IL-7 Up-Regulates TNF-α-Dependent Osteoclastogenesis in Patients Affected by Solid Tumor

Ilaria Roato1,3*, Giacomina Brunetti3, Eva Gorassini1, Maria Grano2, Silvia Colucci2, Lisa Bonello4, Lucio Buffoni1, Roberto Manfredi4, Enrico Ruffini5, Davide Ottaviani6, Libero Ciuffreda5, Antonio Mussa3, Riccardo Ferracini6

1 Center for Experimental Research and Medical Studies (CeRMS), University and AZO San Giovanni Battista, Turin, Italy, 2 Department of Human Anatomy and Histology, University of Bari, Bari, Italy, 3 Department of Medical Oncology, University and AZO San Giovanni Battista, Turin, Italy, 4 Department of Medical Oncology, Ospedale Civile, Asti, Italy, 5 Department of Orthopaedics, AZO San Giovanni Battista, Turin, Italy, 6 Department of Orthopaedics, AZO San Giovanni Battista, Turin, Italy

Background. Interleukin-7 (IL-7) is a potent regulator of lymphocyte development, which has also significant effects on bone; in fact it is a potent osteoclastogenic factor. Some human solid tumors produce high IL-7 levels, suggesting a potential IL-7 role on tumor development and progression. Methodology. We studied 50 male patients affected by solid tumors, and their blood samples were collected at tumor diagnosis. PBMCs were isolated and cultured with/without IL-7 to study its influence on osteoclastogenesis. Serum and cell culture supernatant IL-7 levels were measured by ELISA. The quantitative analysis of IL-7 expression on T and B cells was performed by Real-Time PCR. Principal Findings. Serum IL-7 levels were highest in osteolytic cancer patients, followed by cancer patients without bone lesions, and then healthy controls. We showed the IL-7 production in PBMC cultures and particularly in monocyte and B cell co-cultures. A quantitative analysis of IL-7 expression in T and B cells confirmed that B cells had a high IL-7 expression. In all cell culture conditions, IL-7 significantly increased osteoclastogenesis and an anti-IL-7 antibody inhibited it. We demonstrated that IL-7 supports OC formation by inducing the TNF-α production and low RANKL levels, which synergize in promoting osteoclastogenesis. Conclusions. We demonstrated the presence of high serum IL-7 levels in patients with bone metastasis, suggesting the use of serum IL-7 level as a clinical marker of disease progression and of bone involvement. Moreover, we showed the capability of IL-7 to stimulate spontaneous osteoclastogenesis of bone metastatic patients and to induce osteoclastogenesis in cancer patients without bone involvement. These findings add further details to the disclosure of the mechanisms controlling bone metastasis in solid tumors.

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INTRODUCTION

Interleukin-7 (IL-7) is a pleiotropic immune regulatory protein predominantly produced by stromal cells and by cells at the inflammatory sites [1]. IL-7 is fundamental for the early development of lymphocytes and is a regulator of peripheral T cell homeostasis by modulating the expansion of peripheral T-cell populations in states of T cell depletion [2]. The production of IL-7 by some human solid tumors suggests its potential impact on the process of tumorigenesis [3,4], but it is unclear how IL-7 is involved in solid tumor development and progression. IL-7 stimulates the progression of some types of lymphomas and leukemias [5,6] and in non-small cell lung cancer, it was reported that IL-7 targeted gene therapy may be effective in modifying host cell homeostasis by modulating the expansion of peripheral T-cell lymphocytes and is a regulator of peripheral T cell homeostasis [7]. Recent studies on breast cancer describe a quantitative association between the IL-7 signalling complex and some clinico-pathological parameters: there is a trend towards a higher expression of IL-7 and molecules of its signalling pathway in breast cancer patients with poor prognosis [4]. Moreover, IL-7/IL-7R mRNA was detected in different tumors, such as colorectal [8], renal [9], lung and central nervous system cancers [10].

IL-7 is involved in the control of osteoclastogenesis, however depending on the model considered, it displays either an inhibitory or an activator effect on OCs [11], [12]. The OCs derive from cells of the monocytic-macrophagic lineage, which fuse to form bone-resorbing cells in the presence of Macrophage Colony Stimulating Factor (M-CSF) and Receptor Activator of Nuclear factor kB Ligand (RANKL) [13] or Tumor Necrosis Factor-α (TNF-α) [14,15]. Weitzmann et al. demonstrated that systemic administration of IL-7 stimulates osteoclastogenesis through T cells by RANKL and TNF-α [16]. In addition, they showed that TNF-α produced by T cells synergises with RANKL increasing bone destruction in murine models of estrogen deficiency [17,18].

The addition of exogenous cytokines is necessary to stimulate osteoclastogenesis in patients without osteolysis and in healthy controls. Previously, we demonstrated that peripheral blood mononuclear cells (PBMCs) from cancer patients with osteolysis differentiate spontaneously into OCs. In addition, we showed that in patients with osteolysis the activation of osteoclastic precursors depends on circulating tumor cells or on factors released from the tumor site, such as TNF-α [15].

To better identify the factors involved in the spontaneous osteoclastogenesis, present in bone metastatic patients, we focused our studies on IL-7, since it has been associated with haematological malignancies and inflammatory diseases characterized by a local and/or systemic bone loss [19–21].
IL-7 involvement in the bone metastasis formation and in the spontaneous osteoclastogenesis of cancer patients adds a further detail to the mechanisms of bone resorption and might be useful to design novel therapeutic approaches for treatment and prevention of bone metastasis.

RESULTS
IL-7 levels dosed in supernatants and sera are higher in cancer patients than in healthy controls
We measured the IL-7 levels in 25 patient and healthy control PBMC supernatants, collected at days 5 and 10 of culture. We observed that IL-7 levels were significantly higher in patients than in healthy controls at day 5, \( p<0.0006 \) (Fig. 1A). At day 10, the IL-7 concentration was comparable to the one at day 5, both in patients and in healthy controls (data not shown). To identify the IL-7 source we performed co-culture experiments of monocytes plus B or T cells and we measured its levels in supernatants at day 5 and 10. We showed that IL-7 levels were significantly higher in cancer patients’ co-cultures of both monocytes plus T or B cells than in healthy controls (\( p<0.0006 \)) and remained nearly steady between day 5 and 10. In our model, the purified B cell population is responsible for IL-7 production: co-cultures of monocytes and B cells showed the highest IL-7 levels. Both in PBMC cultures and in monocytes plus T and B cells derived from patients without bone lesions we measured low levels of IL-7, comparable to healthy controls.

Besides, we tested serum levels of IL-7 in 50 patients affected by solid tumors with or without metastasis at the time of diagnosis. Fig. 1B showed that IL-7 serum levels were significantly higher in patients with bone metastasis compared to patients without bone lesions and healthy controls, \( p<0.0001 \). The mean IL-7 value was 17.69±1.24 pg/ml for osteolytic patients, 10.73±0.61 pg/ml for patients without bone lesion and 5.56±0.30 pg/ml for healthy controls. We examined the potential differences in serum IL-7 levels according to the various types of tumor and specifically we considered prostate cancer, NSCLC and SCLC. We identified significant differences in the mean value of IL-7 (\( p<0.02 \)), which were 19.89±6.30 pg/ml for prostate cancer, 14.6±5.95 pg/ml for NSCLC and 7.56±4.09 pg/ml for SCLC.

Evaluation of IL-7 expression in T and B cells with (RQ-PCR)
We analysed the expression of IL-7 on freshly purified T and B cells by RQ-PCR. The analyzed transcripts exhibited high linearity amplification plots (\( r>0.99 \)) and the efficiencies of both PCR reactions were quite similar. As shown in Fig. 2, we did not measure significant differences in the expression of IL-7 in both patients and healthy controls’ T cells, while in healthy controls’ B cells the IL-7 expression was five-fold lower than in patients. The relative expression of IL-7 was seven-fold higher in B cells than in T cells in cancer patients. We did not appreciate any significant differences in IL-7 expression in cancer patients with or without bone metastasis (Fig. 2).

Effects of IL-7 on osteoclastogenesis
The OC differentiation was verified by the presence of multinucleated/TRAP positive cells from cancer patient and healthy control PBMCs (Fig. 3A–B lines, respectively). PBMCs were cultured at different doses of IL-7, that resulted in significantly increased osteoclastogenesis with 2.5 and 10 ng/ml of IL-7 in osteolytic patients and in osteoclastogenesis stimulation for patients

Figure 1. IL-7 levels in cell culture medium and serum. IL-7 levels in cancer patients with/without bone metastases and in healthy controls were analysed by ELISA. At day 5, supernatants of patients’ PBMC cultures and monocyte plus T and B cell co-culture had IL-7 levels higher than healthy controls (A). Bone metastatic patients had significantly higher serum levels of IL-7 compared to patients without bone metastasis and healthy controls (B). Samples were assayed in duplicate and data were expressed as mean values.

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Figure 2. Quantitative analysis of IL-7 expression in T and B cells. IL-7 expression was evaluated by RQ-PCR using \( 2^{-\Delta\Delta Ct} \) method. B cells derived from cancer patients expressed significantly higher levels of IL-7 compared to T cells. Both T and B cells from healthy controls expressed less IL-7 than patients. The control bar represents the negative control: IL-7 expression in HS22 cell line. Data are means±SE of nine independent experiments.

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without bone lesions and healthy controls, \( p < 0.0001 \). At 15 ng/ml, IL-7 had not a stimulatory effect on osteoclastogenesis (Fig. 3C). By adding a neutralizing anti-IL-7 antibody on osteolytic patients’ PBMC, we observed a dose-dependent inhibition of spontaneous osteoclastogenesis, \( p < 0.01 \) (Fig. 3D). We characterized OCs also for the expression of \( \alpha_v\beta_3 \), a typical OC marker. Both OCs derived from culture with and without IL-7 expressed \( \alpha_v\beta_3 \), as shown in Fig. 3E.

The ability of IL-7 to induce OC formation was tested in cultures of purified monocytes alone and in co-cultures with purified T or B cells treated or untreated with IL-7 at 2.5 and 15 ng/ml. Our results showed that monocytes alone were not able to support significant OC formation in either the presence or absence of IL-7, in both cancer patients and healthy controls (data not shown). Moreover, we demonstrated that co-cultures of monocytes and T cells both from bone metastatic patients and healthy controls resulted in a significant induction of OC formation by adding 2.5 ng/ml compared to basal condition, \( p < 0.001 \), while the dose of 15 ng/ml did not elicit a significant stimulatory effect on osteoclastogenesis (Fig. 4A). Co-cultures of monocytes and B cells in presence of IL-7 showed an increase in OC number, but it was not statistically significant (Fig. 4B). In all culture conditions, we observed a comparable osteoclastogenesis in patients without bone metastases and in healthy controls (data not shown).

Since we showed that spontaneous osteoclastogenesis depends on contact between monocytes and lymphocytes [15], we investigated whether T and B cells were necessary for IL-7-dependent OC differentiation. We cultured purified monocytes with IL-7-treated conditioned medium from T and B cells and we did not observe OC differentiation (data not shown). Thus, cell-to-cell interaction between lymphocytes and monocytes are required for solid tumor dependent osteoclastogenesis.
Figure 4. IL-7 stimulates osteoclastogenesis in co-cultures of monocytes and T or B cells. The different subpopulations were isolated from PBMCs and co-cultures of monocytes and T or B cells (M+T; M+B) were made. By adding 2.5 ng/ml of IL-7 in co-cultures we observed an increase in osteoclastogenesis, which was statistically significant for T cells (A) and not for B cells (B). At 15 ng/ml of IL-7, there were not significant effects in both the co-cultures.
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Figure 5. IL-7 increases OC bone resorbing activity. PBMC resorption activity with/without IL-7 were showed for bone metastatic patients (line A) and healthy controls (line B). The basal resorbing activity of OCs derived from patients was significantly higher compared to healthy controls, *p<0.0001 (C). The percentage of resorption area was significantly increased with 2.5 ng/ml of IL-7, both in patients and in healthy controls, *p<0.002 (C). At 15 ng/ml IL-7 did not elicit a significant variation of resorption area. The data represent the means±SE from ten independent experiments.
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IL-7 stimulates OC resorbing activity
Since we showed that IL-7 had effects in stimulating osteoclastogenesis, we verified whether there was a similar stimulatory action on bone resorbing activity. By analysing the resorption area on biocoated slices, we demonstrated that in presence of IL-7 at 2.5 ng/ml the OCs increased the bone resorbing activity of osteolytic patients and stimulated the healthy controls' resorption (Fig. 5A–B lines, respectively). The basal resorption activity of OCs derived from patients was significantly higher compared to healthy controls, *p≤0.0001 (Fig. 5C). After stimulation with IL-7 at 2.5 ng/ml, the bone resorbing activity of OCs was significantly augmented both in patients and in healthy controls, *p≤0.002 (Fig. 5C). At 15 ng/ml of IL-7, we did not observe any statistically significant variation on bone resorbing areas.

IL-7 affects TNF-α and RANKL release in culture
We assessed the IL-7 ability to induce TNF-α and RANKL secretion in PBMC cultures and monocyte plus T or B cell co-cultures at day 5 and 10. The basal levels of TNF-α were similar in PBMC media at day 5 and 10 and they significantly increased by adding 2.5 ng/ml of IL-7, p<0.002 (Fig. 6A). Co-cultures of monocytes and T or B cells showed basal TNF-α levels higher at day 10 than at day 5. Monocytes plus T cells displayed an IL-7 dose-dependent increase of TNF-α level both on days 5 and 10, p<0.01 (Fig. 6B). At 15 ng/ml of IL-7, TNF-α values reached a plateau level both in PBMC cultures and in monocytes plus T cell co-cultures (Fig. 6A–B). At day 5, co-cultures of monocytes and B cells showed a significant enhancement of TNF-α amount at 15 ng/ml, whereas at day 10 we measured a significant increase in TNF-α levels at 2.5 ng/ml, that was unchanged at 15 ng/ml of IL-7, p<0.01 (Fig. 6C).

We found low RANKL levels in culture media collected from PBMC and monocyte plus T or B co-cultures. In particular, in the IL-7 treated cells there were not significant differences compared to unstimulated controls (Table 1). To further study the involvement of TNF-α and RANKL we cultured cancer patients’ PBMC in presence of IL-7 and a neutralizing anti-TNF-α antibody or osteoprotegerin (OPG) in different concentrations. Both the inhibitors determined a dose-dependent inhibition of bone resorbing activity (data not shown) and of osteoclastogenesis, that was stronger in cultures treated with anti-TNF-α (Fig. 7A) than with OPG (Fig. 7B). Thus, our data demonstrated that IL-7 supports osteoclastogenesis by inducing mainly the TNF-α production and low levels of RANKL.

**DISCUSSION**
Over the last years, many studies investigated the cross-talk between immune system and bone, and IL-7 appeared as an important factor in these interactions [12,22,23]. IL-7 is known to be a powerful inducer of T and B lymphopoiesis, moreover many studies link IL-7 to inflammatory diseases [21,24,25] and bone loss in estrogen deficiency conditions [22,26]. Our study focused on IL-7 involvement in the spontaneous osteoclastogenesis occurring in cancer patients with osteolytic metastasis. We showed significantly higher IL-7 levels in supernatants derived from PBMC cultures and co-cultures of monocytes and T or B cells of cancer patients compared to healthy controls. Our data describe for the first time the presence of elevated IL-7 levels in sera of male patients affected by solid tumors, in fact the only data available in literature, concerning IL-7 serum levels in patients affected by solid tumors are related to gynecologic cancers [27–29], while elevated serum IL-7 levels were previously detected in other pathologies with bony involvement, such as multiple myeloma or osteoporosis.

**Table 1. RANKL levels in cell culture media.**

| RANKL pg/ml | Day 5 | Day 10 |
|-------------|-------|--------|
| PBMC        |       |        |
| 0 ng/ml IL-7| 1.91±(1.00) | 6.34±(2.85) |
| 2.5 ng/ml IL-7| 0.1±(0.1)    | 2.28±(2.28)  |
| 15 ng/ml IL-7| 2.6±(1.31)   | 3.94±(3.94)  |
| M+B         |       |        |
| 0 ng/ml IL-7| 0.64±(0.84)  | 5.86±(3.26)  |
| 2.5 ng/ml IL-7| 2.14±(2.14)  | 6.21±(0.23)  |
| 15 ng/ml IL-7| 2.32±(2.32)  | 9.53±(6.45)  |
| M+B         |       |        |
| 0 ng/ml IL-7| 0.40±(0.40)  | 0.83±(0.53)  |
| 2.5 ng/ml IL-7| 0.62±(0.23)  | 1.53±(1.53)  |
| 15 ng/ml IL-7| 0.84±(0.14)  | 1.19±(0.65)  |

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inhibition of IL-7-induced osteoclastogenesis. The anti-TNF-α antibody caused a strong osteoclastogenesis inhibition, about 90% while OPG caused a 50% inhibition (B).

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Figure 7. Effect of anti-TNF-α and OPG on IL-7-induced osteoclastogenesis. The addition of both OPG and anti-TNF-α caused a dose-dependent inhibition of IL-7-induced osteoclastogenesis. The anti-TNF-α antibody caused a strong osteoclastogenesis inhibition, about 90% (A) while OPG caused a 50% inhibition (B).

In this study we demonstrated that IL-7 at 2.5 ng/ml increased osteoclastogenesis in patients' cultures of PBMCs and co-cultures of monocytes plus T or B cells, while at the dose of 15 ng/ml, IL-7 did not affect osteoclastogenesis. To explain this result we hypothesize that, at high doses, IL-7 stimulates a differentiation switch from OCs towards dendritic cells since in cultures with 15 ng/ml of IL-7 we observed only few OCs and mainly undifferentiated macrophages or cells with dendritic cells morphology. This hypothesis is supported by some studies showing that IL-7 regulates dendritic cells differentiation from PBMC cultures [33,34]. Since literature data link IL-7 to TNF-α and RANKL-dependent osteoclastogenesis [16,19,35], we measured their levels in culture media. In detail, we showed that IL-7 enhances in a dose-dependent manner TNF-α levels in PBMC culture media and in co-cultures of monocytes and T or B cells. We hypothesize that TNF-α levels reached maximal values in the PBMC cultures probably due to a cooperative release of TNF-α by monocytes [21], T and B cells stimulated by IL-7. We observed a little and not statistically significant dose-dependent increase of RANKL release from day 5 to day 10 of culture compared to unstimulated condition. According to literature data [36,37] we think that these low levels of RANKL are synergistic with TNF-α in promoting osteoclastogenesis. In fact we observed that both anti-TNF-α antibody and OPG caused a dose-dependent inhibition of IL-7-induced osteoclastogenesis. The anti-TNF-α antibody reached an OC inhibition plateau at 90% while OPG treatment of cell cultures reached a plateau of OC differentiation inhibition at 50%. Further increase in OPG concentrations, in an attempt to reach an higher level of inhibition, was found to be toxic for the cultured cells. This observation suggests that in our model TNF-α might have a stronger role in OC formation than RANKL; it is conceivable that both factors may synergize in OC differentiation.

These last results demonstrate that TNF-α plays a fundamental role in IL-7-induced osteoclastogenesis in patients affected by solid tumor. Moreover, the pivotal role exerted by TNF-α in our systems was also supported by the fact that in freshly isolated T and B cells from patients we did not find RANKL expression, while TNF-α was strongly present. TNF-α enhancement by IL-7 is also present in other pathological conditions, characterized by a local and/or systemic bone loss, such as rheumatoid arthritis [30] or postmenopausal osteoporosis [38].

In conclusion, we propose a new physiopathologic mechanism with a key role of IL-7 in the formation of solid tumor bone metastasis. We demonstrated that IL-7, produced mainly by B cells in cell culture, directly sensitizes T cells to produce pro-osteoclastogenic factors, such as TNF-α and RANKL, and enhances spontaneous osteoclastogenesis. It could be useful to analyse a large number of patients for each different tumor type, in order to set an IL-7 cut off value, representative of a warning threshold for clinicians. This could allow to follow the bone metastatic disease both at diagnosis and during treatments.

MATERIAL AND METHODS

Patients

Samples from peripheral blood (PB) were obtained from 50 male patients affected by solid tumors and 50 healthy controls, matched for age and sex. The main clinical characteristics of patients, aged from 50 to 84 years (median 65±11.01 SD), are listed in Table 2. The patients' samples were collected at diagnosis to exclude biases, including recent chemo/hormono-therapy and pre-existing or non-related bone pathologies. We decided not to include breast...
cancer patients to avoid bias deriving from women treated with steroids or hormonal therapies.

Healthy controls showed a normal bone metabolism (evaluated by bone densitometry) and were not under medications, such as steroids that could increase osteoclastogenesis. Informed consent from patients and healthy controls was obtained to comply with institutional policies.

Cell cultures
PBMCs, collected from cancer patients and healthy controls, were isolated after centrifugation over a density gradient, Lymphoprep (Nycomed Pharma, Norway) and cultured in α-MEM, supplemented with 10% FBS, penicillin 100 U/ml and streptomycin 100 μg/ml (Cambrex, Walkersville MD). To obtain fully differentiated human OCs, PBMCs were cultured with/without rhIL-7 at different doses (0.5–1–2.5–10 and 15 ng/ml), R&D Systems (Abingdon, UK). Culture supernatants were collected on days 5 and 10, when medium was either refreshed and supplemented or not with IL-7. For some experiments, patient PBMCs were cultured with different doses of anti-IL-7 (50–100–300 ng/ml), R&D Systems (Abingdon, UK). Cultures were stopped after 15 days, mature OCs were identified as multinucleated cells containing three or more nuclei and positive for the expression of TRAP (Tartrate-resistant acid phosphatase, Sigma Aldrich, St. Louis MO) and αvβ3 (vitronectin receptor) kindly provided by Prof. G. Tarone. To evaluate bone resorbing activity, for 21 days, PBMCs were cultured on Biocoat Osteologic bone cell culture system (BD Biosciences Bedford, MA) with/without IL-7, then cells were removed and resorption lacunae were identified by light microscopy.

For some experiments PBMCs were cultured in presence of increasing concentration of a neutralizing anti-TNFα (4–5 μg/ml) antibody and osteoprotegerin (OPG 10–20 ng/ml), PeproTech (London, UK).

Isolation of monocytes, T and B cells
Monocytes, T and B cells were isolated from PBMCs by an immunomagnetic selection using MACs microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The purity of monocytes, T and B cells, obtained after separation, was 95–98%, as assessed by flow cytometry. Isolated cells were plated according to the human physiological concentrations: 10% monocytes and 30% lymphocytes (70% for T and 30% for B) with/without IL-7.

ELISA (Enzyme-Linked Immunosorbert Assay)
The amount of IL-7 in cell culture supernatant and serum was determined by ELISA kit (sensitivity was 0,156 to 20 pmol/L, PeproTech (London, UK)).

Quantitative analysis of IL-7 gene expression
Total RNA was extracted from T and B cells and the first-strand cDNA synthesis was performed as previously described [15]. Quantitative analysis of IL-7 was performed with Real-Time Quantitative PCR (RQ-PCR) using β-actin as housekeeping control. RT-PCR was carried out using the iCycler iQ™ system (Bio Rad, CA, USA). PCR primers and TaqMan probes were designed using Primer Express v1.0 software and synthesized by Applied Biosystems (Warrington, UK). TaqMan probe specific for

| Patient | Cancer | Metastasis site | Age | TNM 1 | Stage 2 |
|---------|--------|-----------------|-----|-------|---------|
| 1       | SCLC   | none            | 56  | Extended disease 1 | –       |
| 2       | SCLC   | liver           | 79  | Extended disease 1 | –       |
| 3       | SCLC   | adrenal gland   | 52  | Extended disease 1 | –       |
| 4       | SCLC   | bone            | 64  | Extended disease 1 | –       |
| 5       | NSCLC  | none            | 84  | T1N0M0          | I       |
| 6       | NSCLC  | none            | 69  | pT1N0M0         | I       |
| 7       | NSCLC  | none            | 78  | pT1N0M0         | I       |
| 8       | NSCLC  | bone            | 52  | T1N0M1          | IV      |
| 9       | NSCLC  | none            | 74  | T2N0M0          | I       |
| 10      | NSCLC  | none            | 50  | T2N0M0          | I       |
| 11      | NSCLC  | none            | 76  | T2N0M0          | I       |
| 12      | NSCLC  | none            | 56  | pT2N0M0         | I       |
| 13      | NSCLC  | none            | 66  | pT2N1M0         | II      |
| 14      | NSCLC  | none            | 62  | T2N2M0          | IIIA    |
| 15      | NSCLC  | none            | 72  | T2N2M0          | IIIA    |
| 16      | NSCLC  | none            | 58  | T2N3M0          | IIIB    |
| 17      | NSCLC  | adrenal gland   | 63  | T2N0M1          | IV      |
| 18      | NSCLC  | bone            | 70  | T2N0M1          | IV      |
| 19      | NSCLC  | bone            | 57  | T2N2M1          | IV      |
| 20      | NSCLC  | adrenal gland, bone | 50  | T2N2M1          | IV      |
| 21      | NSCLC  | bone            | 61  | T2N2M1          | IV      |
| 22      | NSCLC  | bone, liver     | 50  | T2N3M1          | IV      |
| 23      | NSCLC  | adrenal gland   | 71  | T2N3M1          | IV      |
| 24      | NSCLC  | bone            | 57  | T2N3M1          | IV      |
| 25      | NSCLC  | none            | 53  | T3N0M0          | IIIA-B  |
| 26      | NSCLC  | none            | 56  | T3N2M0          | IIIA    |
| 27      | NSCLC  | none            | 81  | pT3N2M0         | IIIA    |
| 28      | NSCLC  | lung            | 76  | T3N2M1          | IV      |
| 29      | NSCLC  | bone            | 71  | T3N2M1          | IV      |
| 30      | NSCLC  | bone            | 66  | T3N3M1          | IV      |
| 31      | NSCLC  | adrenal gland, brain | 54  | T3N3M1          | IV      |
| 32      | NSCLC  | none            | 75  | T4N0M0          | IIIB    |
| 33      | NSCLC  | brain, bone     | 59  | T4N3M1          | IV      |
| 34      | NSCLC  | surreal         | 45  | T4N3M1          | IV      |
| 35      | NSCLC  | liver           | 77  | T4N3M1          | IV      |
| 36      | NSCLC  | brain           | 63  | T4N3M1          | IV      |
| 37      | NSCLC  | prostate        | 62  | T3N0M1          | IV      |
| 38      | prostate | bone         | 84  | pT3N1M1         | IV      |
| 39      | prostate | bladder       | 71  | T3N2M1          | IV      |
| 40      | prostate | bone         | 64  | T3N2M1          | IV      |
| 41      | prostate | none          | 68  | T4N0M0          | IIIB    |
| 42      | prostate | bone         | 66  | T4N0M1          | IV      |
| 43      | prostate | bone         | 64  | T4N0M1          | IV      |
| 44      | prostate | bone         | 64  | T4N0M1          | IV      |
| 45      | colon   | bone           | 59  | pT3N1M1         | IV      |
| 46      | colon   | bone           | 66  | pT4N0M1         | IV      |
| 47      | bladder | bone          | 86  | pT3N0M1         | IV      |
| 48      | bladder | bone          | 75  | pT3N2M1         | IV      |
| 49      | pancreas | multiple     | 36  | T4N2M1          | IV      |
| 50      | kidney  | bone           | 74  | pT1N0M1         | IV      |

1TNM international classification: T(primary tumor), N(primary regional lymphonodes), M (metastasis), p (post-surgery)
2tumor histologic grading
3Veterans Administration Lung Cancer Study Group (VALCSG)

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IL-7 (5'-TGAGAAGTGGTCTAATGTTGACATCGGTCAATT-3') and for β-actin (5'-GTGCTAAACAGGGAGGCCCAC-3') were both labelled at the 5' end with 6-carboxy fluorescein (FAM) and the 3' end with 6-carboxy-tetramethyl rhodamine (TAMRA). The IL-7 primer sequences were: sense 5'-TGAGAAC-TGAGAAGTGGTCCAGGCAAA-3', antisense 5'-CAATTCTTCTTCTGTTGCCTCAA-3'. The β-actin primer sequences were: sense 5'-CCCTTGATAAGTTCCATCTGACAAG-3', antisense 5'-AAGGTGTGGTGGCCAGATTTTC-3'. Reactions for IL-7 and β-actin quantification were performed in a 25 μl final volume with 2 μl of sample cDNA, 1X IQ Supermix (Bio Rad), 0.4 μM of each primer and 0.4 μM of the IL-7 probe. The amplification conditions for quantification were: 95°C for 15 minutes and 50 cycles of 95°C for 15 seconds and 58°C for 1 minute.

We tested a series of cell lines for the IL-7 expression and we chose, as positive control, MG63, an osteosarcoma cell line that expressed at high level IL-7. As negative control we used H522, a non-small cell lung cancer cell line. Relative IL-7 quantization in T and B lymphocytes, expressed as fold variation over control and over β-actin, was calculated after determination of the difference between CT (threshold cycle) of the given gene A (IL-7) and that of β-actin. A "42" method was used to perform the calculations [39]. Ct values are means of duplicate measurements. To validate the use of the 2^(-ΔΔCt) method, serial dilutions (ranging from 10^0 to 10^-5) of MG63 cDNA were used to obtain a standard curve.

Statistical analyses
Statistical analyses were performed with the Statistical Package for the Social Sciences (spssx/pcc software (SPSS, Chicago, IL). We compared the results by means of student's paired t test. The results were considered statistically significant for p<0.05.

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Author Contributions
Conceived and designed the experiments: IR RF. Performed the experiments: IR EG. Analyzed the data: IR GB MG SC RF. Contributed reagents/materials/analysis tools: LB RM ER DO LC AM. Wrote the paper: IR GB MG RF.

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