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Over-expression of pro-osteoclastogenic cytokine receptors and chemokines by bone marrow $CD14^+$ monocytes of Multiple Myeloma (MM) patients as compared to Smoldering MM (SMM) and Monoclonal Gammopathy of Uncertain Significance (MGUS): role of IL-21R/IL-21 axis in MM-induced osteoclastogenesis.

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In ogni cosa è salutare,
di tanto in tanto,
mettere un punto
interrogativo a ciò che
a lungo si era dato per
scontato.

(B. Russell)

Summary:

Abstract

Multiple myeloma (MM) is characterized by the uncoupled increase in bone marrow (BM) of osteoclast formation and activation, which lead to bone destruction, as compared to patients with smoldering MM (SMM) and monoclonal gammopathy of uncertain significance (MGUS). Although the molecular analysis of clonal plasma cells (PCs) identified several genes whose overexpression is associated with the occurrence of bone lesions, a clear transcriptional fingerprint able to distinguish the different PC dyscrasias is lacking. As the close relationship between PCs and BM microenvironment plays a pivotal role in MM pathogenesis, ongoing studies are focusing on the presence of potential molecular alterations in the microenvironment. Among the different cell types of BM microenvironment, monocytes are known to be primarily involved in osteoclastogenesis, angiogenesis and immune function. The aim of this study was to analyze the transcriptional and proteomic profiles of the BM $CD14^+$ cells across the different types of monoclonal gammopathies and to identify alterations potentially involved in the pathogenesis of the increased osteoclastogenesis.

The expression profiles of $CD14^+$ samples were evaluated by Affymetrix GeneChip HG-U133Plus 2.0 arrays in 25 MM, 11 SMM and 8 MGUS patients. The proteomic analysis of $CD14^+$ cells of 5 MM, 5 SMM and 5 MGUS was run on Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) and the data analyzed with Proteome Discoverer 1.4 software.

A multiclass analysis identified 18 differentially expressed genes in MGUS, SMM and MM. The comparison of MM with both SMM and MGUS samples identified 61 genes differentially expressed in $CD14^+$ cells (37 up-regulated and 24 down-regulated). Interestingly, we found specific cytokine receptors (*IL21R* and *IL-15R*) and pro-osteoclastogenic chemokines (*CXCL10*) that were over-expressed in $CD14^+$ of MM patients, as compared to SMM and MGUS. Similarly, the proteomic analysis reinforced that different $CD14^+$ monocyte protein profiles were found comparing MM patients with MGUS and SMM ones. Interestingly, MM monocytes over-expressed proteins involved in cell adhesion and inflammation and down-regulated molecules implicated in antimicrobial functions.

Because recent data indicate that IL-21 is a growth factor for MM cells and may promote osteoclastogenesis in some pathological conditions such as rheumatoid arthritis, we further investigate the potential role of *IL-21R* over-expression by MM $CD14^+$ cells in osteoclastogenesis. Firstly, we confirmed the *IL-21R* up-regulation by $CD14^+$ of MM patients at both mRNA and protein level as compared to both SMM and MGUS. On the other hand any significant difference was not observed in the BM plasma IL-21 levels between MM, SMM and MGUS. The treatment with rhIL-21, at the concentration observed in the BM plasma, increased the size of osteoclasts in $CD14^+$-derived *in vitro* osteoclastogenesis assays, in MM patients but not in SMM and MGUS; this suggested higher sensitivity to the IL-21-dependent pro-osteoclastogenic differentiation effect in MM patients. Finally the overexpression of IL-21R by lentiviral vectors, as well as the use of an IL-21R signaling inhibitor, was analyzed in our *in vitro* model to delineate a new anti-osteoclastogenic strategy.

In conclusion our results indicate that different expression fingerprints characterize BM $CD14^+$ monocytes of patients with MM as compared to those with SMM and MGUS, including over-expression of IL-21R, putatively involved in MM-induced osteoclast formation and activation through an increased sensitivity to IL-21.

Riassunto

Il mieloma multiplo (MM) è caratterizzato da un rimodellamento osseo alterato, con aumento del numero e dell'attività degli osteoclasti, che portano alla distruzione ossea, rispetto ai pazienti con smoldering MM (SMM) e gammopatia monoclonale di significato incerto (MGUS). Sebbene l'analisi molecolare delle plasmacellule (PC) clonali abbia identificato diversi geni la cui over-espressione è associata con la presenza di lesioni ossee, non è ancora stato identificato un profilo in grado di distinguere tra loro, in modo chiaro, le diverse discrasie plasmacellulari. Dal momento che la stretta relazione tra PC e microambiente midollare gioca un ruolo fondamentale nella patogenesi del MM, attualmente diversi gruppi di ricerca si sono focalizzati sullo studio della presenza di eventuali alterazioni molecolari presenti nel microambiente midollare. Tra i diversi tipi di cellule che compongono il microambiente midollare, i monociti sono noti per essere primariamente coinvolti nei processi di osteoclastogenesi, angiogenesi e funzione immunitaria. Lo scopo di questo studio è stato quello di analizzare i profili trascrizionali e proteomici dei monociti $CD14^+$ midollari dei diversi tipi di gammopatie monoclonali e di identificare le alterazioni potenzialmente coinvolte nella patogenesi dell'osteoclastogenesi indotta da MM.

I profili di espressione delle cellule $CD14^+$ sono stati valutati attraverso *array GeneChip HG-U133Plus 2.0* su piattaforma Affymetrix, utilizzando campioni ottenuti da 25 pazienti affetti da MM, 11 SMM e 8 MGUS. L'analisi proteomica dei monociti midollari è stata invece eseguita con lo spettrometro di massa *Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer* (Thermo Scientific) ed il software Proteome Discoverer 1.4, utilizzando monociti provenienti da 5 MM, 5 SMM e 5 MGUS.

L'analisi multiclasse dei profili di espressione genica dei monociti midollari ha identificato 18 geni differenzialmente espressi in MGUS, SMM e MM. Il confronto delle cellule $CD14^+$ di MM con le forme indolenti (SMM e MGUS), ha identificato 61 geni differenzialmente espressi (37 up-regolati e 24 down-regolati). Tra questi geni, abbiamo trovato recettori (*IL-21R e IL-15R*) e chemochine pro-osteoclastogeniche (*CXCL10*) che risultano over-espressi nei monociti $CD14^+$ dei pazienti con MM, rispetto a SMM e MGUS. Analogamente, l'analisi proteomica ha identificato diversi profili per i monociti dei pazienti con MM rispetto alle forme indolenti. È interessante notare che, i monociti di MM over-esprimono

proteine coinvolte nell'adesione cellulare e nell'infiammazione, e molecole implicate in funzioni antimicrobiche.

Poiché i dati recenti di letteratura indicano che IL-21 è un fattore di crescita per le cellule di MM e può promuovere l'osteoclastogenesi in alcune condizioni patologiche come l'artrite reumatoide, abbiamo focalizzato la nostra attenzione sul ruolo potenziale dell'asse IL-21/IL-21R nell'osteoclastogenesi indotta da MM. In primo luogo, abbiamo confermato l'over-espressione di IL-21R da parte dei monociti di MM, sia a livello di mRNA che di proteina, rispetto a SMM e MGUS. D'altra parte, non è stata trovata una differenza significativa nei livelli plasmatici midollari di IL-21 tra MM, SMM e MGUS. Il trattamento con IL-21 ricombinante umana, alla concentrazione osservata nei plasmi midollari, determina un aumento delle dimensioni degli osteoclasti in esperimenti di osteoclastogenesi *in vitro*, a partire da precursori ottenuti dal midollo di pazienti con MM ma non con SMM e MGUS. Questo dato suggerisce una maggiore sensibilità all'effetto pro-osteoclastogenico di IL-21 dei pazienti affetti da MM. Infine, abbiamo testato, nel nostro modello *in vitro*, l'over-espressione di IL-21R attraverso vettori lentivirali, nonché l'uso di un inibitore della via di segnalazione di IL-21, per delineare una nuova strategia anti-osteoclastogenica per il MM.

In conclusione i nostri risultati indicano che i monociti midollari dei pazienti con MM, rispetto a quelli con SMM e MGUS, sono caratterizzati da diversi profili sia trascrizionali che proteomici, tra cui l'over-espressione di IL-21R, putativamente coinvolta nella formazione degli osteoclasti MM-indotta.

Introduction

Multiple myeloma (MM) is a malignant disorder characterized by multifocal proliferation of clonal, long-lived plasma cells (PCs) within the bone marrow (BM).[1-3] MM presents clinically with a broad range of manifestations, including skeletal lytic lesions, anemia, immunodeficiency, renal failure and hypercalcemia.[1-3] MM is preceded by a premalignant condition termed monoclonal gammopathy of undetermined significance (MGUS), and in some patients, an intermediate but more advanced premalignant stage, termed smoldering myeloma (SMM), is clinically recognized.[4] Several studies evaluating the Gene Expression Profiles (GEPs) of $CD138^+$ PCs from the BM of healthy donors, MGUS, SMM, newly diagnosed and relapsed MM, had delineate a comprehensive picture of the gene expression changes associated with the neoplastic transformation of human PCs, although MGUS was indistinguishable from MM.[5-8]

As the close relationship between PCs and BM microenvironment plays a crucial role in MM pathogenesis, ongoing studies are focusing on the presence of potential molecular alterations in the microenvironment cells. Alterations of GEPs have been reported in mesenchymal stromal cells (MSCs) and osteoblasts of MM patients in comparison with healthy donors, discriminating MSCs in MM and healthy subjects.[9] Otherwise, MGUS MSC genes were equally distributed between healthy and MM groups,[9] failing to elucidate the biological alterations that lead from MGUS to symptomatic disease.

In MM BM microenvironment, monocytes have a pivotal role because of their primarily involvement in osteoclastogenesis[10-12], angiogenesis[13-15] and immune system dysfunction[16,17], that are the hallmarks of symptomatic MM compared to SMM and MGUS. It is well established that in MM patients in the BM microenvironment there is an increase of the osteoclastogenic process with an enhanced osteoclast formation from monocytes in close contact with MM cells.[11] Different factors produced by MM PCs can stimulate osteoclastogenesis including Interleukin (IL)-6, IL-3, and Macrophage inflammatory protein (MIP)-1α, moreover the contact between MM cells and BM MSCs stimulates the production by MSCs of Receptor activator of nuclear factor kappa-B ligand (RANKL), the main pro-osteoclastogenic cytokine.[10-12] It has been reported that MIP-1α, a chemokine secreted also by monocytes and BM MSCs, is a potent inducer of osteoclast formation.[18]

Recently, we found that IL-3 is increased in BM plasma from MM patients and that induces the production of Activin A by BM monocytes, which stimulates osteoclastogenesis in a RANKL-independent mechanism.[19]

Many studies suggest that monocytes and macrophages may give rise to vascular endothelial cells, accumulate around tumors, and stimulate blood vessel formation.[13-15] Monocytes induce vascular endothelial cell gene expression and develop tube-like structures when they are cultured with bone marrow from patients with MM.[20] Moreover, when co-injected with human MM cells into SCID mice, green fluorescent protein-marked human monocytes were found incorporated into tumor blood vessels and expressed human vascular endothelial cell protein markers.[21] A subset of monocytes that express the angiopoietin receptor Tie-2 were found to be inducers of angiogenesis in tumor models, thus suggesting a mechanism by which Ang-1-secreting MM cells may promote the recruitment of Tie2-expressing monocytes.[22] Interestingly, it has been reported that the contribution of monocytes to the increased vasculature that occurs in the BM of MM patients is higher in MM patients with active disease as compared to those with MGUS or healthy subjects.[23]

Actually the presence of potential alterations in the transcriptional and proteomic profiles of $CD14^+$ monocytes in MM patients as compared to MGUS and SMM are not known and were investigated in this study in order to identify either new genes and molecules potentially involved in the pathophysiology of MM-induced osteoclastogenesis, angiogenesis and immune dysfunction that characterized the progression from MGUS to MM, and druggable as therapeutic targets.

Aims

Monocytes are primarily involved in osteoclastogenesis, angiogenesis and immune function, processes that resulted altered in MM patients as compared with MGUS and SMM ones. On the basis of these evidences the main goals of this project were to isolate BM $CD14^+$ monocytes from a cohort of MM, SMM and MGUS patients and to characterize their transcriptional and proteomic profiles in order to identify alterations potentially involved in the pathogenesis of the MM-induced increased osteoclastogenesis.

The specific aims of this study were:

- To purify $CD138^+$ and $CD14^+$ cells from BM aspirates by an immunomagnetic method and identify the presence of the main genetic abnormalities by FISH analysis.
- To analyze the GEPs of BM monocytes among the different types of PC dyscrasias.
- To analyze the proteomic profiles of BM monocytes among the different types of PC dyscrasias.
- To correlate different expression fingerprints with patient clinical characteristics.
- To validate GEP and proteomics data by Real Time PCR and by western blot on a larger cohort of patients.
- To perform functional studies focusing on the main over- and down-regulated genes, and correlated proteins, arisen from GEP and proteomics data, by lentiviral vectors.

Material and Methods

$CD14^+$, $CD138^+$ and $CD3^+$ cell purification: $CD14^+$ cells were purified from a total cohort of 59 patients with plasma cell disorders including 30 patients with symptomatic MM, 16 patients with SMM and 13 patients with MGUS, sex and age-matched. All patients gave informed consent according to the Declaration of Helsinki. Monocytes were isolated from patient BM samples by an immunomagnetic method with anti-CD14 mAb conjugated with microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). With the same protocol, $CD138^+$, $CD3^+$, $CD4^+$, $CD8^+$ cells were isolated from BM samples. The presence of potential contaminating cells in each fraction were excluded by FACS analysis. Cell pellets were re-suspended in RLT with β-mercaptoethanol and stored at -20°C for mRNA analysis or collected and stored dry at -80°C for proteomic analysis.

Cells culture: Primary BM MSCs were obtained from the $CD14^-CD138^-$ fraction of BM mononuclear cells from MM patients. Cells were incubated in a-minimum essential medium (MEM) supplemented with 15% Fetal Bovine Serum (FBS) until confluence for 2 weeks. $CD3^+$ cells were activated by ionomycin (1uM) and phorbol myristic acetate (5ng/mL), (both from Sigma Aldrich, Milan, Italy) for 72 hrs and then cell pellets were re-suspended in RLT with β-mercaptoethanol and stored at -20°C for mRNA analysis.

Flow cytometry: $CD14^+$, $CD138^+$ or $CD3^+$ cells were resuspended in phosphate-buffered saline (PBS) and stained for 20 minutes at room temperature in the dark, with fluorochrome-conjugated anti-human CD14, anti-human CD138 or anti-human CD3, respectively. After staining, 10.000 gated vital cell events were recorded. Flow cytometry analysis were performed using a fluorescence-activated flow cytometer (BD FACSCanto II, BD Biosciences, San Jose, CA). An example of monocytes purity analysis is shown in Fig.1. Same routinely protocol was used to check CD16 expression by BM monocytes.

Microarrays analysis: Total RNA was extracted from BM $CD14^+$ cells using the RNeasy total RNA isolation kit (Qiagen). Gene expression profiling of BM monocytes was performed with the Affymetrix microarray platform by the GeneChip® HG-U133 Plus 2.0 arrays. Biotin-labeled cRNA was prepared according to the Affymetrix GeneChip Expression Analysis Technical Manual protocol. Unsupervised and supervised analyses of GEPs were carried out using the Significant Analysis of Microarrays (SAM) software version 4.00. Data were analyzed as previously reported.[24]

Proteomic profiling: Total cell lysates were obtained from dry-pellets of $CD14^+$ cells by UREA-based lysis buffer. Then reduction and alkylation were done, followed by protein digestion into peptide fragments. Then peptides were labeled with TMT sixplex Isobaric Mass Tagging kit (Thermo Scientific) according to manufacturer protocol. Labeled peptides were then run on Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific®) and data analyzed with Proteome Discoverer 1.4 Software. Identification of modulate protein was also supported by the analysis platform ProteinQuest (BioDigitalValley), Cytoscape, KEGG and DAVID.

Real Time PCR: 0,5 µg of RNA was reverse-transcribed with 400 U Moloney murine leukemia reverse transcriptase (Applied) in accordance with the manufacturer's protocol. Real Time PCR was performed by adding complementary DNA to an universal master Mix primers and TaqMan probes (Applied Biosystem) for the following genes: *IL21R*: Hs00222310_m1, *CXCL10*: Hs01124251_g1, *IFI27*: Hs01086373_g1, *IFI44*: Hs00951349_m1, *SLAMF7*: Hs00221793_m1 and *ABL*: Hs01104728_m1. The expression of selected genes was check by Real Time PCR by ICycler (BioRad). To normalize the differences in RNA quality and reverse transcription efficiency, we applied the comparative Ct method using the endogenous reference gene ABL. The relative mRNA quantification of each gene was performed by the ΔCt method (Δ=mean Ct–mean Ct ABL). ΔCt was evaluated as the difference between the ΔCt of a sample and the ΔCt of the control. The fold change in mRNA expression (n-fold) was calculated as $2^{-\Delta\Delta Ct}$.

Western blot: Cytosolic extracts were obtained from BM $CD14^+$ cells using a commercial kit (Nuclear Extraction Kit, Active Motif, Carlsbad, CA) and protein levels determined using a standard procedure (Bio-Rad protein assay). We used a specific monoclonal antibody directed to IL-21R (R&D system, Minneapolis, MN) and anti-β-actin monoclonal antibody (Merck Millipore, Darmstadt, Germania) was used as internal control.

ELISA assay: BM IL-21 levels were detected by Human Interleukin-21 ELISA kit (BioVendor, Czech Republic), following the manufacturer's protocol, in a large cohort of 76 newly diagnosed (ND) MM, 42 SMM and 41 MGUS patients.

Lentiviral infections: Lentiviral vectors were prepared following standard procedures as previously reported.[25] Primary $CD14^+$ cells were transduced following published protocols.[26] Briefly, $8x10^6$ $CD14^+$ cells purified from peripheral blood buffy coats of healthy donors were placed in well plate in 2 ml of aMEM 10% FBS and 25ng/ml recombinant human (rh) M-CSF (Peprotech, London, UK) in the presence of empty or IL21R vectors. As control, $CD14^+$ cells were also seeded in the same conditions without adding the lentiviral vector. After 18hrs, 2 ml of new aMEM 10% FBS and 25ng/ml rhM-CSF were added. After 3 days, cells were collected and seeded for osteoclastogenesis assays. Cell pellets for Real Time PCR assays were also made to check *IL21R* expression.

In vitro osteoclastogenesis assay: For the in *vitro* osteoclastogenesis was were used total BM mononuclear cells (MNCs) or $CD14^+$ cells purified from BM aspirates of 3 MM, 3 SMM and 1 MGUS patients. 400.000 MNCs or 200.000 $CD14^+$ cells/well were seeded in 96well plates in αMEM medium with 10% FBS, rhM-CSF 25ng/ml and rhRANKL 60 or 20ng/ml in presence or absence of rhIL-21 (30pg/ml) and then cultured for 28 days, replacing half medium each 2-3 days. Osteoclasts were identified and counted by light microscopy at the end of the culture period as multinucleated (>3) cells positive for tartrate resistant acid phosphatase (TRAP) assay (Sigma Aldrich, Milan, Italy).

200.000 lentiviral transduced $CD14^+$ cells/well were seeded in 96well plates in αMEM medium with 10% FBS, rhM-CSF 10ng/ml and rhRANKL 50ng/ml and then cultured for 28 days, replacing half medium each 2-3 days. Osteoclasts were identified and counted

by light microscopy at the end of the culture period as multinucleated (>3) cells positive for TRAP assay.

Results

GEP analysis.

The BM monocyte samples included in the GEP analysis were 16 MM, 11 SMM and 8 MGUS, thus excluding samples contaminated by the presence of plasma cells.

Different types of GEP analysis were conducted. Firstly we failed to find any GEP related to the presence of the main cytogenetic abnormalities in $CD14^+$ from MM patients (data non shown).

An unsupervised analysis between all the MM, SMM and MGUS monocyte samples was not able to cluster patients according to their diagnosis (data not shown). A multiclass analysis identified 18 differentially expressed genes in monocytes from MGUS *vs* SMM *vs* MM, as shown in Fig.2a. As shown in Fig.2b, the comparison of MM with both SMM and MGUS samples identified 61 genes differentially expressed in $CD14^+$ cells. 37 were genes up-regulated in MM *vs* (MGUS+SMM) and 24 ones were down-regulated (Fig.2b). Interestingly, among the differentially expressed genes we found cytokine receptors (*IL21R* and *IL-15R*), chemokines with pro-osteoclastogenic properties (*CXCL10*), interferon-inducible proteins (*IFI27* and *IFI44*) and *SLAM7* that were up-regulated in $CD14^+$ of MM patients as compared to SMM and MGUS. By means of Real Time quantitative PCR, we confirmed a significant up-regulation of *IL21R*, *SLAMF7*, *CXCL10*, *IFI27* and *IFI44* genes in $CD14^+$ cells from MM patients in comparison to SMM and/or MGUS in a cohort of 10 MM, 7 SMM and 5 MGUS patients, including primary monocytes used for GEP analysis and ones purified from additional patients (Fig.3).

Proteomic analysis.

For the final proteomic analysis, $CD14^+$ cells of 5 MM, 3 SMM and 3 MGUS were used. Our proteomic analysis results showed that 71 proteins were modulated in MM monocytes as compared to SMM and MGUS ones: 47 proteins were up-regulated, among these molecules involved in cell adhesion and inflammation, and 24 were down-

regulated, including proteins involved in antimicrobial functions. The main proteins modulated are listened in Table 1.

Monocyte immunophenotype.

Our GEP and proteomic data highlighted an activated profile of MM monocytes, as compared to those of asymptomatic patients. Thus, we evaluated the immunophenotype of BM monocytes in a cohort of patients comprising 3 MGUS, 8 SMM and 9 MM. As shown in Fig.4, the median percentage of $CD14^+CD16^+$ cells in BM samples increased among the different types of monoclonal gammopathies, resulting significantly higher in MM *vs* MGUS (p:0.0040; Mann-Withney test) and in SMM *vs* MGUS (p:0.0242; Mann-Withney test).

IL-21/IL-21R axis in MM BM microenvironment.

Because literature data indicate that IL-21 is growth factor for MM cells[27,28] and may promote osteoclastogenesis in pathophysiological conditions such as rheumatoid arthritis[29], we further investigate the potential role of IL-21R over-expression in MM $CD14^+$ cells.

IL21R up-regulation in MM monocytes was validated by Real Time PCR, as shown in Fig.5a. MM $CD14^+$ cells expressed significantly higher levels of *IL21R* compared to MGUS, such as symptomatic myeloma as compared to asymptomatic ones. IL-21R over-expression in MM monocytes was demonstrated also at protein level by western blot in a large MM patients cohort, as shown in Fig.5b for two example patients.

Then, we have evaluated *IL21* expression in BM microenvironment. As reported in Table 2, BM MSCs, monocytes and plasma cells did not express *IL21* gene, that is otherwise expressed by T lymphocytes, checked by Real Time PCR. *IL21* was over-expressed after T cell *in vitro* activation, with ionomycin and phorbol myristic acetate. We lack to find a significant difference between MGUS, SMM and MM $CD3^+$ cells, as reported in Fig.6. Between the T lymphocytes, *IL21* is expressed from the $CD4^+$ ones (Table 2).

Consistently with these data, we lacked to find a significant difference in the BM levels of IL-21 across the different monoclonal gammopathies. As reported in Table 3, the median

BM IL-21 level was of 34 pg/ml for MGUS samples, 31 pg/ml for SMM and 34 pg/ml for ND MM.

Subsequently, we performed *in vitro* osteoclastogenesis assays from BM mononuclear cells or from purified monocytes in presence of rhRANKL (20 and 60 ng/ml) and rhM-CSF (25ng/ml) and in the presence or the absence of the rhIL-21 at concentration found in patient BM plasma (30pg/ml). The presence of rhIL-21 did not affect the number of TRAP positive osteoclasts either at the lower and higher concentration of rhRANKL (Fig.7a). Otherwise the presence of rhIL-21 leaded to the differentiation of MM BM precursors in larger osteoclasts, as shown in the Fig.7b. This effect was not seen for SMM and MGUS precursors.

Modulation of IL-21/IL-21R axis.

To further validate IL-21 involvement in MM-induced osteoclast activation, we induced IL-21R over-expression in $CD14^+$ cells purified from peripheral blood buffy coats of healthy donors.

$CD14^+$ cell infected with IL-21R vector, as compared to those infected with the empty vector, over-expressed IL-21R as evaluated by Real Time PCR and reported in Fig.8a. Then *in vitro* osteoclastogenesis assays were performed and the over-expression of IL21R significantly increased the number of TRAP positive osteoclasts (p=0.0093, t-test) (Fig.8b and 8c).

Finally, we have tested Janex1, an inhibitor of IL21 signaling through JAK3/STAT.[30] As shown in Fig.9, Janex1 suppress IL-21 pro-osteoclastogenic effect in the *in vitro* osteoclastogenesis assays.

Discussion

MM is an incurable hematologic malignancy of PCs that accumulate and proliferate in the BM.[1-3] Clinical MM manifestations include skeletal lytic lesions, anemia, immunodeficiency, renal failure and hypercalcemia.[1-3] MM is preceded by two premalignant condition defined MGUS and the advanced stage SMM.[1-4] Several studies evaluated the GEPs of $CD138^+$ PCs obtained from patients with newly diagnosed MM and with MGUS, and from normal healthy subjects, to identify genes that might play a role in the initiation and progression of MM.[5-6] However, whereas MGUS and MM could be distinguished from normal PC, these two conditions were difficult, if not impossible, to differentiate from each other. These data suggest that changes in the BM microenvironment or the failure of immune surveillance, rather than a genetic change in the tumor cell, may account for the malignant conversion of this benign PC dyscrasia. Otherwise, GEPs were used to segregate MM patients into prognostic categories and identify new therapeutic targets, especially, in the pathogenesis of the osteolysis.[7] For example, a study revealed that 57 genes, including *DKK-1*, were differently expressed by PC of patients with or without bone lesions.[8]

MM pathophysiology and progression is strictly dependent on the interactions between MM cells and BM microenvironment. Actually, different studies are focusing on the presence of potential molecular alterations in the BM microenvironment cells and the mechanisms by which MM cells modified their microenvironment. It has been reported that BM MSCs and pre-osteoblasts of MM patients have typical GEPs in comparison with healthy donors. GEP independently classified the BM MSCs analyzed in a normal and in an MM group, with MGUS MSC genes equally distributed between those 2 groups.[9] However, are not yet clear all the BM biological alterations that lead from MGUS to SMM and, finally, to symptomatic MM.

In this study the attention was focused on BM monocytes because their primarily involvement in osteoclastogenesis[10-12], angiogenesis[13-15] and immune system dysfunction[16,17], hallmarks that distinguish symptomatic MM from SMM and MGUS. It is well established that in the MM BM microenvironment there is an increase of the

osteoclastogenic process with an enhanced osteoclast formation from monocytes in close contact with MM cells. Different factors produced by MM PCs and, after the interaction with PCs, BM MSCs and monocytes, can stimulate osteoclastogenesis including IL-6, IL-3, MIP-1α and Activin A.[10-19]

In this study we have investigated the presence of potential alterations in the transcriptional and proteomic profiles of BM $CD14^+$ monocytes in MM patients as compared to MGUS and SMM. Our study revealed that transcriptome and proteome of $CD14^+$ in MM patients, as compared to SMM and MGUS, showed an up-regulation of genes involved in immune response, chemotaxis and osteoclastogenesis and proteins belonging to cell adhesion and inflammation families. These are new genes and molecules potentially involved in the pathophysiology of MM-induced osteoclastogenesis, angiogenesis and immune dysfunction that characterized the progression from MGUS to MM, and potentially druggable as therapeutic targets.

In line with these results, we found that the median percentage of $CD14^+CD16^+$ cells in BM samples increased among the different types of monoclonal gammopathies, resulting significantly higher in MM *vs* MGUS. It has been reported that the $CD16^+$ monocytes represent 5–10% of peripheral blood monocytes in normal individuals and are dramatically expanded in several pathological conditions including sepsis, human immunodeficiency virus 1 infection, and cancer.[31-33] In addition, $CD14^+CD16^+$ cells were found to be increased in Psoriatic arthritis, compared to controls, and they are the main source of OCs.[34]

Between the genes arisen from GEP analysis, we found that *IL21R* is over-expressed by monocytes in MM patients, as compared to SMM and MGUS ones, suggesting a possible role of IL-21 in pathogenesis and progression of MM. Literature data have shown that IL-21 is a growth factor for MM cells[27,28] IL-21 is a cytokine with pleiotropic effects on the immune system, including promote proliferation, differentiation, and effector functions of B, T, natural killer, and dendritic cells.[35-37] IL-21 was identified as a four-helix-bundle cytokine that is most homologous to IL-15 and also has significant homology to IL-2 and IL-4. The human IL-21 gene was mapped on 4q26-q27.[35] The binding of IL-21 to its receptor, IL-21R, leads to the activation of the Jak–STAT pathway, in particular Jak1, Jak3, STAT1, and STAT3.[30,36-39] In addition to the Jak–STAT pathway, IL-21 also activates MAPKs and PI3K pathways.[40,41]

Furthermore, the potential therapeutic capacity of IL-21 in the treatment of cancers has been widely investigated. Conducted phase I trials in metastatic malignant melanoma and renal cell carcinoma have shown that rhIL-21 has a favorable antitumor activity.[42-43] IL-21, which is considered as an immunoregulatory cytokine, plays diverse effects in hematological malignancies, mainly depending on the neoplastic cell type, ad it has been suggested for a potential immunotherapeutic approach to cancer.[41] IL-21 induced apoptosis of Chronic Lymphocytic Leukemia,[44-47] Follicular Lymphoma,[48] Diffuse Large B Cell Lymphoma[50,51] and Mantle Cell Lymphoma,[51] indicating that it could be used to treat these tumors, especially in combination with molecules which upregulate its activity. Combination of IL-21 with Rituximab or other tumor-targeted antibodies that can mediate antibody-dependent cell-mediated cytotoxicity (ADCC), and in combination with agents that debulk tumors and aid the generation of tumor-specific T cells may be useful.[41] The potential role of IL-21 as a growth factor for certain hematological tumors, such as MM[27,28] and adult T-cell Leukemia/Lymphoma,[52,53] may suggest that targeting the IL-21/IL-21R system or blocking the downstream JAK/STAT pathways could be of benefit in these conditions. Since systemic IL-2 administration can mediate long-term complete responses in some patients with widespread metastatic melanoma and renal cell cancer, in the future, using or blocking IL-21/IL-21R system could provide a new strategy to treat hematological malignancies.

A recent study have also shown that IL-21 is a pro-osteoclastogenic cytokine in rheumatoid arthritis (RA), and that this cytokine was up-regulated in the synovium, synovial fluid, and serum of patients with RA.[29] On the contrary, in our study, we lack to find a significantly difference in BM IL-21 levels across the different monoclonal gammopathies. Otherwise, the treatment with rhIL-21, at the concentration found in BM patient plasma (30pg/ml), increased the size of BM $CD14^+$-derived osteoclasts from MM patients. These positive effect on *in vitro* osteoclastogenesis was not seen in assays performed from SMM and MGUS BM precursors. These data suggest that MM monocytes showed an higher sensitivity to IL-21, as compared to MGUS and SMM.

Our data showed a possible involvement of IL-21/IL-21R axis in MM-induced osteoclastogenesis, further supported by the IL-21R over-expression by a lentiviral vector in $CD14^+$ monocytes obtained from healthy donors. IL-21R over-expression was

able to increase the number, as well the size, of $TRAP^+$ cells in *in vitro* osteoclastogenesis assays.

Finally, we modulated in our *in vitro* model IL-21/IL-21R axis, blocking its signaling by Janex-1.[30] The treatment of MM OC-precursors with Janex-1 blunted the pro-osteoclastogenic effect of rhIL-21, confirming a role of IL-21/IL-21R axis in MM-induced osteoclastogenesis.

In conclusion, our results revealed that IL-21/IL-21R axis is involved in MM-induced osteoclast formation from $CD14^+$monocytes and could be a target in MM bone disease.

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Tables

TABLE 1: Main proteins up- or down-regulated in MM monocytes as compared to MGUS and SMM ones.

UP-REGULATED	
Accession	**Description**
K7EP23	Splicing factor 3A subunit 2 (Fragment)
O43242	26S proteasome non-ATPase regulatory subunit 34
P01834	Ig kappa chain C region
P01857	Ig gamma-1 chain C region
P05109	Protein S100-A8
Q00839-2	Isoform Short of Heterogeneous nuclear ribonucleoprotein U
Q08170	Serine/arginine-rich splicing factor 4
Q9H1E3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1
DOWN-REGULATED	
Accession	**Description**
B4DTY9	Integrin beta
P02768	Serum albumin
P02792	Ferritin light chain
P49913	Cathelicidin antimicrobial peptide
P59665	Neutrophil defensin 1

TABLE 2: IL-21 expression by BM microenvironment cells.

	-ΔCt
BM MSCs	NA
$CD14^+$ cells	NA
$CD138^+$ cells	NA
$CD3^+$ cells	-8,3
Activated $CD3^+$ cells	-1
$CD8^+$ cells	NA
$CD4^+$ cells	-8,6

TABLE 3: IL-21 BM plasma levels of patients with monoclonal gammopathies. All values were reported as pg/ml.

	median	average	standard deviation	range
MGUS	34	345	970	0-5360
SMM	31	857	2142	0-8496
ND MM	34	789	3061	0-19966

Legend of Figures

Figure 1: Monocytes purity. Representative flow cytometry plots to assess the purity of BM monocytes after purification with anti-CD14 Ab conjugated microbeads. Monocytes were identified as $CD14^{+}CD45^{+}CD138^{-}$ cells.

Figure 2: GEP analysis. Heatmaps of the differentially expressed genes resulted **(a)** from three classes SAM supervised analysis of the monocytes samples clustered according to the diagnosis of the patients and **(b)** from the supervised analysis MM *vs* (MGUS+SMM) of $CD14^{+}$.

Figure 3: Real Time PCR validation. Quantitative Real Time PCR of *IL21R*, *SLAMF7*, *CXCL10*, *IFI27* and *IFI44* genes performed on BM monocytes samples. Values represent the median of the -ΔCt of the reactions. (*=Fold change ≥2)

Figure 4: Monocyte immunophenotype. (a) Example plot of flow cytometry data on BM monocytes expression of CD14 and CD16. **(b)** Box plot of percentage values of $CD14^{+}CD16^{+}$ cells evaluated in BM samples obtained from patients with MGUS, SMM and MM.

Figure 5: IL-21R expression by BM monocytes. (a) Box plot of *IL21R* expression reported as –ΔCt, evaluated by Real-time PCR in monocyte purified from MGUS, SMM and MM patients. **(b)** IL-21R expression, evaluated by western blot, on BM monocytes of two representative patients, one SMM and one MM. DOHH2 cell line was use as positive control and $CD14^{+}$ purified from healthy donors buffy coats (Buffy coat CD14+) as negative control.

Figure 6: *IL21* expression by T cells. Bar chart of *IL21* expression reported as average –ΔCt, evaluated by Real Time PCR in $CD3^+$ cells purified from MGUS, SMM and MM patients.

Figure 7: IL-21 effect on *in vitro* osteoclastogenesis. (a) Total MNCs or $CD14^+$ monocyte, obtained from BM patient aspirates, were seeded 400.000 or 200.000 cells/well, respectively, in 96well plates in αMEM medium with 10% FBS, rhM-CSF 25ng/ml and rhRANKL 60 or 20ng/ml in presence or absence of rIL21 (30pg/ml). After 28 days of culture, osteoclasts were identified as multinucleated (>3) TRAP positive cells and counted by light microscopy. Bar graph showed the median variation of the osteoclast number in the presence of rhIL-21 as compared to control (vehicle) either in the presence of rhRANKL at lower (20ng/ml) and at higher concentration (60ng/ml). SMM: osteoclast precursors obtained from a SMM patient; MM: osteoclast precursors obtained from a MM patient. **(b)** Representative images of osteoclastogenesis assays with or without (control) rhIL-21 of osteoclast precursors obtained from a SMM and a MM patient (Original magnification 4x).

Figure 8: Effect of IL-21R over-expression on *in vitro* osteoclastogenesis. (a) IL21R over-expression by $CD14^+$ cell transduced with lentiviral vector for the over-expression of IL-21R (CD14+ cells IL21R) as compared to those infected with the empty control vector (CD14+ cells Empty) or not infected (CD14+ cell w/o vector), checked by Real Time PCR. Bar graph represented mean –ΔCt. **(b)** 200.000 $CD14^+$ cells/well were seeded in 96well plates in αMEM medium with 10% FBS, rhM-CSF 10ng/ml and rhRANKL 50ng/ml. After 28 days of culture, osteoclasts were identified as multinucleated (>3) TRAP positive cells and counted by light microscopy. Bar graph showed the mean number of osteoclasts of each condition, reported with standard deviation. **(c)** Representative images of osteoclastogenesis assays of $CD14^+$ cell transduced with lentiviral vector for the over-expression of IL-21R (CD14+ cells IL21R) or the empty control vector (CD14+ cells Empty) (Original magnification 4x).

Figure 9: IL-21/IL-21R signaling inhibition effect on *in vitro* osteoclastogenesis: **(a)** MM BM $CD14^{+}$ monocyte were seeded 200.000 cells/well in 96well plates in αMEM medium with 10% FBS, rhM-CSF 25ng/ml, rhRANKL 20ng/ml and rhIL-21(30pg/ml) in presence or absence of Janex 1 (10uM). After 28 days of culture, osteoclasts were identified as multinucleated (>3) TRAP positive cells and counted by light microscopy. Bar graph showed the mean number of osteoclasts of each condition, reported with standard deviation. **(b)** Representative images of osteoclastogenesis assays with or without (control) rhIL-21 in the presence or absence of Janex 1 (Original magnification 4x).

Figures

FIGURE 1

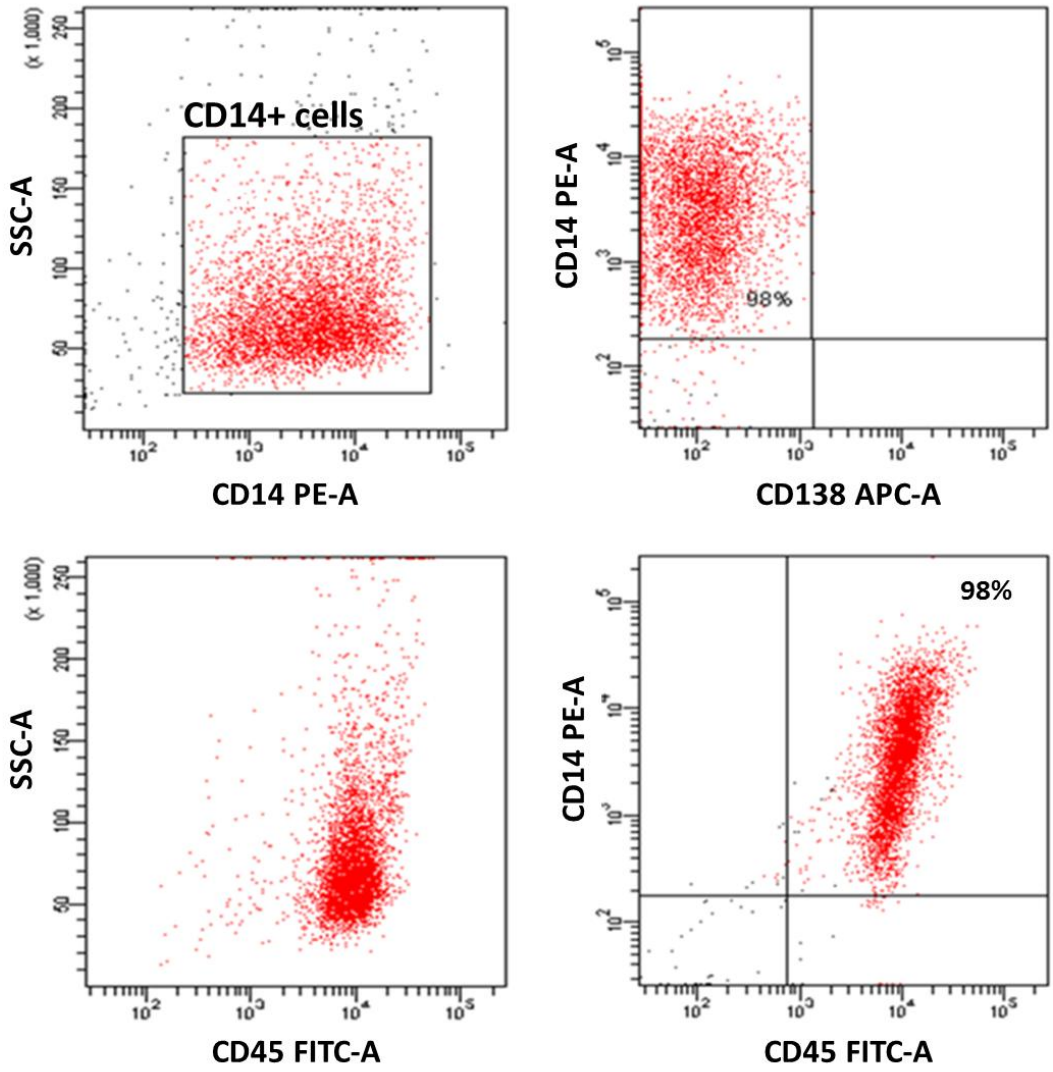


FIGURE 2

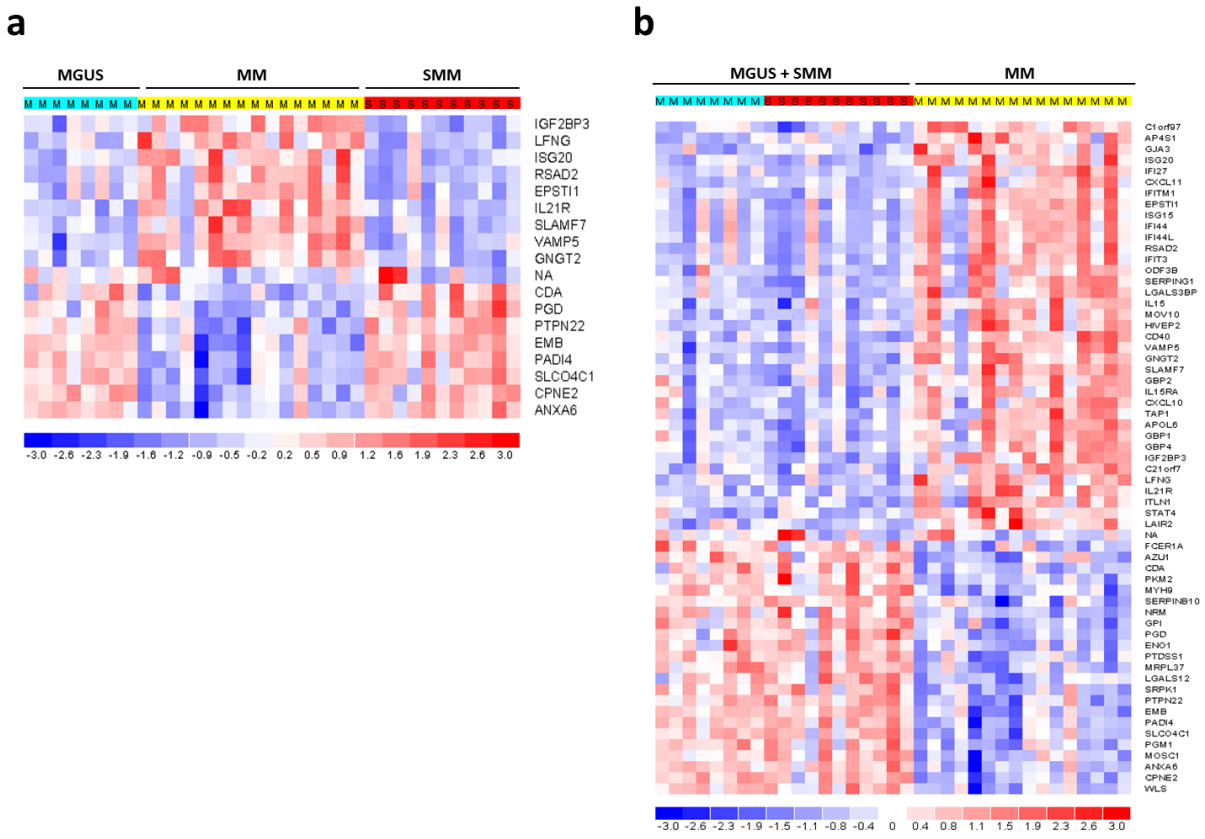

a
MGUS
MM
SMM
IGF2BP3
LFNG
ISG20
RSAD2
EPSTI1
IL21R
SLAMF7
VAMP5
GNGT2
NA
CDA
PGD
PTPN22
EMB
PADI4
SLCO4C1
CPNE2
ANXA6
-3.0 -2.6 -2.3 -1.9 -1.6 -1.2 -0.9 -0.5 -0.2 0.2 0.5 0.9 1.2 1.6 1.9 2.3 2.6 3.0
b
MGUS + SMM
MM
C1orf97
AP4S1
GJA3
ISG20
IFI27
CXCL11
IFITM1
EPSTI1
ISG15
IFI44
IFI44L
RSAD2
IFIT3
ODF3B
SERPING1
LGALS3BP
IL15
MOV10
HIVEP2
CD40
VAMP5
GNGT2
SLAMF7
GBP2
IL15RA
CXCL10
TAP1
APOL6
GBP1
GBP4
IGF2BP3
C21orf7
LFNG
IL21R
ITLN1
STAT4
LAIR2
NA
FCER1A
AZU1
CDA
PKM2
MYH9
SERPINB10
NRM
GPI
PGD
ENO1
PTDSS1
MRPL37
LGALS12
SRPK1
PTPN22
EMB
PADI4
SLCO4C1
PGM1
MOSC1
ANXA6
CPNE2
WLS
-3.0 -2.6 -2.3 -1.9 -1.5 -1.1 -0.8 -0.4 0 0.4 0.8 1.1 1.5 1.9 2.3 2.6 3.0

FIGURE 3

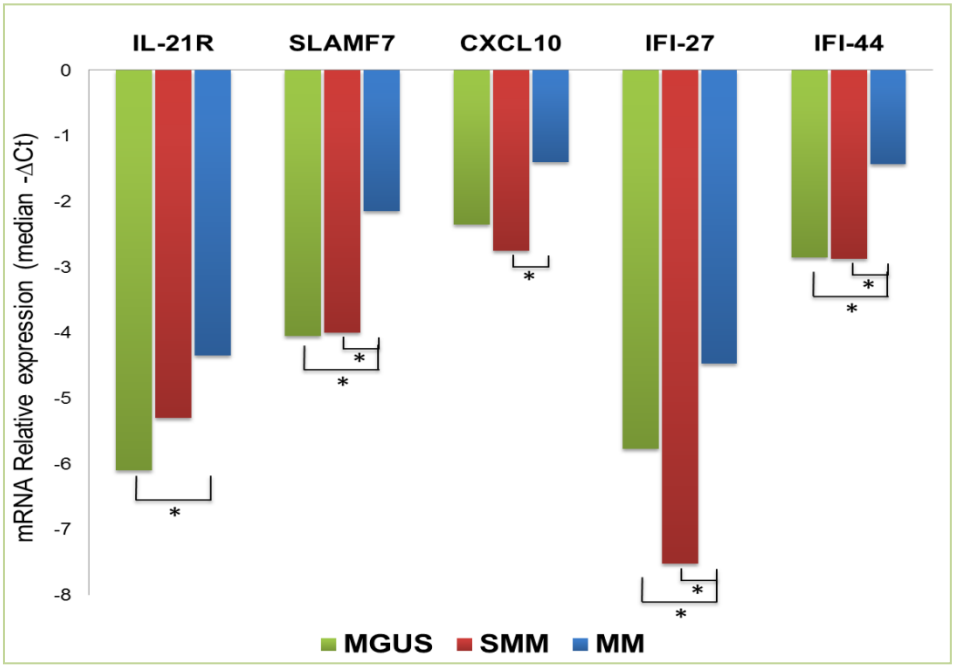

IL-21R
SLAMF7
CXCL10
IFI-27
IFI-44
mRNA Relative expression (median -ΔCt)
0
-1
-2
-3
-4
-5
-6
-7
-8
*
MGUS
SMM
MM

FIGURE 4

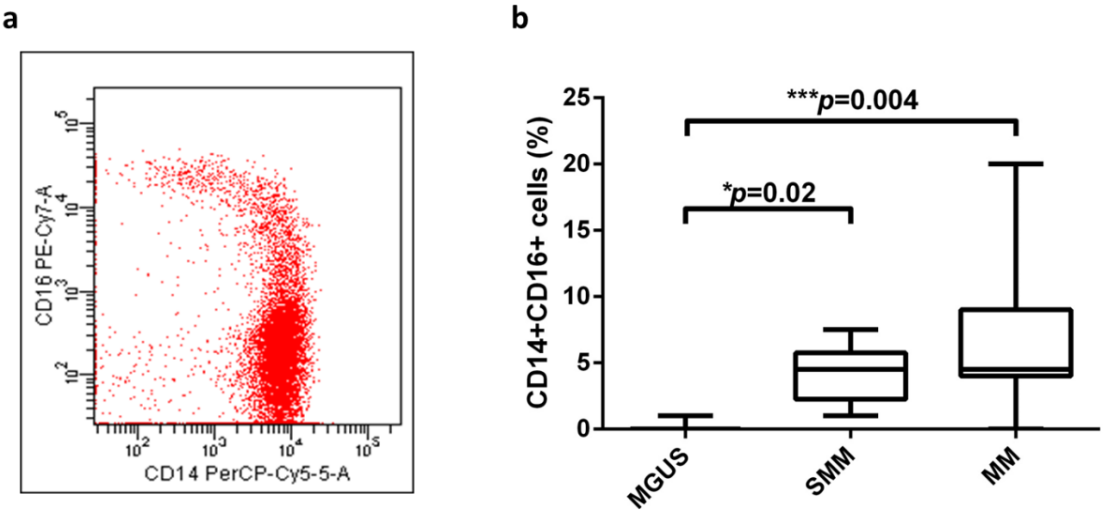

a
CD16 PE-Cy7-A
CD14 PerCP-Cy5-5-A
b
CD14+CD16+ cells (%)
***p=0.004
*p=0.02
MGUS
SMM
MM

FIGURE 5

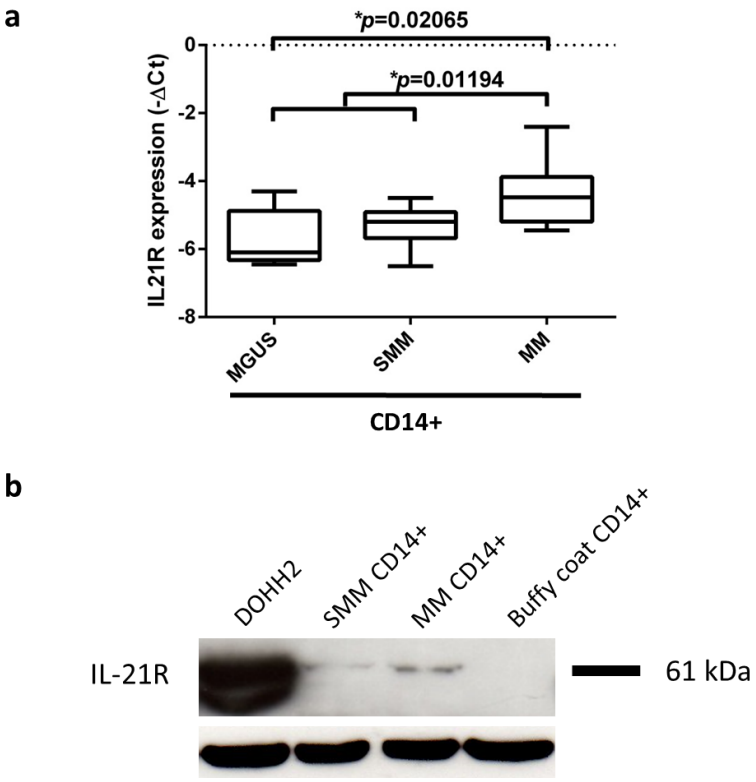

a
*p=0.02065
*p=0.01194
IL21R expression (-ΔCt)
0
-2
-4
-6
-8
MGUS
SMM
MM
CD14+
b
DOHH2
SMM CD14+
MM CD14+
Buffy coat CD14+
IL-21R
61 kDa

FIGURE 6

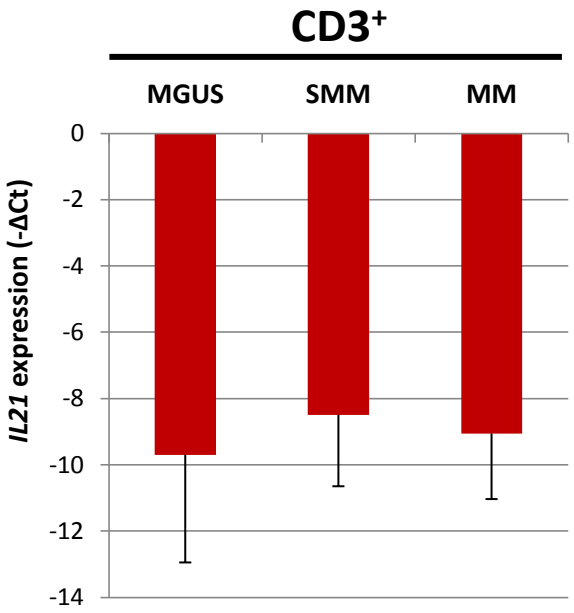


FIGURE 7

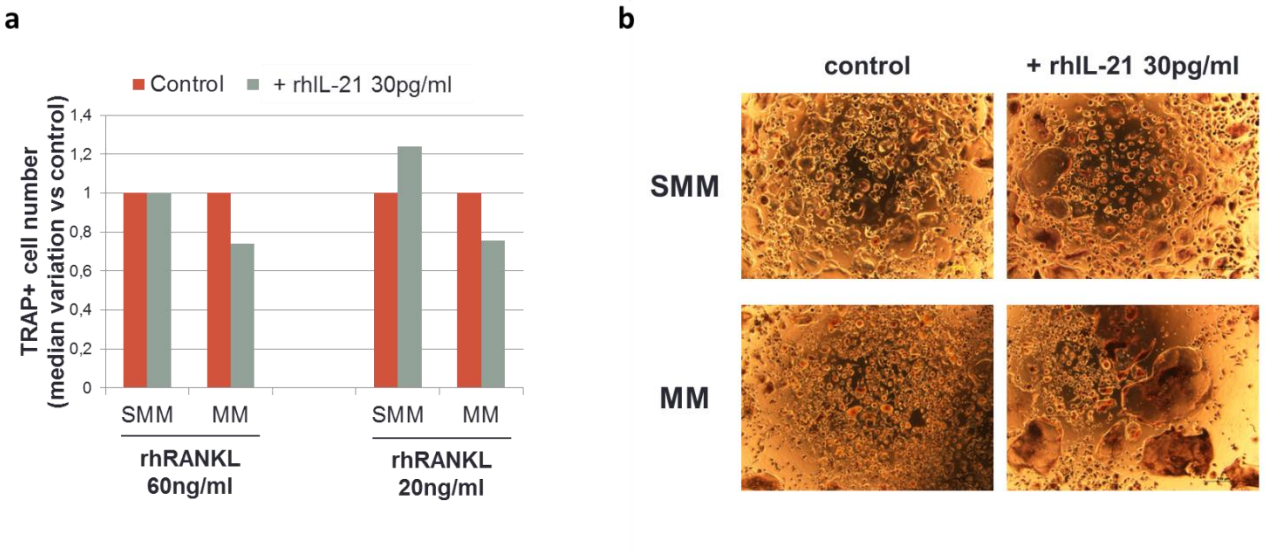

a
Control
+ rhIL-21 30pg/ml
TRAP+ cell number
(median variation vs control)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
SMM
MM
SMM
MM
rhRANKL
60ng/ml
rhRANKL
20ng/ml
b
control
+ rhIL-21 30pg/ml
SMM
MM

FIGURE 8

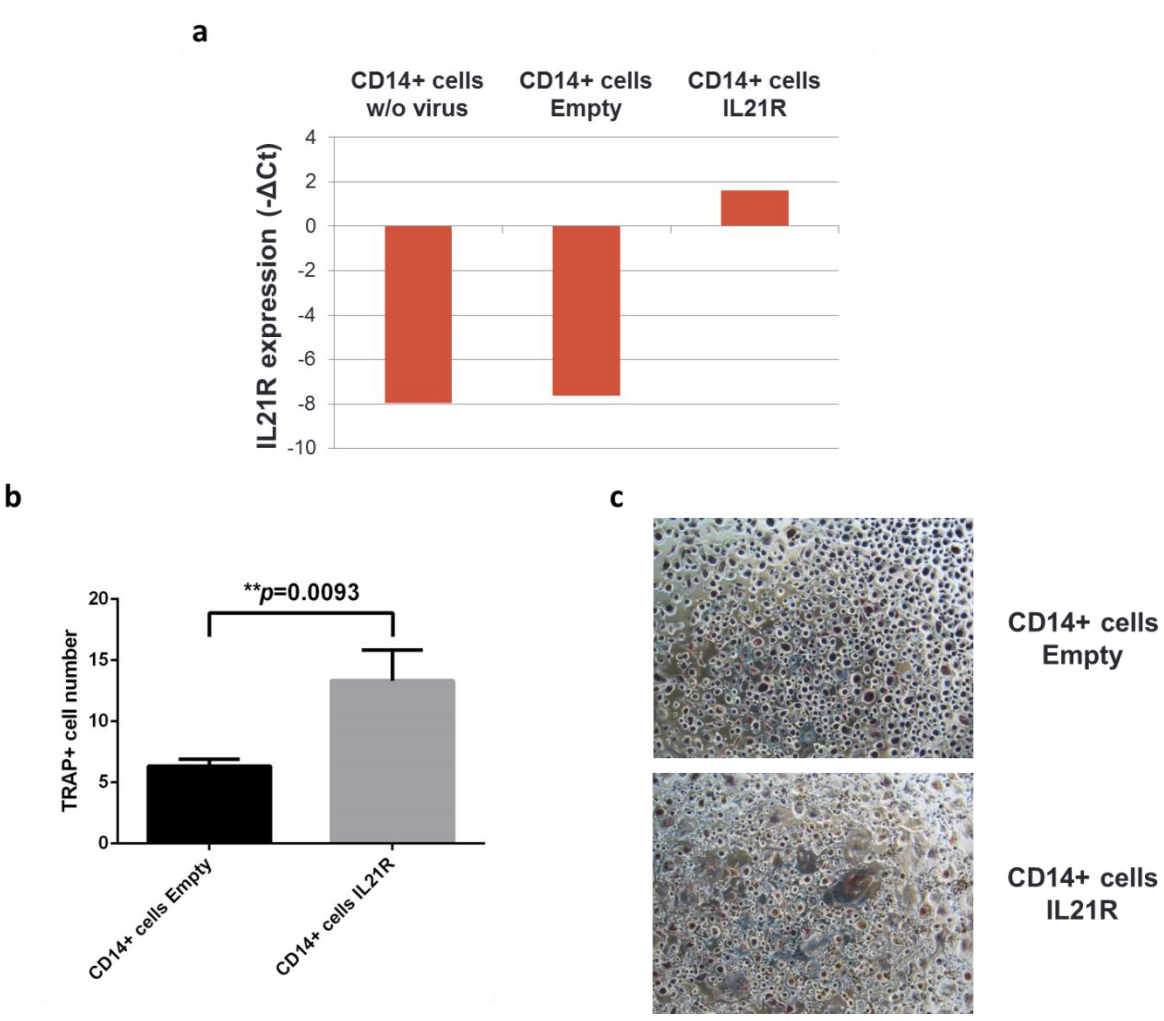
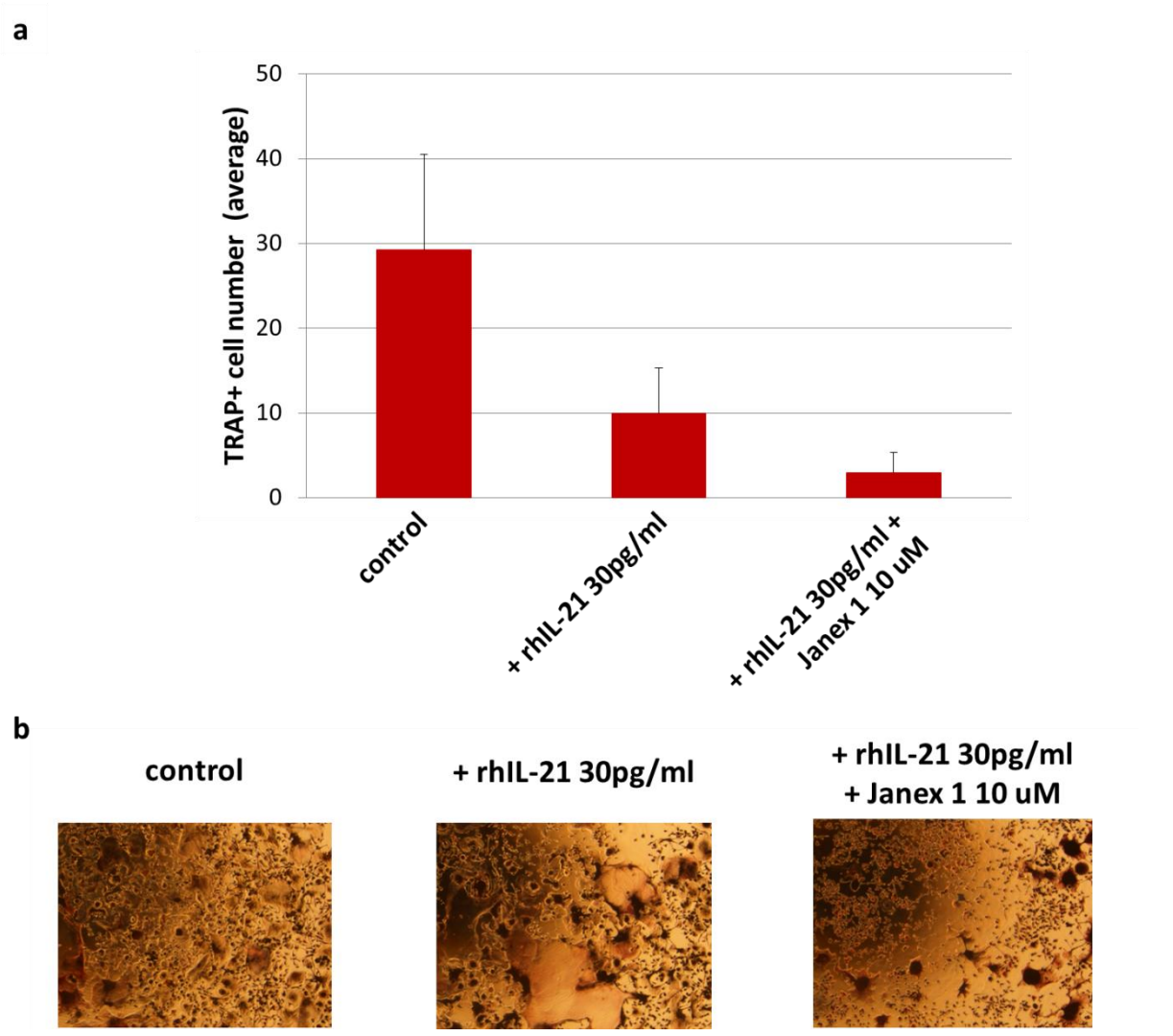

a
CD14+ cells w/o virus
CD14+ cells Empty
CD14+ cells IL21R
IL21R expression (-ΔCt)
4
2
0
-2
-4
-6
-8
-10
b
**p=0.0093
TRAP+ cell number
20
15
10
5
0
CD14+ cells Empty
CD14+ cells IL21R
c
CD14+ cells Empty
CD14+ cells IL21R

FIGURE 9


a
TRAP+ cell number (average)
50
40
30
20
10
0
control
+ rhIL-21 30pg/ml
+ rhIL-21 30pg/ml + Janex 1 10 uM
b
control
+ rhIL-21 30pg/ml
+ rhIL-21 30pg/ml
+ Janex 1 10 uM

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