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## Chemokines in Ewing sarcoma

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# Chapter 3

## **Expression of CCL21 in Ewing sarcoma shows an inverse correlation with metastases and is a candidate target for immunotherapy**

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## ABSTRACT

Ewing sarcoma is an aggressive neoplasm predominantly occurring in adolescents and has a poor prognosis when metastasized. For patients with metastatic disease in particular, immunotherapy has been proposed as possible additive therapy with benefit. CCL21 activation-based immunotherapy was successful in preclinical studies in other tumor types, therefore we investigated CCL21 expression in EWS as potential target for immunotherapy. The *CCL21* RNA expression was determined in 21 Ewing sarcoma cell lines and 18 primary therapy-naïve Ewing sarcoma samples. In the tumor samples this was correlated to the number and CD4<sup>+</sup>/CD8<sup>+</sup> ratio of infiltrating T-cells and clinical parameters. Higher RNA expression levels of *CCL21* significantly correlated with a lower CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio (P=0.009), good chemotherapeutic response (P=0.01) and improved outcome (P<0.001). In patients with metastases *CCL21* expression was significantly lower than in patients without (P<0.0005). *CCL21* expression was significantly higher in Ewing sarcoma tissue samples as compared to cell lines (P<0.01), implying the involvement of a stromal factor. Protein expression analysis of CCL21 and its receptor CCR7 in 24 therapy-naïve tumor demonstrated overall no expression in Ewing sarcoma cells. In conclusion, CCL21 is expressed in clinical Ewing sarcoma samples by non-tumor infiltrating stroma cells. The observed positive correlation with survival implies that CCL21 might be a potential prognostic marker for EWS and marks the potential of CCL21 immunotherapy in EWS.

## KEYWORDS

bone tumor; soft tissue tumor; immunotherapy; tumor microenvironment; immune response

## INTRODUCTION

Ewing sarcoma (EWS) is the third most common primary bone sarcoma predominantly occurring in children and adolescents [1]. It is characterized by aggressive/destructive local growth and has a high-grade malignant behavior, with (micro-) metastases at the time of presentation being common. Patients with metastases or recurrent disease have a poor outcome with 15-30% long-term survival [2,3].

To date, after the initial introduction of multimodal chemotherapy, no further improvement in survival of these patients has been accomplished and besides the classical parameters such as tumor site, resectability, response to chemotherapy and size no prognostic markers are in clinical use for decision making. EWS contains very low number of mutations compared to other tumors, which suggests that corrective apoptosis pathways are still functional, like the TNF-related apoptosis-inducing ligand (TRAIL) pathway [4-6]. The death receptor pathways and other apoptotic pathways are active in EWS and consequently the tumor is sensitive for activation of these pathways by natural killer (NK)-cells and cytotoxic T-cells [7-9]. Immunotherapy in Ewing sarcoma has been shown to have a promising potential role in vitro and is being tested in two clinical trials by administrating donor NK-cells (NCT01287104, NCT02100891) [7,8,10].

We previously investigated the immune microenvironment in EWS and demonstrated a relation between the number of infiltrating cytotoxic T-cells and patient outcome [11]. Expression levels of pro-inflammatory chemokines (particularly CXCL9, CXCL10 and CCL5) correlated positively with the number of infiltrating CD8<sup>+</sup> T-cells [11]. Another potent T-cell

chemoattractant is CCL21, which acts via its receptor CCR7 as a single attractant or in combination with CXCL9 and CXCL10 [12,13]. In addition, CCL21 may increase dendritic cell-provoked T-cell responses, leading to more efficient anti-tumor immune responses [14,15]. As immunotherapy, the use of CCL21 has successfully been tested and a trial with dendritic cells expressing CCL21 showed even better results than CCL21 alone in non-small lung cancer [16]. Because of the immunogenic role of CCL21 and its immunotherapeutic potential, we studied the CCL21 expression in primary therapy-naïve Ewing sarcoma samples and EWS cell lines by analyzing the RNA expression levels of *CCL21*. The measured RNA expression levels were correlated with the number of infiltrating T-cells and the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio in Ewing sarcoma samples. A reversed CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio has been reported as predictor of improved outcome in other tumors [17,18]. In our study the CD4<sup>+</sup>/CD8<sup>+</sup>T-cell ratio showed inverse correlation with the *CCL21* expression level and increased *CCL21* expression levels were associated with better survival. This relation suggests that testing for CCL21 levels in therapy-naïve EWS tumor samples might be used as a prognostic marker and supports a potential role for this cytokine in anti-tumor immunity.

## MATERIALS AND METHODS

### Clinical information on patient samples

18 cryopreserved primary therapy-naïve samples from 18 EWS patients, all containing more than 80% tumor cells as assessed by light microscopy, and a validation tissue microarray of formalin fixed paraffin embedded (FFPE) specimens of 16 tumors of 16 patients were obtained from the Department of Pathology, Leiden University Medical Center and were handled in a coded fashion, according the Dutch national ethical guidelines ('Code for Proper Secondary Use of Human Tissue'). Ewing sarcoma diagnosis was established according to WHO criteria, including immunohistochemistry (IHC) and *EWSR1* translocation detection either by real-time quantitative-reverse transcriptase PCR (RT-Q-PCR) or interphase FISH. Good chemotherapeutic response was defined by less than 10% morphologically viable tumor cells upon histopathologic evaluation of the post-chemotherapy resection specimen [20,21]. Median patient age at diagnosis of the cohort was 17.5 years (range of 5-35 years) (**Table S1**).

### Ewing sarcoma cell lines

Ewing sarcoma cell lines (n=21) were obtained from multiple sources: L-1062 and L-872 were established in-house [22]; CHP100, RM-82, IARC-EW7, TC32 and 6647, CHP100, RM-82, IARC-EW-7, WE-68, IARC-EW-3, STA-ET-2.1, TTC-466, STA-ET-10, CADO-ES1, TC-71, VH-64, COH and STA-ET-1 were obtained from the EuroBoNeT consortium collection (Institute of Pathology, University Medical Center, Düsseldorf, Germany) [23] and SK-ES-1, SK-NM-C, A-673 and R-D-ES from the American Type Culture Collection (ATCC). All cell lines and primary culture L-4027 were cultured in a monolayer under equal conditions and in Iscove's Modified Dulbecco's Medium containing GlutaMAX supplement, supplemented with 1% streptomycin/penicillin and 10% heat-inactivated FCS (all from Life Technologies, Bleiswijk, The Netherlands). Authentication of cell lines using Powerplex 1.2 and CellID STR (Promega, Leiden, The Netherlands) and mycoplasma DNA Q-PCR screening were regularly performed on all cell lines.

### RNA isolation

Total RNA was isolated using TRIzol Reagent (Life Technologies, Bleiswijk, The Nether-

lands) according to manufacturer's instructions. RNA concentration was measured using Nanodrop and quality of the RNA was determined using Bioanalyzer2000 RNA Nano chip (Agilent Technology, Amstelveen, The Netherlands). Samples with a RNA integrity number  $\geq 5$  were included for RT-Q-PCR analysis.

### RT-Q-PCR analysis and Fluidigm

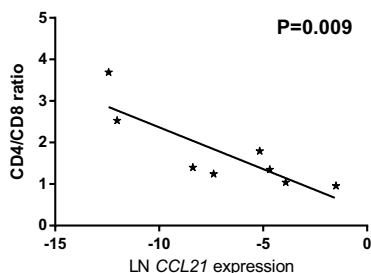
cDNA generation and RT-Q-PCR using Fluidigm BioMark system was performed according to the H format protocol of the manufacturer (QIAGEN, Venlo, The Netherlands). Samples were prepared for RT-Q-PCR using a 96x96 dynamic array chip and performed using BioMark HD system (Fluidigm, San Francisco, CA, USA). All primers for this array chip were obtained from QIAGEN (Venlo, The Netherlands) including nine control genes: RPL13A, BTF3, YWHAZ, UBE2D2, ATP6V1G1, IPO8, HBS1L, AHSP and TBP. Samples were measured in duplicates and analyzed using BioMark software, delivered with the HD system.

### Detection of infiltrating T-lymphocytes

Number of CD4 and CD8 positive T-cells were determined according to Berghuis *et al.* [11]. In brief, FFPE tumor sections were stained for CD3 (Dako, Heverlee, Belgium), CD4 and CD8 (Novocastra, Newcastle upon Tyne, United Kingdom) and scanned with Zeiss LSM-510 confocal microscope (Carl Zeiss AG, Göttingen, Germany). In each section 10 areas were selected, digital photographed and lymphocytes were counted.

### Immunohistochemistry

Tumor sections were stained with anti- CCL21 (Sigma-Aldrich, Steinheim, Germany) and CCR7 (Abcam, Cambridge, United Kingdom) antibodies. Extensive validation data for anti-CCL21 antibody (HPA051210) using IHC on various tissue microarray and western blot are accessible at the Human Protein Atlas portal [23]. Sections were dewaxed, rehydrated and were subjected to citrate pH6.0 (CCL21) or Tris-EDTA pH9 (CCR7) antigen retrieval. Sections stained for CCL21 expression were incubated with 5% ELK milk for 30 min at room temperature and incubated with anti-CCL21 (1:600) in 5% ELK overnight at 4 °C. Sections stained for CCR7 expression were incubated 1.5% BSA with anti-CCR7 (1:2000) overnight at 4 °C. Afterwards sections were incubated with Immunologic Poly-HRP-GAM/R/R IgG (Leica Biosystems, Eindhoven, The Netherlands) and Dako liquid DAB<sup>+</sup> Substrate Chromogen System (Dako, Heverlee, Belgium). Scanning of the slides was performed by Philips Ultra Fast Scanner (Philips Healthcare, Eindhoven, Netherlands). Tonsil tissues, both regular and



**Figure 1: Increased CCL21 RNA expression correlates with reversed CD4+/CD8+ ratio of infiltrating T-cells.** CCL21 RNA expression levels of samples with available high quality RNA and high quality FFPE material (n=8) were natural log transformed and correlated to the ratio between the total counted CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> infiltrating T-cells. P-value of the linear regression analysis is demonstrated.

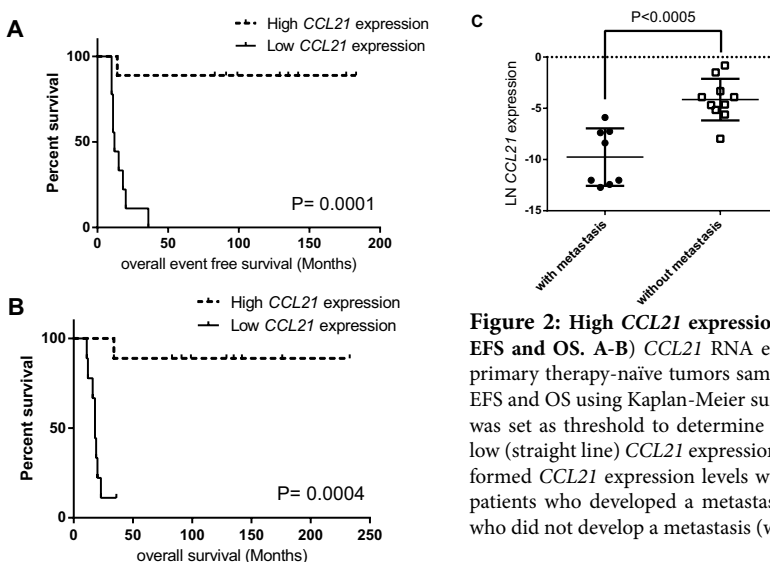
decalcified FFPE processed, were used as a control. All slides were evaluated by at least two experienced persons of which one was a reference pathologist (PCWH).

## Statistical analysis

Survival curves were calculated using the Kaplan-Meier method and P-values were calculated using the log-rank test using SPSS 20 (IBM Inc. Amsterdam, The Netherlands) and Prism Graphpad 6 (Graphpad Software Inc. La Jolla, CA, USA). Multivariate analysis of the parameters could not be performed due to the limited number of samples. Correlations were calculated with SPSS 20 using Pearson or Spearman correlation. Linear correlations were calculated with Prism Graphpad 6. High RNA expression was set as expression above the median. Student t-tests P-value was calculated using Prism Graphpad assuming non parametric distribution due to limited numbers of samples and was corrected using Manley-Welch correction.

## RESULTS

RNA expression of *CCL21* was analyzed in 18 primary therapy-naïve tumor samples and the expression levels were correlated with the immunohistochemical staining of the CD4<sup>+</sup> and CD8<sup>+</sup> infiltrating T-cells in eight tissue samples for which sufficient FFPE material was still available (Table S2). In these samples, the *CCL21* expression was inversely correlated to CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio (Figure 1). However, the absolute numbers of CD8<sup>+</sup> or CD4<sup>+</sup> T-cells did not correlate with *CCL21* expression and varied widely between the samples (data not shown). Since a high CD8<sup>+</sup> T-cells infiltration was associated in Ewing sarcoma with a better outcome, we correlated *CCL21* RNA expression levels in therapy-naïve tumor samples with development of metastases, survival and chemotherapeutic response. Kaplan-Meier survival analysis demonstrated that an increased *CCL21* expression correlated significantly both with improved EFS and OS (P=0.0001; P=0.0004) (Figure 2A-B). Moreover, natural logarithm transformed *CCL21* expression was significantly higher in patient samples who did not develop a metastasis compared to patients who did (P<0.0005) (Figure 2C). However, no correla-

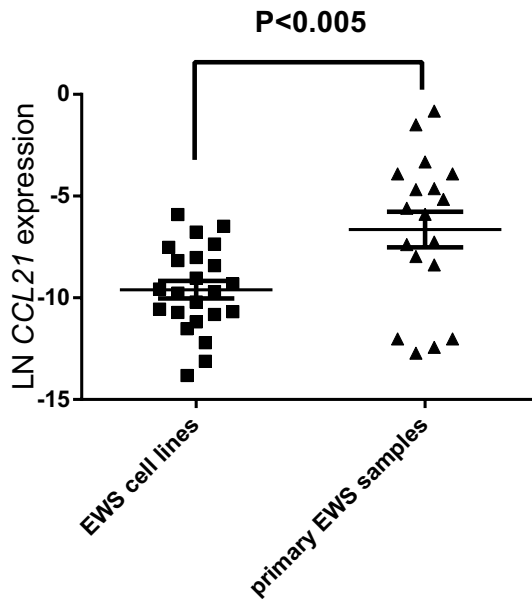


**Figure 2: High *CCL21* expression correlated to better EFS and OS.** A-B) *CCL21* RNA expression levels of the primary therapy-naïve tumors samples were correlated to EFS and OS using Kaplan-Meier survival analysis. Median was set as threshold to determine high (dotted line) and low (straight line) *CCL21* expression. C) Natural log transformed *CCL21* expression levels were compared between patients who developed a metastasis (with) and patients who did not develop a metastasis (without).

tion with metastasis at diagnosis was observed (data not shown). The improved survival may be linked to a better chemotherapeutic response as correlation between good response and increased *CCL21* expression was observed ( $P=0.02$ ). It should be noted that good response to chemotherapy was correlated with improved outcome ( $P=0.008$ ).

In addition, we investigated the *CCL21* RNA expression in 21 cell lines and 1 primary culture. The *CCL21* expression level in the cell lines was significantly lower than the in therapy-naïve tumor samples (Figure 3), with a large variation of expression levels between tumor samples as compared to cell lines.

To show that the expression difference of *CCL21* is derived from infiltrating immune cells in the tumor tissues we studied *CCL21* expression at protein level. The eight cases of which sufficient FFPE material was available were stained for *CCL21* using IHC. In addition, the tumor samples were stained for CCR7, the receptor of *CCL21*. In the tumor tissue Ewing sarcoma cells were negative for *CCL21* and CCR7 while infiltrating stromal cells did show expression of both *CCL21* and CCR7. An additional tissue micro array contacting 16 EWS cases was included for validation of the CCR7 and *CCL21* expression. Similarly to the other cases, all but one of the cases included in the TMA block were all EWS cells were negative for both CCR7 and *CCL21* (Figure 4).

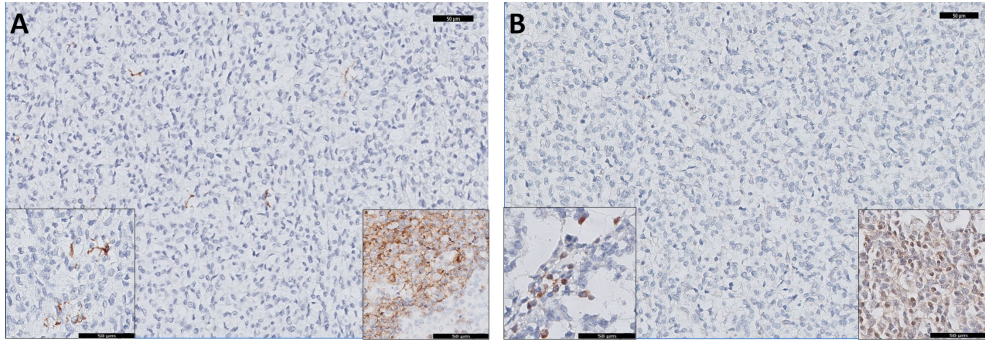


**Figure 3:** RNA expression levels of *CCL21* were significantly higher in tumors compared to cell lines. *CCL21* expression levels of 21 cell lines and 1 primary culture were compared to expression levels of the primary therapy-naïve tumor samples.



## DISCUSSION

Previously, we demonstrated that pro-inflammatory chemokines CXCL9 and CXCL10 were associated with an increase in tumor infiltrating CD8<sup>+</sup> T-cells [11]. CCL21 is, like CXCL9 and CXCL10, a CD8<sup>+</sup> T-cell chemoattractant and its potency is enhanced by the interaction with CXCL9, CXCL10 and interferon gamma (IFN $\gamma$ ) [13]. This prompted us to further investigate the role of CCL21 in Ewing sarcoma. We observed that an increased *CCL21* RNA expression was correlated with an decreased CD4<sup>+</sup>/CD8<sup>+</sup> ratio. It is likely that these CD3<sup>+</sup>CD8<sup>+</sup> positive



**Figure 4: Neither CCR7 nor CCL21 expression observed in Ewing sarcoma cells by immune-histochemical detection.** Eight tumor samples included in the RNA expression analysis and a TMA with 16 samples in duplicate were stained for CCR7 and CCL21 (20 times magnification). **A)** Tumor cells showed no expression, while infiltrating stromal cells showed expression of CCR7 (left inset, 40 times magnification), positive control is in the right inset from tonsil. **B)** Tumor cells showed no expression, while infiltrating stromal cells showed expression of CCL21 (left inset, 40 times magnification), positive control is in the right inset from tonsil. A scale bar of 50  $\mu$ m is included in the images.

lymphocytes are T-cells but the presence of CD3<sup>+</sup>CD8<sup>+</sup> NK T-cells cannot be excluded [24]. In addition, increased *CCL21* expression correlated with both better EFS and OS and inversely correlated with the development of metastasis. These observations may point to a role of *CCL21* in the anti-tumor immune response related to the proportion and type of immune cells present in or around the tumor in EWS patients [25-27]. Even though the presence of infiltrating immune cells in pediatric sarcomas, particularly in Ewing sarcoma, was found to be limited [28], the effect of these cells with regards to therapy response is significant.

A second factor which might have had an influence on the observed correlation with patient survival is the chemotherapeutic response of the tumor, which is known to be a marker for survival [29,20,30]. Patients with a good chemotherapeutic response had a higher *CCL21* expression in the tumor sample compared to patients with a poor response. Although it is generally believed that chemotherapy has an immunosuppressive effect by decreasing the number of leukocytes, by now it has become clear that certain chemotherapeutic agents can augment the tumor immunogenicity and stimulate dendritic cell maturation [31-33]. In mice combining *CCL21* immunotherapy with the chemotherapeutic agent paclitaxel had a synergistic effect [34]. *CCL21* attracts dendritic cells and is suggested to improve the T-cell activation of mature dendritic cells [35,14]. Increased expression of *CCL21* might be associated with increased number of dendritic cells or an improved immunologic response upon tumor cell death. In patients with *CCL21* producing cells present chemotherapy could enhance the antitumor immunity and would lead to a better chemotherapeutic response. We note that our

study based on a small patient cohort, therefore a larger study would be needed to validate the observed correlations using therapy-naïve samples.

The significantly higher *CCL21* expression in primary therapy-naïve tumors compared to EWS cell lines suggests the involvement of a stromal factor in *CCL21* expression. *CCL21* expression can be enhanced by the interaction with CXCL9, CXCL10 and IFN $\gamma$  [13]. However, we reported earlier the absence of *CCL21* expression in cell lines even after IFN $\gamma$  stimulation indicating that this might be regulated by the EWSR1-FLI1 transcription factor [11,3]. In this study some cell lines had, although low, *CCL21* expression. The difference in *CCL21* expression between tumor samples and cell lines might be not only caused by a stromal factor but could also be due to selective *in vitro* culture conditions. Therefore, the protein expression levels and localization of *CCL21* was determined in EWS patients samples using IHC and demonstrated that *CCL21* expression was restricted to tumor infiltrating cells and not present in Ewing sarcoma cells. In addition, only in one sample CCR7 expression was detected in EWS cells. EWSR1-ETS is known to downregulate many chemokines and chemokine receptors, directly or indirectly, for example by altering regulatory miRNA expression levels and pattern[3]. Of these the let-7 miRNA family is known to regulate expression of the *CCL21*-CCR7 [36]. The tumor suppressor let-7a is, for example, known to be directly downregulated by EWSR1-ETS but this decrease of let-7a does not lead to increased CCR7 expression like in breast cancer cells [37,38]. In several studies have investigated the role of the let-7 family in EWS and demonstrated increase of some members, mainly let-7g, in tumor samples. These studies also revealed various pathways in which these let-7 family members play a role, however none of them have could establish a direct connection between *CCL21* or CCR7 and let-7 [39-41,37,38].

It is important to note that high *CCL21* expression observed in the tumor cell reported in other tumors, for example bladder cancer and breast cancer, were found to be associated with an increased proliferation, number of metastases and a suppressive immune reaction. These tumors expressed high levels of *CCL21* and/or CCR7, which might have led to paracrine or autocrine activation of a pro-tumorigenic *CCL21*/CCR7 axis [42,25,27]. However, as no *CCL21* expression was detected in the EWS cells and CCR7 expression only in one sample, an active *CCL21*/CCR7 axis in EWS cells is unlikely. In studies correlating *CCL21* and CCR7 expression not only the expression level but also the source tumor derived versus infiltrating stromal cells should be considered and recorded.

The potency of immunotherapy to treat EWS has been demonstrated by a number of studies [7,8,10,43]. *CCL21* is a chemoattractant for dendritic cells, cytotoxic T-cells and natural killer cells and can improve the immune response, therefore it has been tested as an immunotherapeutic in preclinical and clinical settings [15,34,16]. It has been tested as a single agent and combining it with chemotherapy can have a synergistic effect [34]. This might be true for EWS as well, considering the increased expression *CCL21* in patients with a good chemotherapeutic response. However, prior to administration of *CCL21* immunotherapy determination of CCR7 expression of in EWS samples may be needed, as increased expression of *CCL21* and CCR7 expression in tumor cells was found to have negative effect and, one out the 24 tested EWS samples showed high CCR7 expression. For this case *CCL21* administration might have resulted in an adverse effect, but further studies are needed to draw a firm conclusion on that. In addition, the potential of *CCL21* treatment in not *CCL21* primed tumors, meaning no *CCL21* expression was present, should be further investigated.

In conclusion, in this study we showed that patients with increased *CCL21* RNA expression have a better EFS and OS. In addition, protein expression of *CCL21* and its receptor CCR7

were overall not detected in EWS cells, indicating the absence of pro-tumorigenic paracrine and autocrine loops in most EWS samples. This tumor entity might therefore serve as a good target for an immunotherapy approach based on the use of CCL21. Furthermore, expression levels of *CCL21* might be used as a potential prognostic marker for survival.

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## REFERENCES

- 1 De Alava, E., Lessnick, S. L. & Sorensen, P. H. in *WHO Classification of Tumors of Soft Tissue and Bone* (eds C.D.M. Fletcher, J.A. Bridge, P. C. W. Hogendoorn, & F. Mertens) 306-9 (IARC, 2013).
- 2 Ladenstein, R. *et al.* Primary disseminated multifocal Ewing sarcoma: results of the Euro-EWING 99 trial. *J Clin Oncol* **28**, 3284-91, (2010).
- 3 Sand, L. G. L., Szuhai, K. & Hogendoorn, P. C. W. Sequencing overview of Ewing sarcoma: a journey across genomic, epigenomic and transcriptomic landscapes. *Int J Mol Sci* **16**, 16176-215, (2015).
- 4 Lawrence, M. S. *et al.* Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* **499**, 214-8, (2013).
- 5 Kontny, H. U. *et al.* Sensitivity of Ewing's sarcoma to TRAIL-induced apoptosis. *Cell Death Differ* **8**, 506-14, (2001).
- 6 Lissat, A. *et al.* Interferon- $\gamma$  sensitizes resistant Ewing's sarcoma cells to tumor necrosis factor apoptosis-inducing ligand-induced apoptosis by up-regulation of caspase-8 without altering chemosensitivity. *Am J Pathol* **170**, 1917-30, (2007).
- 7 Verhoeven, D. H. J. *et al.* NK cells recognize and lyse Ewing sarcoma cells through NKG2D and DNAM-1 receptor dependent pathways. *Mol Immunol* **45**, 3917-25, (2008).
- 8 Pahl, J. W. *et al.* Antibody-dependent cell lysis by NK cells is preserved after sarcoma-induced inhibition of NK cell cytotoxicity. *Cancer Immunol Immunother* **62**, 1235-47, (2013).
- 9 de Hooge, A. S. K. *et al.* Expression of cellular FLICE inhibitory protein, caspase-8, and protease inhibitor-9 in Ewing sarcoma and implications for susceptibility to cytotoxic pathways. *Clin Cancer Res* **13**, 206-14, (2007).
- 10 Evans, C. H. *et al.* EWS-FLI-1-targeted cytotoxic T-cell killing of multiple tumor types belonging to the Ewing sarcoma family of tumors. *Clin Cancer Res* **18**, 5341-51, (2012).
- 11 Berghuis, D. *et al.* Pro-inflammatory chemokine-chemokine receptor interactions within the Ewing sarcoma microenvironment determine CD8+ T-lymphocyte infiltration and affect tumour progression. *J Pathol* **223**, 347-57, (2011).
- 12 Lo, J. C. *et al.* Differential regulation of CCL21 in lymphoid/nonlymphoid tissues for effectively attracting T cells to peripheral tissues. *J Clin Invest* **112**, 1495-505, (2003).
- 13 Sharma, S. *et al.* SLC/CCL21-mediated anti-tumor responses require IFN $\gamma$ , MIG/CXCL9 and IP-10/CXCL10. *Mol Cancer* **2**, 22-, (2003).
- 14 Hong, C. Y., Lee, H. J., Kim, H. J. & Lee, J. J. The lymphoidchemokine CCL21 enhances the cytotoxic T lymphocyte-inducing functions of dendritic cells. *Scand J Immunol* **79**, 173-80, (2014).
- 15 Lin, Y., Sharma, S. & John, M. S. CCL21 Cancer Immunotherapy. *Cancers (Basel)* **6**, 1098-110, (2014).
- 16 Lee, J. M. *et al.* Phase I trial of trans-thoracic injection of CCL21 gene modified dendritic cells in human non-small cell lung carcinoma. *J Surg Res* **186**, 558, (2014).
- 17 Shah, W. *et al.* A reversed CD4/CD8 ratio of tumor-infiltrating lymphocytes and a high percentage of CD4(+)/FOXP3(+) regulatory T cells are significantly associated with clinical outcome in squamous cell carcinoma of the cervix. *Cell Mol Immunol* **8**, 59-66, (2011).
- 18 García-Martínez, E. *et al.* Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. *Breast Cancer Res* **16**, 488, (2014).
- 19 van der Woude, H. J. *et al.* Monitoring the effect of chemotherapy in Ewing's sarcoma of bone with MR imaging. *Skeletal Radiol* **23**, 493-500, (1994).

- 20 Picci, P. *et al.* Prognostic significance of histopathologic response to chemotherapy in nonmetastatic Ewing's sarcoma of the extremities. *J Clin Oncol* **11**, 1763-9, (1993).
- 21 Szuhai, K., Ijszenga, M., Tanke, H. J., Rosenberg, C. & Hogendoorn, P. C. W. Molecular cytogenetic characterization of four previously established and two newly established Ewing sarcoma cell lines. *Cancer Genet Cytogenet* **166**, 173-9, (2006).
- 22 Ottaviano, L. *et al.* Molecular characterization of commonly used cell lines for bone tumor research: A trans-European EuroBoNet effort. *Genes Chromosomes Cancer* **49**, 40-51, (2010).
- 23 Pontén, F., Jirstrom, K. & Uhlen, M. The Human Protein Atlas—a tool for pathology. *J Pathol* **216**, 387-93, (2008).
- 24 Kronenberg, M. TOWARD AN UNDERSTANDING OF NKT CELL BIOLOGY: Progress and Paradoxes. *Annu Rev Immunol* **23**, 877-900, (2005).
- 25 Tutunea-Fatan, E., Majumder, M., Xin, X. & Lala, P. K. The role of CCL21/CCR7 chemokine axis in breast cancer-induced lymphangiogenesis. *Mol Cancer* **14**, 35, (2015).
- 26 Shields, J. D. *et al.* Chemokine-mediated migration of melanoma cells towards lymphatics - a mechanism contributing to metastasis. *Oncogene* **26**, 2997-3005, (2007).
- 27 Shields, J. D., Kourtis, I. C., Tomei, A. A., Roberts, J. M. & Swartz, M. A. Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science* **328**, 749-52, (2010).
- 28 Vakkila, J., Jaffe, R., Michelow, M. & Lotze, M. T. Pediatric cancers are infiltrated predominantly by macrophages and contain a paucity of dendritic cells: a major nosologic difference with adult tumors. *Clin Cancer Res* **12**, 2049-54, (2006).
- 29 Picci, P. *et al.* Chemotherapy-induced tumor necrosis as a prognostic factor in localized Ewing's sarcoma of the extremities. *J Clin Oncol* **15**, 1553-9, (1997).
- 30 Huang, H.-Y. *et al.* Ewing sarcomas with p53 mutation or p16/p14ARF homozygous deletion: a highly lethal subset associated with poor chemoresponse. *J Clin Oncol* **23**, 548-58, (2005).
- 31 Zitvogel, L., Apetoh, L., Ghiringhelli, F. & Kroemer, G. Immunological aspects of cancer chemotherapy. *Nat Rev Immunol* **8**, 59-73, (2008).
- 32 Ma, Y. *et al.* Anticancer Chemotherapy-Induced Intratumoral Recruitment and Differentiation of Antigen-Presenting Cells. *Immunity* **38**, 729-41, (2013).
- 33 Emens, L. A. & Middleton, G. The Interplay of Immunotherapy and Chemotherapy: Harnessing Potential Synergies. *Cancer Immunol. Res.* **3**, 436-43, (2015).
- 34 Chen, P. *et al.* Low-dose paclitaxel improves the therapeutic efficacy of recombinant adenovirus encoding CCL21 chemokine against murine cancer. *Cancer Sci* **105**, 1393-401, (2014).
- 35 Murphy, P. M. Double Duty for CCL21 in Dendritic Cell Trafficking. *Immunity* **32**, 590-2, (2010).
- 36 Kim, S.-J. *et al.* MicroRNA let-7a suppresses breast cancer cell migration and invasion through down-regulation of C-C chemokine receptor type 7. *Breast Cancer Res* **14**, R14, (2012).
- 37 De Vito, C. *et al.* Let-7a Is a Direct EWS-FLI-1 Target Implicated in Ewing's sarcoma Development. *PLoS One* **6**, e23592, (2011).
- 38 Zhang, Z. *et al.* Let-7a Functions as a Tumor Suppressor in Ewing's Sarcoma Cell Lines Partly by Targeting Cyclin-Dependent Kinase 6. *DNA Cell Biol* **33**, 136-47, (2014).
- 39 Karnuth, B. *et al.* Differentially Expressed miRNAs in Ewing sarcoma Compared to Mesenchymal Stem Cells: Low miR-31 Expression with Effects on Proliferation and Invasion. *PLoS One* **9**, e93067, (2014).
- 40 Hameiri-Grossman, M. *et al.* The association between let-7, RAS and HIF-1 $\alpha$  in Ewing Sarcoma tumor growth. *Oncotarget; Vol 6, No 32*, (2015).
- 41 Sohn, E. J., Park, J., Kang, S.-i. & Wu, Y.-P. Accumulation of pre-let-7g and downregulation of mature let-7g with the depletion of EWS. *Biochem Biophys Res Commun* **426**, 89-93, (2012).
- 42 Mo, M. *et al.* CCL21/CCR7 enhances the proliferation, migration, and invasion of human bladder cancer T24 cells. *PLoS One* **10**, e0119506, (2015).
- 43 Berghuis, D. *et al.* Histone deacetylase inhibitors enhance expression of NKG2D ligands in Ewing sarcoma and sensitize for natural killer cell-mediated cytotoxicity. *Clin Sarc Res* **2**, 8, (2012).



## SUPPLEMENTARY DATA

Table S1: Clinical details of the primary therapy-naïve tumor samples.

Patient number	Age (yrs)	Sex	Primary tumor site	Metastasis at diagnosis <sup>a</sup>	Starting treatment protocol	Response to chemo <sup>b</sup>	Translocation	Metastasis later <sup>c</sup>	Re-lapse <sup>d</sup>	EFS Time (month)	EFS <sup>e</sup> OS Time (month)	OS <sup>f</sup>
L318	35	male	proximal radius	0	CESS86	1	EWS-FLI1	0	0	183	0	233
L463	24	male	thorax wall	0	CESS86	ND	EWS-FLI1	1	1	12	1	20
L469	19	female	distal fibula	0	EICES	0	EWS-FLI1	1	1	20	1	23
L513	11	male	pelvis	1	EICES	ND	EWS-FLI1	0	ND	18	1	18
L629	5	male	tibia + fibula	1	EuroEwing99	1	Clinical Ewing sarcoma *	0	0	135	0	135
L683	17	male	tibia	0	EICES	0	EWS-FLI1	1	0	10	1	16
L848	15	female	humerus	1	EuroEwing99	1	EWS-FLI1	0	0	142	0	142
L1034	18	male	pelvis	1	EuroEwing99	0	EWS-FLI1	1	0	11	1	18
L1098	10	male	femur	0	EuroEwing99	1	EWS-FLI1	0	0	129	0	129
L1220	19	male	os pubis	1	EuroEwing99	ND	EWS-FLI1	1	0	10	1	11
L1232	14	male	humerus	0	EuroEwing99	ND	EWS-FLI1	0	ND	14	1	34
L1379	13	male	fibula	1	EuroEwing99	0	EWS-FLI1	0	0	99	0	99
L1489	25	male	pelvis	0	EuroEwing99	1	EWS break by FISH	0	0	91	0	91
L1570	12	male	humerus	0	EuroEwing99	1	EWS-FLI1	0	0	83	0	83
L1722	18	male	humerus	0	EuroEwing99	1	EWS-FLI1	1	1	36	1	36
L2154	11	female	femur	1	EuroEwing99	1	EWS-FLI1	0	0	176	0	176
L2161	19	male	pelvis	0	EuroEwing99	0	EWS-FLI1	1	0	11	1	12
L2162	19	male	pelvis	1	EuroEwing99	ND	EWS-FLI1	1	0	15	1	19

ND: Not determined; EFS: Event free survival; OS: Overall survival <sup>a-c,d,e</sup> 1: Event reported or 0: No event reported; <sup>b</sup> 1: < 10% tumor vitality or 0 > 10% tumor vitality  
<sup>f</sup> 1: Dead or 0: Alive.

\*: a case with RT-Q-PCR negativity for *EWSR1-FLI1*, *EWSR1-ERG* in diagnostic settings, negativity for *BCOR-CCNB3* RT-Q-PCR and the lack of *EWSR1*, *FUS* and *CIC* split apart signals from FFPE sections. Clinical as well as morphological and immunohistochemically consistent with Ewing Sarcoma.

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**Table S2: Counts of T-cells and CCL21 RNA expression levels**

Patient	Normalized CCL21 RNA expression	Tumor infiltrating T-cells (cells/mm <sup>2</sup> )		
		CD3+CD4+	CD3+CD8+	CD4+/CD8+ ratio
L2162	0.000004	118	32	3.68
L1722	0.000006	96	38	2.53
L1220	0.00023	49	35	1.40
L1034	0.00063	61	49	1.24
L1570	0.0058	138	77	1.79
L2154	0.0093	155	115	1.35
L1098	0.020	26	25	1.04
L1489	0.22	91	95	0.96

