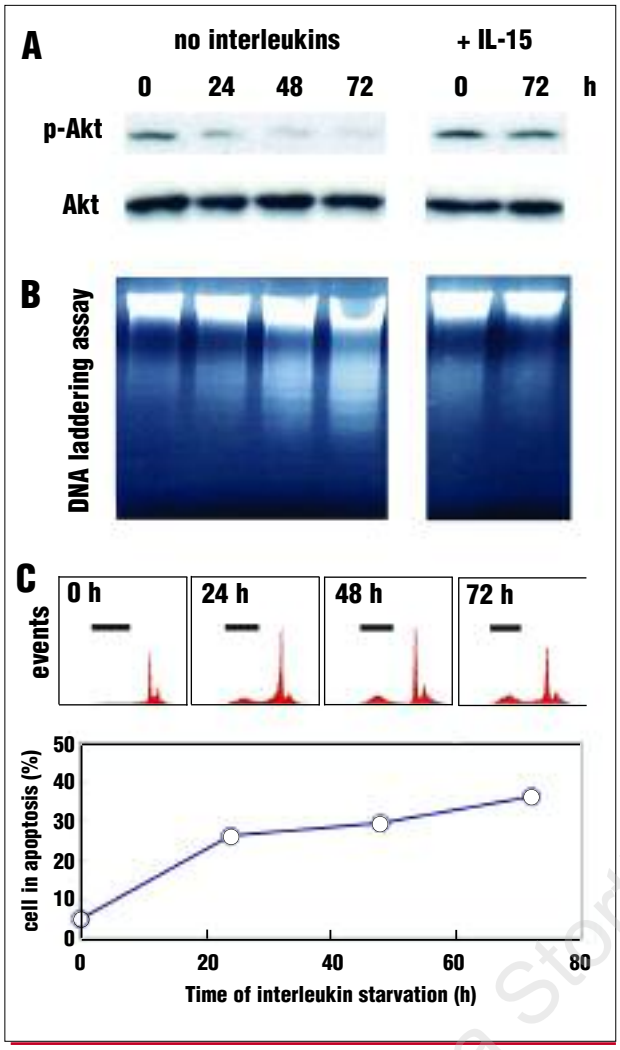

Figure 2. Apoptosis was induced after starving NK-92 cells of interleukins (IL-2 and IL-15). Western blotting analysis (A) for NK-92 deprived of interleukins revealed progressive dephosphorylation of AKT (A, upper panel, p-Akt) at different time points (0, 24, 48 and 72 hours, respectively). Total AKT (A, lower panel, Akt) remained unchanged. Starving NK-92 cells of interleukins led to apoptosis as shown by the progression to laddering of genomic DNA (B) and the progressive increase in the portion of cells in the sub-G1 peaks (C) in flow cytometry analyses. Supplementing NK-92 with IL-15 maintained phosphorylation of AKT and prevented cells undergoing apoptosis (right column, A and B, +IL-15 at 0 and 72 hours, respectively).

tion with interleukin-15 (IL-15, 5 ng/mL). AKT phosphorylation was also preserved by the IL-15 supplementation (Figure 2). These results suggest that, besides IL-2, IL-15 can also maintain the survival of NK-92 cells and AKT activation. The serum levels of IL-15 were significantly higher in ENTL patients (mean±standard deviation [SD]=3.22±0.53 pg/mL, n=8) than in normal healthy individuals (mean±SD=1.46±0.12 pg/mL, n=6; Student's t-test, p=0.011). The serum level of IL-2, on the other hand, was not significantly different (mean ± SD=0.159±0.012 IU/mL and 0.154±0.013 IU/mL in ENTL and normal subjects, respectively). These data suggest that while both IL-2 and IL-15 can maintain ENTL cell survival, IL-15 might play a significant role in ENTL patients *in vivo*. IL-2 and IL-15 share 2 common βγ receptors.[6] The mechanisms of IL-15 stimulation *in vivo* and the downstream pathways are, however, not clear. In a mouse model, prolonged stimulation with IL-15 resulted in the development of NK lymphoma.[7] Our data suggest that AKT may be one of the downstream pathways mediating the action of IL-15 in ENTL. The systemic increase of IL-15 in ENTL patients suggests that IL-15 may be important for the increase in tumor load of ENTL. All our cases are EBV positive, but the causes of raised IL-15 remain to be determined. Investigation along these pathways in ENTL may allow further understanding of this rare lymphoma.

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Multiple Myeloma

Lack of receptor activator of nuclear factor-κB ligand (RANKL) expression and functional production by human multiple myeloma cells

The direct expression and production of the critical osteoclastogenic factor, the receptor activator of NF-κB ligand (RANKL), is a matter of controversy. In this study we definitively demonstrate by both qualitative and quantitative polymerase chain reaction analysis that human myeloma cells do not express significant levels of RANKL mRNA or produce RANKL able to stimulate osteoclast formation.

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Recent data indicate that up-regulation of the critical osteoclastogenic factor, receptor activator of NF-κB ligand (RANKL), occurs in cells of the bone marrow (BM) microenvironment such as stromal-osteoblastic cells[1] and T lymphocytes,[2] triggering osteoclast formation in patients with multiple myeloma. However the direct expression and production of RANKL by human myeloma cells and the potential role of RANKL derived myeloma cells in osteoclastogenesis is still controversial since RANKL mRNA and protein have variably been reported to be absent[1-3] and present in human myeloma cells.[4-7] In order to clarify this issue, we first investigated RANKL mRNA expression in human myeloma cells by reverse transcription polymerase chain reaction (RT-PCR) using two different primer pairs able to amplify both the soluble and the trans-membrane isoform (RANKL: sense: 5'-TGGATCACAGCACATCAGAGCAGAG-3'; antisense: 5'-ATACTCTGTAGCTAGGTCTCCTGAAG-3') by Ampli Taq Gold DNA polymerase (Applied Biosystems, UK) and comparing different PCR conditions. We also used the same primer pair (sense: 5'-AACAGGCCTTTC-AAGGAGCTG-3'; antisense: 5'-TAAGGAGGGGTTG-GAGACCTCG-3') and PCR conditions as used by other authors[6] in order to be able to compare the results. When 1 μg of total RNA and 35 cycles of amplification with Ampli Taq Gold DNA polymerase (Applied Biosystems, UK) were used, all the human myeloma cell lines (HMCL) tested and normal CD138+ plasma cells (PC) obtained from reactive tonsils were negative for RANKL expression (Figure 1A). Using all the RNA extracted from 10^6 cells and 30 cycles of amplification a band of slight intensity was detected in normal PC and at very low intensity in RPMI-8226 (Figure 1B). Only performing the PCR with 35 cycles of amplification, as previously described,[4-6] did we find that RPMI-8226 cells were positive for RANKL expression, as were PC. All the other HMCL presented a slight band (Figure 1C). With the same very sensitive PCR conditions, CD138+ plasma cells purified from 40 MM patients at diagnosis or relapse by an immunomagnetic method[1-2] were negative for RANKL expression as shown in Figure 2D for 8 representative

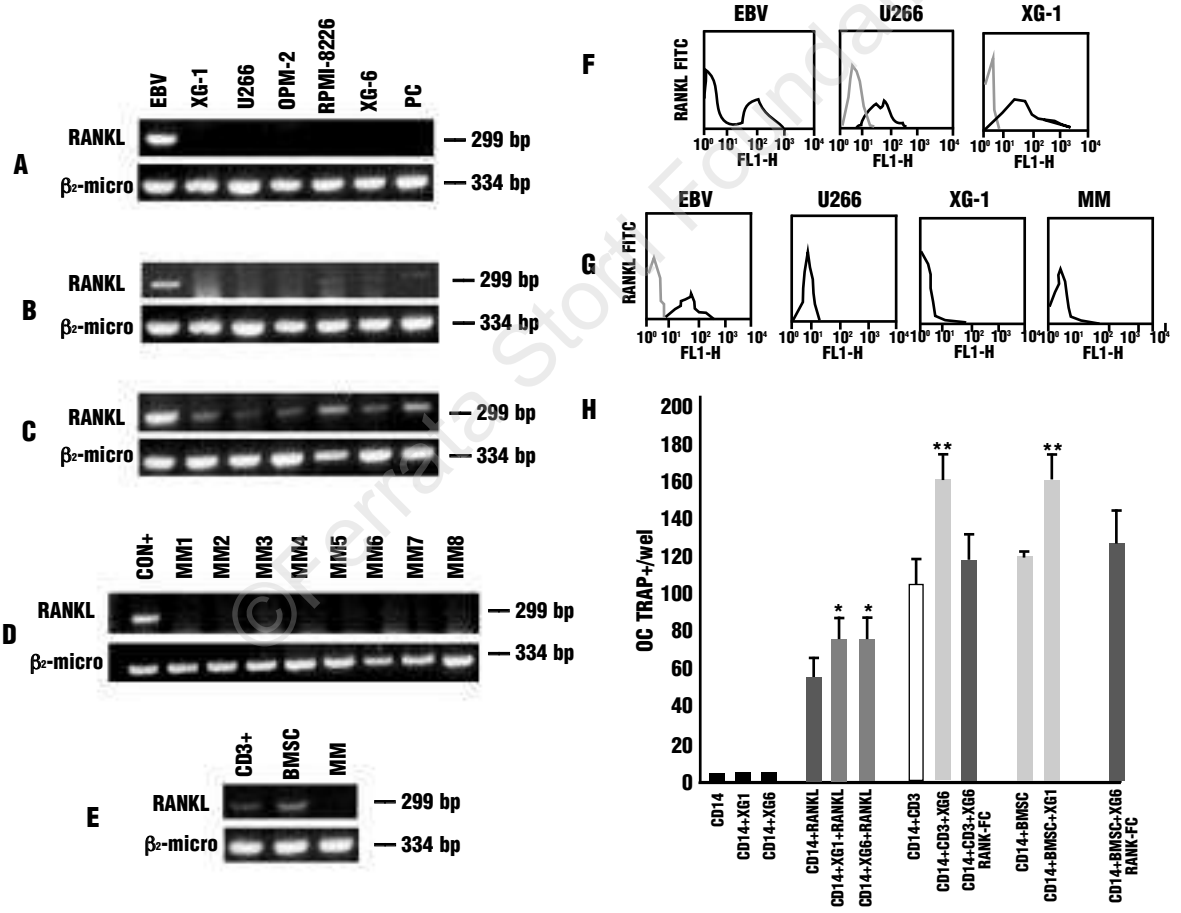

Figure 1. Lack of functional RANKL expression in human myeloma cells. RT-PCR was performed in order to test RANKL mRNA expression in HMCL (XG-1, U266, OPM-2, RPMI-8226, XG-6), an EBV-positive cell line and normal plasma cells (PC) obtained from reactive tonsils using different PCR conditions: 1 μg of total RNA and 35 cycles of amplification with Ampli Taq Gold DNA polymerase (A) or all the RNA extracted from 10^6 cells amplified for both 30 cycles (B) and 35 cycles (C). BM CD138+ MM cells of 8 representative patients (D), BMSC and BM purified CD3+ lymphocytes (E) were tested for RANKL mRNA expression using the most sensitive conditions (all mRNA extracted and 35 cycles of amplification). RANKL membrane expression was evaluated by flow cytometry with two different anti-RANKL monoclonal antibodies, clone 70525 (F) and clone 75707 (G). (H) Peripheral human CD14+ cells were incubated with HMCL XG-1 or XG-6, in the presence or absence of soluble RANKL or with activated CD3+ cells or with BMSC in the presence or absence of RANK-Fc (20 ng/mL). Multinucleated (nuclei >3) TRAP+ cells were identified as osteoclastic cells. Graphs represent the mean levels ±SE of 3 repeated experiments. *= $p<0.05$, **$p<0.01$.

Table 1. Quantitative evaluation of RANKL mRNA in multiple myeloma.

Cell lines	ΔC_t	$\Delta\Delta\Delta C_t$	*n-fold expression*
A			
EBV	-0.55	0	1
RPMI-8226	7.21	7.76	4.61×10^{-3}
XG-1	8.65	9.2	1.70×10^{-3}
XG-6	8.31	8.86	2.15×10^{-3}
OPM-2	9.33	9.88	1.06×10^{-3}
U266	10.20	10.75	5.80×10^{-4}

ΔC_t: mean $C_{t,RANKL}$ - mean $C_{t,ABL}$, $\Delta\Delta C_t$= mean ΔC_t - mean $\Delta C_{t,EBV}$; n-fold expression: normalized RANKL amount relative to EBV ($2^{-\Delta\Delta Ct}$).

Cell type	ΔC_t	$\Delta\Delta\Delta C_t$	*n-fold expression*
B			
BMSC	3.17±0.3	0.0±0.3	1 (0.81-1.23)
BM $CD3^+$	3.93±0.4	0.76±0.4	0.59 (0.45-0.78)
$CD138^+$ MM	9.05±1.5	5.88±1.5	1.7×10^{-2} (4.8×10^{-2}-$6.\times10^{-3}$)

ΔC_t: mean $C_{t,RANKL}$- mean $C_{t,ABL}$; $\Delta\Delta C_t$: mean ΔC_t- mean ΔC_t, EBV; n-fold expression: normalized RANKL amount relative to BMSC ($2^{-\Delta\Delta Ct}$).

samples. In contrast, both BMSC and BM-purified $CD3^+$ lymphocytes, obtained from MM patients with osteolytic lesions as previously described,[1-2] expressed RANKL mRNA (Figure 1E).

Subsequently we evaluated the level of RANKL mRNA by quantitative PCR in HMCL, myeloma cells, BMSC and BM $CD3^+$ lymphocytes from 10 MM patients. Two microliters of c-DNA, prepared from 1 μg of total RNA, were used in a total volume of 25 μL of a universal master Mix (Applied Biosystems), 300 nM of a RANKL-specific primer pair (sense: CGTTGGATCACAGCACATCAG, antisense: TGCTCCTCTTGGCCAGATCT) and 200 nM of TaqMan probe (6-FAM-CAGAGAAAGCGATGGTGGATGGCT-CAT-MGB). To normalize for differences in RNA extraction and efficiency of the reverse transcription, we applied the comparative Ct method using RNA from the endogenous reference gene *ABL*.[8] The relative RANKL mRNA quantification in HMCL was performed by the comparative ΔΔCt method.[9] As shown in Table 1A we found that the levels of RANKL in RPMI-8226, XG-1, XG-6, OPM-2 and U266 were 2.3 log, 2.8 log, 2.7 log, 3 log and 3.2 log lower than in EBV, used as calibrator. Moreover, we found that median RANKL expression in $CD3^+$ lymphocytes and $CD138^+$ plasma cells from 10 MM patients was, respectively, 0.2 log (range 0.1-0.3) and 1.8 log (1.3-2.2) lower than in BMSC (Table 1B). To investigate the previously reported presence of RANKL on the membrane of human myeloma cells,[6-7] we checked RANKL by flow cytometry using two different antibodies from R&D systems (Minneapolis, MN, USA). With the anti-RANKL monoclonal antibody, clone 70525, we found that HMCL and EBV-positive cells were positive for RANKL (Figure 1F), as reported by others.[6-7] In contrast, using the specific anti-RANKL mAb, clone 175707 that does not cross-react with other RANKL family members, such as FAS ligand or TRAIL, we found that only EBV-positive cells expressed RANKL whereas HMCL and freshly purified MM cells did not (Figure 1G). Similar results were obtained by Western blot analysis (*data not shown*) suggesting that the RANKL expression on myeloma cells, observed with the first monoclonal antibody used, could be a false positive signal. Finally we checked whether human myeloma cells produce RANKL in an *in vitro* osteoclastogenesis system using purified $CD14^+$ monocytes from healthy subjects[10] rather than total BM mononuclear cells[4,6] to exclude contaminating cells that produce RANKL. In this system we found that HMCL were not able to induce osteoclast formation, evaluated as TRAP-positive mononucleated cells,[2,10] without the presence of soluble RANKL (100 ng/mL, R&D) (Figure 1H). On the other hand, HMCL significantly increased osteoclast formation from $CD14^+$ cells only in the presence of activated CD3 lymphocytes or BMSC co-cultured in a transwell insert or in a cell-to-cell contact system, respectively (Figure 1H). Our observations are in line with those reported by others showing that HMCL alone have marginal bone resorbing activity as compared to HMCL co-cultured with BMSC[11] while EBV-positive cell lines, such as ARH-77, that express RANKL, are able to stimulate osteoclast formation. On the other hand, the previously reported capacity of HMCL to stimulate osteoclast formation *in vitro*[4,6] was observed using whole BM or peripheral mononuclear cells but not with purified $CD14^+$ monocytes suggesting that the presence of T lymphocytes and/or BMSC could explain the osteoclastogenic effect of HMCL observed in the past.

In conclusion, our data clearly demonstrate that human myeloma cells neither express significant levels of RANKL mRNA nor produce RANKL able to stimulate osteoclast formation.

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Multiple Myeloma

Efficacy of bortezomib therapy for extramedullary relapse of myeloma after autologous and non-myeloablative allogeneic transplantation

We report the successful use of bortezomib to treat a patient with multiple myeloma (MM) who had extramedullary relapse (paraspinal and thoracic masses and multiple cranial nerve palsy) after autologous and non-myeloablative allogeneic hematopoietic stem cell transplantation (HSCT).

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A 50-year woman was diagnosed with stage III IgG lambda MM in October 2001; she was treated with 4 VAD cycles, radiotherapy to the lumbar spine and autologous HSCT after 200 mg/m^2 melphalan in October 2002, followed by a non-myeloablative allogeneic HSCT from her HLA identical brother 3 months later. Conditioning was 2 Gy total body irradiation and graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine A and mycophenolate mofetil. In February 2003, a complete remission was obtained (negative serum immunofixation and no marrow plasma cell infiltration), which lasted 9 months. No significant GVHD developed and GVHD prophylaxis was withdrawn on day +195. In November 2003, the patient complained of spine and right hemithorax pain. Magnetic resonance imaging (MRI) of the spine detected a T9 paraspinal mass, with partial compression of the spinal medulla; the mass proved to be a plasmacytoma at biopsy. Computed tomography (CT) of the thorax showed 3 masses (maximum diameter, 7 cm) involving the second, third, fourth and fifth right ribs. No serum or urine paraprotein was detected and marrow biopsy was normal, with 100% of cells being of donor origin. The patient underwent urgent radiotherapy to T9 and complained of paresthesia of the right cheek, difficulty in speaking, dysphagia, and inability to move her tongue to the right. Palsy of the IX, X and XII cranial nerves was diagnosed. Cerebral MRI with post-contrast T1-weighted images showed abnormal enhancement of the meninges of the right cerebellar hemisphere and of the interhemispheric sickle. Cerebrospinal fluid (CSF) showed a protein and cell content within the normal limits. CSF polymerase chain reaction for cytomegalovirus, herpesvirus and neurotropic viruses was negative. High-dose dexamethasone therapy and one intrathecal administration of corticosteroids and cytosine-arabinoside did not modify any neurological sign. In January 2004, the patient started treatment with bortezomib (VELCADE) at the dose of 1.3 mg/m^2 on days 1, 4, 8, 11, every 21 days. WHO grade III thrombocytopenia and WHO grade I intestinal toxicity were reported. At the end of the first cycle, all the neurological symptoms and signs had disappeared and after 3 cycles cerebral MRI and thoracic CT had returned to normal (Figure 1). CSF examination was again normal. This response has persisted up to now, without any clinical sign of GVHD. Our patient had an extramedullary MM relapse after autologous and non-myeloablative allogeneic transplantation, involving a vertebra and 4 ribs. Moreover, she developed multiple cranial nerve palsies. We did not detect plasma cells in the CSF or masses in MRI. However, the MRI showing thick-

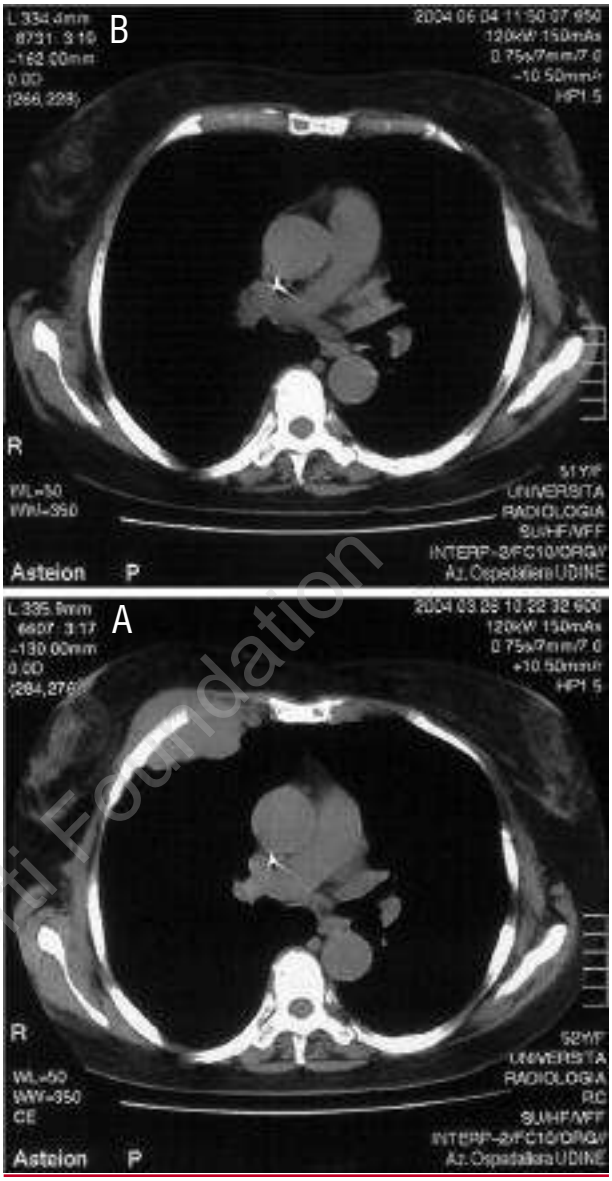

Figure 1. A. The thorax computer thomography (CT) showed a plasmacytoma of the fifth right rib. B. After 3 cycles of bortezomib the mass had disappeared.