multiple myeloma: a review of the current understanding of epidemiology, biology, risk stratification, and management of myeloma precursor disease. Clin Cancer Res. 2013;19(5):985-994.
9. Bataille R, Chappard D, Marcelli C, et al. Mechanisms of bone destruction in multiple myeloma: the importance of an unbalanced process in determining the severity of lytic bone disease. J Clin Oncol. 1989;7(12):1909-1914.
10. Roodman GD. Role of the bone marrow microenvironment in multiple myeloma. J Bone Miner Res. 2002;17(11):1921-1925.
11. Corre J, Mahtouk K, Attal M, et al. Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. Leukemia. 2007;21(5):1079-1088.
12. Garayoa M, Garcia JL, Santamaria C, et al. Mesenchymal stem cells from multiple myeloma patients display distinct genomic profile as compared with those from normal donors. Leukemia. 2009;23(8):1515-1527.
13. Todoerti K, Lisignoli G, Storti P, et al. Distinct transcriptional profiles characterize bone microenvironment mesenchymal cells rather than osteoblasts in relationship with multiple myeloma bone disease. Exp Hematol. 2010;38(2):141-153.
14. Yaccoby S, Wezeman MJ, Henderson A, et al. Cancer and the microenvironment: myeloma-osteoclast interactions as a model. Cancer Res. 2004;64(6):2016-2023.

15. Ribatti D, Vacca A. The role of monocytes-macrophages in vasculogenesis in multiple myeloma. Leukemia. 2009;23(9):1535-1536.
16. Kawano Y, Moschetta M, Manier S, et al. Targeting the bone marrow microenvironment in multiple myeloma. Immunol Rev. 2015;263(1):160-172.
17. Giuliani N, Bataille R, Mancini C, Lazzaretti M, Barille S. Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment. Blood. 2001;98(13):3527-3533.
18. Bataille R, Chappard D, Klein B. The critical role of interleukin-6, interleukin-1B and macrophage colony-stimulating factor in the pathogenesis of bone lesions in multiple myeloma. Int J Clin Lab Res. 1992;21(4):283-287.
19. Choi SJ, Cruz JC, Craig F, et al. Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. Blood. 2000;96(2):671-675.
20. Han JH, Choi SJ, Kurihara N, Koide M, Oba Y, Roodman GD. Macrophage inflammatory protein-1alpha is an osteoclastogenic factor in myeloma that is independent of receptor activator of nuclear factor kappaB ligand. Blood. 2001;97(11):3349-3353.
21. Silbermann R, Bolzoni M, Storti P, et al. Bone marrow monocyte-/macrophage-derived activin A mediates the osteoclastogenic effect of IL-3 in multiple myeloma. Leukemia. 2014;28(4):951-954.
22. Lee HW, Choi HJ, Ha SJ, Lee KT, Kwon YG. Recruitment of monocytes/macrophages in different tumor microenvironments. Biochim Biophys Acta. 2013;1835(2):170-179.
23. Belge KU, Dayyani F, Horelt A, et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J Immunol. 2002;168(7):3536-3542.
24. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J Leukoc Biol. 2007;81(3):584-592.
25. Chiu YG, Shao T, Feng C, et al. CD16 (FcRgammaIII) as a potential marker of osteoclast precursors in psoriatic arthritis. Arthritis Res Ther. 2010;12(1):R14.
26. Sponaas AM, Moen SH, Liabakk NB, et al. The proportion of CD16(+)CD14(dim) monocytes increases with tumor cell load in bone marrow of patients with multiple myeloma. Immun Inflamm Dis. 2015;3(2):94-102.
27. Petitprez V, Royer B, Desoutter J, et al. CD14+ CD16+ monocytes rather than CD14+ CD51/61+ monocytes are a potential cytological marker of circulating osteoclast precursors in multiple myeloma. A preliminary study. Int L Lab Hematol. 2015;37(1):29-35.
28. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol. 2014;15(12):e538-548.
29. Palma BD, Guasco D, Pedrazzoni M, et al. Osteolytic lesions, cytogenetic features and bone marrow levels of cytokines and chemokines in multiple myeloma patients: role of chemokine (C-C motif) ligand 20. Leukemia. 2016;30(2):409-416.
30. Storti P, Donofrio G, Colla S, et al. HOXB7 expression by myeloma cells regulates their pro-angiogenic properties in multiple myeloma patients. Leukemia. 2011;25(3):527-537.
31. Ramnaraine ML, Mathews WE, Clohisy DR. Lentivirus transduction of human osteoclast precursor cells and differentiation into functional osteoclasts. Bone. 2012;50(1):97-103.
32. Markham A. Elotuzumab: first global approval. Drugs. 2016;76(3):397-403.
33. Brenne AT, Ro TB, Waage A, Sundan A, Borset M, Hjorth-Hansen H. Interleukin-21 is a growth and survival factor for human myeloma cells. Blood. 2002;99(10):3756-3762.
34. Menoret E, Maiga S, Descamps G, et al. IL-21 stimulates human myeloma cell growth through an autocrine IGF-1 loop. J Immunol. 2008;181(10):6837-6842.
35. Kwok SK, Cho ML, Park MK, et al. Interleukin-21 promotes osteoclastogenesis in humans with rheumatoid arthritis and in mice with collagen-induced arthritis. Arthritis Rheum. 2012;64(3):740-751.
36. Kim KW, Kim HR, Kim BM, Cho ML, Lee SH. Th17 Cytokines regulate osteoclastogenesis in rheumatoid arthritis. Am J Pathol. 2015;185(11):3011-3024.
37. Mehta DS, Wurster AL, Grusby MJ. Biology of IL-21 and the IL-21 receptor. Immunol Rev. 2004;202:84-95.
38. Ma J, Ma D, Ji C. The role of IL-21 in hematological malignancies. Cytokine. 2011;56(2):133-139.
39. Millet P, Vachharajani V, McPhail L, Yoza B, McCall CE. GAPDH binding to TNF-alpha mRNA contributes to posttranscriptional repression in monocytes: a novel mechanism of communication between inflammation and metabolism. J Immunol. 2016;196(6):2541-2551.
40. Zhan F, Hardin J, Kordsmeier B, et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. Blood. 2002;99(5):1745-1757.
41. Davies FE, Dring AM, Li C, et al. Insights into the multistep transformation of MGUS to myeloma using microarray expression analysis. Blood. 2003;102(13):4504-4511.
42. Tian E, Zhan F, Walker R, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med. 2003;349(26):2483-2494.
43. Lichtenstein A, Berenson J, Norman D, Chang MP, Carlile A. Production of cytokines by bone marrow cells obtained from patients with multiple myeloma. Blood. 1989;74(4):1266-1273.
44. Portier M, Zhang XG, Ursule E, et al. Cytokine gene expression in human multiple myeloma. Br J Haematol. 1993;85(3):514-520.
45. Caprioli F, Sarra M, Caruso R, et al. Autocrine regulation of IL-21 production in human T lymphocytes. J Immunol. 2008;180(3):1800-1807.
46. Asao H, Okuyama C, Kumaki S, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. J Immunol. 2001;167(1):1-5.
47. Zeng R, Spolski R, Casas E, Zhu W, Levy DE, Leonard WJ. The molecular basis of IL-21-mediated proliferation. Blood. 2007;109(10):4135-4142.
48. LaBranche TP, Jesson MI, Radi ZA, et al. JAK inhibition with tofacitinib suppresses arthritic joint structural damage through decreased RANKL production. Arthritis Rheum. 2012;64(11):3531-3542.
49. Giuliani N, Lisignoli G, Colla S, et al. CC-chemokine ligand 20/macrophage inflammatory protein-3alpha and CC-chemokine receptor 6 are overexpressed in myeloma microenvironment related to osteolytic bone lesions. Cancer Res. 2008;68(16):6840-6850.
50. Tucci M, Stucci S, Savonarola A, et al. Immature dendritic cells in multiple myeloma are prone to osteoclast-like differentiation through interleukin-17A stimulation. Br J Haematol. 2013;161(6):821-831.