Serum interleukin-8 reflects tumor burden and treatment response across malignancies of multiple tissue origins

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Running title

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Foot note

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Statement of translational relevance

Tumor burden monitoring is critical in the assessment of response to treatment and the management of cancer patients. Radiological imaging is the most widely used and reliable technology for this purpose, but has important limitations when evaluating tumor response to antiangiogenic drugs and immunotherapy. Serum tumor markers are also useful in carcinomas of certain tissue origins. We found that IL-8 serum concentrations correlate with tumor burden in a variety of human solid
malignancies at baseline and following antitumor treatment, and accurately reflect viability of tumor cells, thereby providing a useful platform to evaluate responses to novel antiangiogenic and immunotherapeutic agents.
ABSTRACT

Background: IL-8 is a chemokine produced by malignant cells of multiple cancer types. It exerts various functions in shaping pro-tumoral vascularization and inflammation/immunity. We evaluated sequential levels of serum IL-8 in preclinical tumor models and in patients to assess its ability to estimate tumor burden.

Methods: IL-8 levels were monitored by sandwich ELISAs in cultured tumor cells supernatants, tumor-xenografted mice serum and in samples from 126 cancer patients. We correlated IL-8 serum levels with baseline tumor burden and with treatment-induced changes in tumor burden, as well as with prognosis.

Results: IL-8 concentrations correlated with the number of IL-8 producing tumor cells in culture. In xenografted neoplasms, IL-8 serum levels rapidly dropped after surgical excision, indicating an accurate correlation with tumor burden. In patients with melanoma (n=16), renal cell carcinoma (n=23), non small cell lung cancer (n=21) or hepatocellular carcinoma (n=30), serum IL-8 concentrations correlated with tumor burden and stage, survival (melanoma, n=16; renal cell carcinoma, n=23; hepatocellular carcinoma, n=33) and objective responses to therapy, including those to BRAF inhibitors (melanoma, n=16) and immunomodulatory monoclonal-antibodies (melanoma, n=8). IL-8 concentrations in urine (n=18) were mainly elevated in tumors with direct contact with the urinary tract.

Conclusions: IL-8 levels correlate with tumor burden in preclinical models and in cancer patients. IL-8 is a potentially useful biomarker to monitor changes in tumor burden following anticancer therapy, and has prognostic significance.
Introduction

Malignant cells and tumor stroma from diverse tissue origins biosynthesize and secrete the interleukin-8 (IL-8) chemokine (1,2), rendering this polypeptide a potential biomarker in cancer.

IL-8 (CXCL8) is a member of the CXC chemokine family with the ELR motif (3). It was originally identified as a proinflammatory chemotactic factor for neutrophils (4). Already known to enhance tumor cell growth and promote angiogenesis (5,6), more recently, IL-8 has been proposed to be a potent protumoral factor. In breast cancer and in glioblastoma it has recently been suggested that IL-8 directly favors cancer stem cells (7,8).

Because of its potent pro-inflammatory properties, IL-8 is tightly regulated, and its expression is low or undetectable in normal tissue. IL-8 expression is primarily regulated by activator protein 1 (AP1) and nuclear factor-κB (NF-κB) as transcription factors. Different stimuli have been shown to up-regulate IL-8 expression including inflammatory signals (i.e., tumor necrosis factor-α and IL-1β) or environmental stresses (i.e., hypoxia and chemotherapy agents). In contrast, steroid hormones (corticosteroids, androgens and estrogens) have been described to down-regulate IL-8 production (9).

Multiple mechanisms are apparently involved in the protumoral actions of IL-8. These include direct effects on endothelial cells, on malignant cell proliferation and migration, as well as indirect effects attracting host immune system cells such as macrophages and neutrophils. Myeloid cells in tumors produce secondary mediators that further promote tumor angiogenesis and growth (10,11). IL-8 exerts its functions through the CXCR1 and CXCR2 receptors. The absence of IL-8 in the mouse genome...
has hampered progress due to the fact that the mouse orthologues are not entirely equivalent, even though human IL-8 is at least partially functional on the corresponding CXCR1 and CXCR2 mouse receptors (12). Therefore the precise function of IL-8 is difficult to address with experiments in search of genetic evidence.

High IL-8 concentrations have been previously detected in serum or tissue specimens from cancer patients and correlated with tumor size, depth of infiltration, stage and prognosis. Reports in melanoma (13–15), non-small-cell lung cancer (NSCLC)(16,17), prostate cancer (18), esophageal squamous cell carcinoma (19), soft sarcoma (20) and bone sarcoma (21), renal cell carcinoma (RCC)(22), urothelial tumors (23,24), hepatocellular carcinoma (HCC)(25,26), colorectal cancer (CRC)(27), gastric cancer (28), pancreatic cancer (29), ovarian cancer (30), breast cancer (31), neurologic tumors (32), lymphoma (33,34) and others (5,35) are available, but its value as a cancer biomarker to predict changes in tumor burden and to monitor response to cancer treatment has been poorly explored.

All in all, preclinical and clinical data suggested that IL-8 could be a good candidate to accurately appraise the numbers of tumor cells producing this chemokine at any given time point. Due to a short serum half-life, serum concentrations ought to closely follow changes in IL-8-producing cells. Therefore, we performed this study to assess the correlation of IL-8 with tumor burden in various malignant diseases and its relation with tumor response following anticancer therapy. IL-8 could become a useful tool to evaluate response to different antitumor treatments, particularly to kinase inhibitors and immunotherapy, in which the value of tumor imaging is limited because of the complex biological mechanisms of action involved in these treatment modalities.
**Material and Methods**

**Patients**

One hundred twenty-six patients with confirmed diagnosis of solid tumors were included in this study from January 2003 to March 2014.

Tissue origins were melanoma (n. 27), RCC (n. 27), NSCLC (n. 24), HCC (n. 33), prostate cancer (n. 6), bladder cancer (n. 3), and others (n. 2). Clinical features are summarized in *Supplementary Table 1*. The protocol for the study was approved by local ethics committee (reference 111/2010) and all patients signed written informed consent.

Tumor burden was measured in all patients by physical examination or imaging studies, according to modified Response Evaluation Criteria in Solid Tumors (RECIST 1.1), with the modification that we measured all lesions and not only RECIST-target lesions. Simultaneously (± 2 weeks), we collected blood or/and urine samples from each patient.

For NSCLC and HCC we estimated tumor stage using the AJCC TNM 2007 edition (36), and the Barcelona Clinic Liver Cancer (BCLC) staging criteria (37) respectively.

To assess variations in IL-8 levels following treatment, we prospectively collected sequential blood samples at baseline and at the moment of evaluation of response from melanoma patients treated with BRAF inhibitors (iBRAF) and ipilimumab.
To assess variation in IL-8 levels following cytoreductive surgery, we prospectively collected sequential blood samples at baseline and 5-7 days after surgery in 7 patients. Clinical features are summarized in Supplementary Table 2.

**Sample collection and biochemical assays**

Peripheral blood samples were obtained by venipuncture (10 ml, BD Vacutainer® glass serum tube) and centrifuged (1,000 g, 15 minutes) to isolate serum which was stored at -80°C in aliquots until analysis. No previous freeze-thaw cycles were performed before analysis. Freezers were monitored and activity recorded in a quality controlled tissue bank that follows standard operation procedures.

LDH was analyzed by a kinetic spectrophotometric method using a kit from Roche on a Modular Analytics P800 analyzer (Roche) with results converted to 30°C.

C-reactive protein (CRP) was measured with an immunoturbidimetric assay using reagents from Roche (Roche Diagnostics GmbH, Mannheim, Germany) on a Hitachi modular P autoanalyzer (Roche/Hitachi).

Serum levels of IL-8 were measured by a commercial enzyme linked immunosorbent assay (ELISA) that detects both the monomer and dimer form (Human IL-8 ELISA set; BD Bioescience Pharmingen, San Diego, US), according to the manufacturer’s instructions. Serum levels of IL-6 were measured by commercial ELISA (Human IL-6 Set, BD Biosciences Pharmigen, San Diego, US) according to the manufacturer’s instructions. All samples were measured in duplicate. The detection cut-off levels of the assay were 3.1 pg/ml and 4.6 pg/ml for IL-8 and IL-6 respectively. The coefficient of variation was always <15%.
Urine samples were collected, centrifuged (1,000g, 15 minutes), supernatant aliquoted and frozen at -80°C until analysis. For analysis, samples were thawed on ice. No previous freeze-thaw cycles were performed before analysis. IL-8 levels were measured in duplicate at multiple serial dilutions using an ELISA specified for urine analyses following the manufacturer’s recommendations (Human IL-8 ELISA Kit, RayBio, RayBiotech, Inc., Norcross, GA).

The optimal cut-off point for LDH and CRP was established by adapting the kit specifications to our laboratory and were respectively LDH = 292 U/L and CRP = 0.3 mg/L. For serum cytokines we used different cut-offs in different malignant pathologies, calculated as a median of all IL-8 or IL-6 values obtained in melanoma, RCC and IL-8 values obtained in HCC. The calculated cut-off values were as follows: melanoma IL-8= 60pg/ml, IL-6= 4.6 pg/ml; RCC IL-8= 10 pg/ml, IL-6= 4.6 pg/ml; HCC IL-8= 40pg/ml.

**Cell lines and cell culture**

The HT-29 human CCR cell line was obtained from American Type Culture Collection (Rockville, MD, USA). UMBY and ICNI human melanoma cell lines were derived at the clinical facility Erlangen from primary surgical samples and were used at early culture passages. The Me275 human melanoma cell line was derived at the Ludwig Institute for Cancer Research, Lausanne Branch, from a surgically excised melanoma and was a kind gift from Dr. Pedro Romero. The 786-O human RCC cell line was kindly provided by Dr Luis del Peso (CSIC-UAM, Madrid, Spain). The human lung cancer cell line A549 was obtained from American Type Culture Collection, Manassas,
VA (cat. CRL-1573). The human hepatocellular carcinoma cell line HUH-7 was kindly provided by Dr. Brechot (INSERM, Paris, France).

**Tumor xenografted mice**

Rag2-/-IL2Rγc-/- mice were obtained from The Jackson Laboratory and bred at our institution. Animal experiments were conducted in accordance with Spanish laws and approval was obtained from the animal experimentation committee of the University of Navarra (reference 034/10 approval).

A total of 5x10^6 HT-29 cells, 2x10^6 HUH7 or A549 cells or 1x10^6 ICNI or UMBY cells were injected subcutaneously into the flank of Rag2-/-IL2Rγc-/- mice in 50 and 100uL of PBS respectively. Thereafter, we sequentially collected blood samples and measured the size of the tumor every three/four days. The blood samples were centrifuged (13,000 rpm, 10 minutes) and supernatant aliquoted and frozen at -80°C until analysis. Serum levels of IL-8 were measured as specified for human blood samples.

**RT-PCR assays**

Total cellular RNA was extracted from cells using Maxwell 16 simply RNA Cells Kit (Promega) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 2 µg of total cellular RNA with M-MLV reverse transcriptase (Invitrogen) in the presence of RNase Inhibitor (Invitrogen). Real-time PCR was carried out with iQ SYBR green supermix in an iQ5 real-time PCR detection system (Bio-Rad). The specific primers used were as follows: IL-8, forward primer 5'-CCAGGAAGAAACCACCGGA -3' and reverse primer 5'-GAAATCAGGAAGGCTGCCAAG -3'; and for β-actin, forward primer 5'-TCCCTGGAGAAGAGCTAACGA -3' and reverse
primer 5'-AGGAAGGAAGGCTGGAAGAG-3'. Samples were analyzed in triplicate and data were normalized by comparison with β-actin as an internal control. The amount of each transcript was expressed according to the formula $2^{Ct(b\text{-}actin)-DCt(IL8)}$, where $C_t$ is the cycle at which the fluorescence increases appreciably above background fluorescence.

**IL-8 mRNA assay in tumor biopsies**

Fresh samples were fixed with formol 10% and immediately after, the tissues were embedded in paraffin blocks. After cooling, the blocks were cut with a microtome making sections from 5 to 10 µm, which were mounted on a microscope slide.

After deparaffination, the samples were rehydrated and stained with hematoxylin to discern tumor tissue from healthy tissue. Then, the slides were washed with distilled water and DEPC-treated water to inactivate RNAse enzymes and with Glicerol 10% in TAE 10X. At this point, the pathologist selected the tumor area of each sample and scraped it with the aid of a fine needle.

Total RNA was extracted with the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer’s protocol. The concentration was quantified with a NanoDrop Spectrophotometer (NanoDrop Technologies). First-strand cDNA was synthesized from 0.5 µg of total cellular RNA with M-MLV reverse transcriptase (Invitrogen) in the presence of RNase Inhibitor (Invitrogen). Real-time PCR assay was performed as described above.
**Statistical analysis**

The non-parametric Mann-Whitney U-tests were applied to compare the concentrations of the tumor markers. Wilcoxon tests were used to compare changes in serum levels of tumor markers during treatment. Response and progressive disease were defined according with RECIST 1.1. Best response was defined as the best objective response (stable disease, partial response, complete response or progressive disease) assessed between the first day of treatment to progression, death or last follow-up. Overall survival (OS) Kaplan-Meier curves were appraised from treatment initiation or IL-8 determination to death or last date of follow-up and compared by the log-rank test. A two-tailed p-value ≤ 0.05 was considered to be statistically significant. Statistical analyses were performed with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).
Results

IL-8 quantitatively reflects the number of tumor cells

We determined concentrations of IL-8 in culture supernatants of 786-0, HT29, HUH7, A549 and Me275 cancer cell lines. Figure 1A represents IL-8 levels in relation to the absolute number of tumor cells seeded to culture wells 48h prior to harvesting the culture media. Importantly, quantitative data were consistent in all cell lines derived from different tissue origins, since 786-0 represents RCC, HT29 CRC, HUH7 HCC, A549 NSCLC and Me275 melanoma. The level of production seems to be an inherent feature of each tumor cell line, since repeated experiments rendered very stable results. However, the lack of linearity in the curves shows that other variables are determining the final outcome. The output of IL-8 into the supernatant is known to change in response to hypoxia. We confirmed that the mRNA encoding for IL-8 increased in CRC (HT29) and melanoma (UMBY and ICNI) cell lines under hypoxia (1% O₂) as shown in Supplementary Figure 1A.

In order to further substantiate the potential of IL-8 as a biomarker, we xenografted Rag2-/-IL2Rγc-/- mice with HT-29 tumor cells and monitored over time both tumor size and serum IL-8 concentrations. Figure 1B shows that IL-8 serum concentrations rose proportionally to subcutaneous tumor volumes. Tumors were surgically removed on day 18 or 26, and as early as 2h, post-surgery blood samples were drawn. As seen in Figure 1B, serum levels of IL-8 that had been rising during tumor progression abruptly dropped to undetectable levels within two hours. These xenograft experiments were also performed with UMBY and ICNI melanoma cell lines (Figure 1B) and with HUH7 (HCC) and A549 (NSCLC) cell lines (Supplementary Figure 1B) that more abundantly express IL-8. Serum IL-8 levels were higher, but the drop
following surgery was also rapid and dramatic, further suggesting the potential of IL-8 to monitor tumor burden in a variety of malignancies.

Our results were interpreted in the sense that IL-8 serum concentrations may allow to follow-up tumor burden also in humans. To explore in the real surgical practice scenario if serum IL-8 decreased following surgery, we have assessed IL-8 serum levels before (day -1) and 5-7 days after either curative or tumor-debulking surgery in a small series of patients (Supplementary Figure 2, Supplementary Table 2). Indeed, surgery induced a dramatic and statistically significant (p=0.01) reduction of IL-8 levels in all cases. Similar results have been observed in a series of ovarian cancer patients following debulking surgery (Dr. Frances Balkwill, personal communication).

**IL-8 serum concentrations indicate tumor burden and extent of disease in human patients**

Since IL-8 expression is shared by multiple human tumors of very different histological origins, we correlated IL-8 levels with tumor burden and disease stage in serum samples from cancer patients. To confirm that IL-8 is produced by tumor cells in some of our patients, IL-8 mRNA expression was determined in the biopsy of three melanoma patients, confirming detectable expression by the dissected malignant cells (Supplementary Figure 3).

To categorize tumor burden, we used in 16 melanoma and 23 RCC patients the median of cumulative tumor diameters (100 mm for metastatic melanoma patients and 150 mm for RCC patients) as the cut-off value to categorize high-tumor burden and low-tumor burden groups. As shown in Figure 2A, IL-8 concentrations were clearly higher in patients with high-tumor burden than in patients with low tumor burden.
(p<0.001 for metastatic melanoma and p<0.01 for RCC). IL-6 and CRP concentrations as biomarkers of inflammation were also quantitated in 14 melanoma and 19 RCC patients’ serum samples, showing a less significant correlation than IL-8 with burden of disease (IL-8 p<0.0001; IL-6 p<0.01; CRP p<0.001, Supplementary Figure 4). Indeed, the sensitivity and specificity of IL-8 to recognize patients with high tumor burden was superior than that of IL-6 and CRP (Specificity: IL-8 77%; IL-6 66%; CRP 33%. Sensitivity: IL-8 100%; IL-6 60%; CRP 100%).

We also correlated IL-8 levels with tumor stage in NSCLC (n=21) and HCC (n=30) patients. For NSCLC, IL-8 was almost undetectable in stage I and II patients, while it increased gradually in stage III and IV patients (p<0.01). As for HCC, IL-8 concentrations also were higher in patients presenting more advanced disease (p<0.01, Figure 2B).

**Objective responses to treatment can be assessed by monitoring IL-8 serum concentrations**

We assessed IL-8 and LDH serum levels in 16 melanoma patients treated with iBRAF before treatment, at best response and upon disease progression (Figure 3A). Clinical characteristics are depicted in Supplementary Table 1. Levels of IL-8 correlated with best clinical response (p<0.01), and with progressive disease (p<0.05). LDH levels also correlated with best clinical response, although with a lower significance (p<0.05), and did not correlate with progressive disease.

We also followed IL-8 serum levels in 8 patients with metastatic melanoma treated in our institution with ipilimumab 3 or 10 mg/kg up to four doses. One patient presented a partial response, 3 patients had stable disease and four progressed rapidly. IL-8 serum values decreased in patients benefiting from ipilimumab, while they
clearly rose in all the patients that progressed (Figure 3B). Overall survival correlated with variations in IL-8 levels (p=0.035) (inset Figure 3B). This finding is not restricted to ipilimumab immunotherapy since in an ongoing series of melanoma patients treated with anti-PD-1 mAbs a similar correlation between IL-8 levels and clinical response has been observed (data not shown).

**Overall survival correlates with serum IL-8 levels**

We assessed the relation between the overall survival of melanoma (n= 16), RCC (n= 23) and HCC (n= 33) patients with IL-8 serum levels. Clinical characteristics are described in Supplementary Table 1. We used the median concentration of IL-8 in each tumor type to categorize patients with high or low IL-8 levels (melanoma 60 pg/ml; RCC 10 pg/ml; HCC 40 pg/ml).

As seen in Figure 4, lower concentrations of IL-8 in serum clearly correlated with longer survival in all tumor types (melanoma; hazard ratio [HR]=0.21; 95% confidence interval [CI], 0.05-0.92; p=0.038/ RCC; HR=0.09; 95% CI, 0.01-0.4; p=0.004/ HCC; HR=0.2; 95% CI, 0.07-0.56; p=0.02).

**Urine IL-8 concentrations can be useful to monitor tumor burden in genitourinary malignancies**

We hypothesized that urine IL-8 levels could be used as a surrogate of blood levels. To study this possibility, we analyzed in parallel the serum and urine concentrations of IL-8 in healthy volunteers, in patients with solid tumors and in patients with urinary tract tumors that were in contact with the urinary tract, as assessed by CT-scans.
Figure 5 represents IL-8 concentrations in serum and urine. We divided the urine concentration by creatinine levels to correct for diuresis. The magnitude of tumor burden is shown between serum and urine concentration graphs. Healthy volunteers (n=12) had either undetectable or very low IL-8 concentrations in blood and urine. Eighteen cancer patients were studied. Clinical characteristics are summarized in Supplementary Table 1. Twelve patients with tumors that did not involve the urinary tract presented increased serum IL-8 levels that correlated with tumor burden (upper inset in Figure 5), but did not present high levels of IL-8 in urine. Accordingly, the correlation of serum and urine IL-8 concentrations was poor (lower inset in Figure 5). However, patients with tumors that were in contact with the urinary tract (n=6), presented very high concentrations of IL-8 in urine.
Discussion

IL-8 is a polypeptide produced by malignant cells of different histologic origins (1). Reasons for deregulated IL-8 biosynthesis in tumor cells are multiple and involve oncogenic transcriptional aberrations (5) and hypoxia (38).

We showed that in tumor cell cultures, the number of tumor cells correlates directly with the recovery of soluble IL-8 in culture supernatants. Moreover, in melanoma and colorectal cancer xenografts, IL-8 concentrations precisely correlated with tumor burden. This supports the hypothesis that IL-8 output is a relatively constant parameter for a single tumor cell and that IL-8 serum levels may accurately reflect the amount of tumor cells. Moreover, when tumor xenografts were excised, serum concentration of IL-8 dropped very rapidly, becoming undetectable in a matter of hours. This likely reflects the rapid renal clearance of IL-8, a consequence of its low molecular weight, which is under the filtration threshold (39). Finally, IL-8 significantly decreased in cancer patients 5-7 days after disease-reduction surgery confirming in patients the results observed in xenografted mice. All in all, our results are in line with previous reports in which stage or tumor load were correlated with serum IL-8 concentrations (18,19,21,28,31).

None of the healthy volunteers that we studied showed increased IL-8 in serum or urine. However serum IL-8 is not only increased in malignancies, since it is also an important acute inflammatory mediator which can increase upon infection or tissue damage (40), thus limiting its value as a biomarker to diagnose the presence of cancer. Therefore, we explored its potential to monitor the evolution of cancer patients.

In our study we made three important observations: (i) IL-8 serum levels correlate with tumor burden in several tumor types; (ii) IL-8 serum levels decrease or
increase respectively following changes in tumor burden upon clinical responses or disease progressions induced by novel treatments, such as iBRAF or ipilimumab, and such variations correlate with changes in tumor burden; and (iii) IL-8 concentrations in urine are increased in tumors that are in contact with the urinary tract, which we interpret as due to a direct output of IL-8.

Regarding the value of serum IL-8 to estimate tumor burden, we observed that there is a correlation between IL-8 serum levels and tumor size and cancer stage in patients with melanoma, RCC, NSCLC and HCC. These tumors lack adequate biomarkers that can be used in clinical practice, with the sole exception of alpha-fetoprotein (AFP) for a fraction of cases of HCC (41). Therefore the development of potential biomarkers for these tumors is an urgent unmet need. We also observed that patients with higher IL-8 levels presented decreased survival, in accordance with previous reports (25–27,29,31,42–44). While this fact may be explained by tumor burden, a contribution by a direct pathogenic role of IL-8 cannot be excluded, since IL-8 is involved in tumor vascularization (5,45) and chemotactically disorients migration of immune cells (12). Guida et al (46) have reported that IL-8 was unrelated with prognoses in metastatic melanoma patients, even though the group with the highest levels of IL-8 presented the lowest median overall survival. Other groups have reported lack of association of IL-8 and prognosis (34,47–49). Complexity of IL-8 functions, technical issues and/or characteristics of the selected patient populations may explain such discrepancies. Therefore, the correlation between serum IL-8 and prognosis deserves further investigation.

To assess response to therapy, a biomarker needs to reflect the amount of cancer cells in the organism. This could be useful to evaluate responses without the
need to perform imaging studies, or to interpret the occurrence of the “pseudo-progressions” that are observed with immunomodulatory antibodies (50). We observed that IL-8 serum levels decreased and increased respectively in patients that presented objective responses or progressive disease following treatment with iBRAF or ipilimumab. This is concordant with three previous reports where decrease of IL-8 levels during treatment showed association with improved response to classical chemotherapy and radiotherapy (51–53). In the case of iBRAF, it must be borne in mind that IL-8 is upregulated by the MAP-kinase pathway and hence decreases in IL-8 may also reflect the pharmacodynamic effects of iBRAF on the control of IL-8 transcription (54). Nonetheless, when patients eventually progress, IL-8 levels increase in spite of continuous therapy with iBRAF. In addition, our data in melanoma patients treated with ipilimumab and with anti-PD-1 blocking monoclonal antibodies (Melero I et al, unpublished data), which are not supposed to directly affect IL-8 transcription provide evidence for IL-8 as an independent biomarker that monitors the burden of tumor cells and thus can be used to monitor the progression of the disease and the response to therapy. In that way, IL-8 showed a better correlation with tumor burden in the progression of the disease in melanoma patients than LDH, the classical marker in this disease.

Urine determinations are more convenient for patients and health care providers. Previous reports have shown that IL-8 is increased in the urine of bladder cancer patients (55–57). Our data suggest that urine IL-8 concentrations warrant further investigation as a biomarker in tumors that are in direct contact with the urinary tract, including bladder and prostate cancer. However, according with our
results urine IL-8 does not seem to be a good marker of tumor burden in tumors that are not in contact with the urinary tract.

In conclusion, our study provides clear evidence that serum IL-8 levels correlate with tumor burden and prognosis in patients with different tumor types, and that, since IL-8 serum concentrations rapidly reflect changes in bioproduction by cancer cells, IL-8 may become a helpful biomarker to monitor the clinical activity of novel cancer therapies, such as iBRAF or immunomodulatory mAbs.

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References


Figure legends:

Figure 1. IL-8 production by cultured and xenografted tumor cells reflects tumor cell number.

(a) Concentrations of IL-8 in the tissue 48h-culture supernatants of the cell lines 786-0 (representing RCC), Me475 (representing melanoma) and HT29 (representing CRC) seeded at the indicated density. (b) Time course of the serum concentrations of IL-8 (upper panels) and tumor volume (lower panels) in Rag2-/-IL2Rγc-/- mice xenografted with the indicated cell lines. Individual mice are represented and at the time point indicated by the arrow the tumor was surgically removed. Serum IL-8 concentrations are depicted at 2h and 24h (ICNI and UMBI) or 2h, 12h, 24h and 72h (HT29) after surgery.

Figure 2: IL-8 serum concentrations reflect tumor burden and extension.

(a) Serum IL-8 concentrations in two series of prospectively banked samples from melanoma (n=16) and RCC (n=23) for which tumor burden was assessed by image studies and physical examination. High (≥100 mm melanoma; ≥150mm RCC) and low (<100mm melanoma; <150mm RCC) tumor burden patients were categorized showing different levels of IL-8 (median IL-8 high tumor burden: melanoma 412 pg/ml; RCC 54 pg/ml and low tumor burden: melanoma 19.57pg/ml; RCC 7.8 pg/ml). (b) Measurements as in A, in a series of serum samples from NSCLC (n=21) and HCC (n=30), in relation to AJCC and BCLC staging classifications respectively. The lines show the median value for each group and statistical comparisons were made with Mann-Whitney tests. (ns, non significant; *, p<0.05; **, p<0.01; ***, p<0.001).
Figure 3. IL-8 serum concentration reflects objective melanoma responses to iBRAF and an anti-CTLA4 mAb.

(A) Serum concentrations of IL-8 were measured in melanoma patients (n=16) treated with iBRAF (vemurafenib or dabrafenib) at baseline, at best response and at the time of progressive disease. The lines show the median value at each moment. Statistical comparisons were made using Wilcoxon tests (Left panel). LDH levels in the same patients also reflect objective response (Right panel). Squares represent nonresponding patients in which only progressive disease was observed, so their IL-8 and LDH levels are therefore only represented at baseline and at the moment of progressive disease. BR, best response; PD, progressive disease.

(B) Sequential follow up IL-8 serum in 8 metastatic melanoma patients treated with ipilimumab (at 3 or 10 mg/kg). Upper panels represent progressors at baseline (prior to treatment) and upon clinical-radiological evidence of progression (12-16 weeks after treatment onset). Lower panels represent four patients in whom stabilization of disease or partial response (as indicated in the corresponding graphs) were observed 12-16 weeks after treatment onset. At the bottom of each panel the time to death (TTD) is indicated in months. The inset shows a survival comparison of both groups (dotted line, patients with raised levels of IL-8; continuous line, patients with decreased/stabilized levels of IL-8). (ns, non significant; *, p<0.05; **, p<0.01).

Figure 4. Serum IL-8 predicts survival in a variety of malignant diseases.

Kaplan-Meier plots representing survival for patients with melanoma (n=16), renal cell carcinoma (RCC) (n=23) and hepatocellular carcinoma (HCC) (n=33) stratified by the
median levels of IL-8 for each tumor type. P-values were calculated using the log-rank tests and are shown in each graph.

**Figure 5. Serum and urine IL-8 in solid cancer patients, healthy volunteers and urogenital tumors.**

Serum (upper panels) and urine (lower panels) IL-8 concentrations were assessed in healthy donors (n=12), cancer patients (n=18) suffering carcinomas of the indicated color-coded histological origins. Numbers between graphs depict tumor size as the sum of longest lesion diameters of each individual. Patients represented in the right panels were CT-scan documented to host tumors in contact with the urinary tract (bladder or urethra) at the time of sample collection. Upper inset shows relation between tumor burden magnitude and serum IL-8 levels. Lower inset shows the relation between urine and serum IL-8 levels in cancer patients.
Figure 1A

![Graph showing IL-8 levels vs. absolute number of cells seeded/well for different cell lines.](image-url)
Figure 2A

**Melanoma**

* Graph showing melanoma data with tumor burden (sum of longest diameter) against IL-6 levels (ng/ml). The bars indicate significant differences with *** indicating p < 0.001.

**RCC**

* Graph showing RCC data with tumor burden (sum of longest diameter) against IL-6 levels (ng/ml). The bars indicate significant differences with ** indicating p < 0.01.
Figure 4.
Figure 5.
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