Immunotherapy by Gene Transfer with Plasmids Encoding IL-12/IL-18 is Superior to IL-23/IL-18 Gene Transfer in a Rat Osteosarcoma Model

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Abstract. *Background: Osteosarcomas are primary malignant tumors of bone or soft parts arising from bone-forming mesenchymal cells. Despite dramatic therapeutic advances, namely neo-adjuvant and adjuvant chemotherapy, progress is at a plateau. Cytokine-mediated gene therapy might represent a further advance in the therapy of the osteosarcoma. Materials and Methods: We transfected UMR 108 osteosarcoma cells with different plasmids encoding IL-12, IL-23, proIL-18 and ICE (Interleukin-converting enzyme). IFN-γ induction, which is known to induce antitumor effects mediated by the immune system, and cytotoxic effects of various cytokine combination were investigated. Results: Our results show that local secretion of IL-12 by UMR 108 cells led to an induction of cytotoxic effects mediated by mononuclear cells, which were enhanced by additional administration of recombinant IL-18. In contrast to IL-18, IL-23 showed a moderate increase of IFN-γ induction when transfected alone and could only slightly increase the IFN-γ induction mediated by IL-12. IL-18 enhanced IFN-γ induction when applied alone and was able to increase the IFN-γ production that was induced by IL-12. Conclusion: IL-23 seems to be a less effective immuno-therapeutic for adjuvant treatment of osteosarcomas than IL-12 and IL-18, when taking only IFN-γ induction into consideration.*

Osteosarcoma is a bone tumor developing mainly in adolescents and young adults. At presentation, the majority of these young osteosarcoma patients have pulmonary

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micrometastases and one-third of these patients will relapse with pulmonary metastases although the primary tumor has been treated with aggressive chemotherapy and surgery (1).

Recent advances in our understanding of the interactions between cytokines, tumor cells and the immune system have opened roads to new cytokine-based anticancer strategies. Several studies have shown that IL-12 and IL-18 have significant antitumor activity, such as reduction of tumor growth or even complete tumor regression, by activating various immune functions.

IL-12 is a structural heterodimer formed of two subunits with a molecular weight of 35 (p35) and 40 kDa (p40). Primarily produced by macrophages and dendritic cells, IL-12 has several antitumor effects being mediated by the activation of cytotoxic T lymphocytes, as well as natural killer cells, which finally induce IFN-γ expression (2-11). IL-12 is one of the first cytokines that eradicates established tumors by itself (12). Tumor regression is not exclusively coupled with an induction of IFN-γ. IFN-γ-antibodies do not completely abrogate the IL-12 effects [unpublished data] (4,13,14). IFN-γ enhances many antigen-nonspecific immune and non-immune mechanisms, which favor tumor regression. These include direct cytotoxicity in combination with TNF-α (15), decrease of cellular proliferation (15), induction of nitric oxide production (16) and inhibition of angiogenesis. *In vivo* experiments have shown that systemic or local application of IL-12 was followed by a reduction of tumor growth and spreading of metastases (17,18). However, particularly the systemic therapy with recombinant IL-12 showed major side-effects, including death, which have been reported recently from clinical trials testing its anticancer potential. Worth *et al.* have shown that nasal application of an adenoviral vector containing murine interleukin-12 eradicates osteosarcoma lung metastasis in mice (17).

IL-23 consists of a heterodimer of a 40 kD protein (p40), which is also a component of the heterodimeric IL-12, as described above and a protein designated p19 subunit. This p19 subunit has recently been identified by Oppman *et al.* when searching sequence databases with a compütionally derived profile of Interleukin-6 (19).This protein shares homology with members of the IL-6/IL-12 family and is closely related to the p35 subunit of IL-12. Like this p35 subunit of IL-12, p19 is poorly secreted when it is expressed alone and needs the co-expression of its heterodimerizing partner p40 for higher expression. Formation of biologically active IL-23 heterodimers requires the synthesis of both subunits within the same cell (19). IL-23 shows biological activities that are similiar to IL-12, such as a proliferative effect on T cells and natural killer cells and a stimulation of IFN-γ production by T cells, although its IFN-γ stimulation is described as moderate. To our knowledge an antitumor effect of IL-23 in contrast to IL-12 has not yet been described. The IL-12-dependent effects depend on activation of the transcription factor STAT 4. Since Oppman *et al.* described that IL-23 also activates the STAT 4 transcription factor, both cytokines share parts of the same pathway.

Interleukin 18 (IL-18), originally designated as IFN-γ-inducing factor, is a pleiotropic cytokine secreted by activated macrophages and Kupffer cells (8,20,21). IL-18 induces IFN-γ production in both T cells and NK cells and enhances Fas ligand expression on Th 1 and natural killer cells (8,22-25). It additionally enhances the cytotoxity of murine and human NK cells (11) and can inhibit tumor growth in some murine tumor systems (26, 27). Similarly to IL-1β, IL-18 is synthesized as a biologically inactive precursor protein lacking a typical signal peptide (20). In order to generate the active form of IL-18, pro-IL-18 needs to be cleaved by the IL-1β-converting enzyme (ICE), an intracellular cysteine protease (28). Cleavage of pro-IL18 by ICE facilitates secretion of the active form of IL-18.

The aim of this study was to establish osteosarcoma cells that produce bioactive IL-12, IL-18 and IL-23 and, furthermore, to demonstrate the effects of these cytokines on the enhancement of IFN-γ production by immuno-competent cells. Since the conventional systemic cytokine-based therapies need high dosage and show severe side-effects, we especially focused on the synergistic effects of these three different cytokines to possibly reduce unwanted actions in clinical application as an immuno-therapy addressing osteosarcomas.

Materials and Methods

Cell culture and cell line. The established rat osteosarcoma cell line UMR 108, which was obtained from the European Collection of Cell Culture, Salisbury, UK, was used for all experiments. Cells were grown in either DMEM or RPMI-1640 Medium supplemented

Figure 1. *Schematic representation of the constructs expressing rat IL-23 and IL-18. Abbreviations: CMV-Prom, Cytomegalovirus immediate early promoter; IL-12-p40-rat, rIL-23-p19, IL-18_rat, rnICE, cDNA of subunits of rat IL-23 and IL-18 + ICE; IRES, internal ribosome entry side of polio virus; SV40An, polyA signal of Simian Virus 40; SV40 PROM, immediate early promoter of Simian Virus 40; neo, neomycin- resistant gene which is used in eukaryotes for selection with neomycin (G418) and in prokaryotes for selection with kanamycin; ori, origin of replication of E.coli plasmid Col E1; An, Herpes Simplex Virus Thymidine Kinase Polyadenylation Signal.*

with 10% Fetal Calf Serum (Biochrom, Berlin, Germany), 2.5 μg/ml Amphotericin B and 10 μg/ml Gentamicin (both Gibco, Karlsruhe, Germany).

Isolation and production of stimulated mononuclear cells. Spleens taken from Wistar rats were cut into pieces and passed through a cell strainer (mesh size 100 μm). Mononuclear (MN) cells were isolated by a Ficoll-Paque gradient centrifugation (Pharmacia, Erlangen, Germany) and then plated.

For the later PCR amplification, MN cells were cultured with 1000 U/ml IL-2 and 100 U/ml IFN-γ for 24 h. The activated cells were lysed and mRNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany). mRNA was reversed transcribed using a cDNA Synthesis Kit (Promega, Madison, USA) with oligo-dT as primer.

PCR amplification. The rat IL-12 p35 and IL-12 p40 cDNA were amplified by polymerase chain reaction as described earlier (29).

For amplification of IL-18, ICE and the p19 subunit of IL-23, the following primers were used under standard PCRs-conditions with the PCR Core Kit (Boehringer, Mannheim, Germany).
IL-18:
IL-18_3': 5'-GGATCCTAACTTTGATGTAAGTTAG-3'
IL-18_5': 5'-GAATTCACCATGGCTGCAATACCAGAAGAAG-3'
ICE:
ICE_3': 5'-GGGCCCTAATGTCCTGGGAAGAGG-3'
ICE_5': 5'-CTCGAGACCATGGCCGACAAGGTCCTGAG-3'
p19 subunit of IL-23:
p19_3': 5'-GGGCCCTTAAGCCGTTGGCACTAAGGGCTCAGT-3'
p19_5': 5'-CTCGAGACCATGCTGGATTGCAGAGCAATAATACTC-3'
The final PCR products were analyzed by agarose gel electrophoresis and cloned into the TA cloning vector pCR 2.1 (Invitrogen, Groningen, The Netherlands). Individual clones were subsequently sequenced to confirm normal coding potential and were subcloned in the different expression plasmids.

Vector construction and cell transduction. Several different plasmid vectors were used in this study. In order to establish OS cells expressing bioactive IL-12(pCMVIL-12), IL-18(pCMVIL-18+ICE), proIL-18 (pCMVproIL-18) and IL-23(pCMVIL-23), we cloned the different genes into a plasmid that contains a neomycin-resistant gene and put the genes under the control of the CMV-promoter. In the case of pCMVIL-12, pCMV-IL18+ICE and pCMVIL-23, the different genes needed for the subunits are linked by an internal ribosome entry site of the poliovirus. The IRES fragment used in our constructs produces a ratio of 3:1 of the upstream gene compared to the second chain (30). The vector for IL-12 expression has been published earlier (29). The expression vectors pCMVIL-18+ICE and pCMVIL-23 are shown in Figure 1. As a control vector for interleukin expression, the same construct was used after omitting the different cytokine genes and religation of the vector (pCMV).

In every setting $1x10^5$ osteosarcoma cells (OS) were seeded in a 12-well plate and transfected with the different plasmids. Transfection was performed with Fugene® according to the instructions of the manufacturer (Boehringer). The transfection efficiency was assayed with the pEGFP plasmid (Fa.Clontech, Heidelberg, Germany). The supernatants of the different settings were harvested after 48 h and were subsequently used in the IFN-γ bioassay. For settings where the combination of cytokines was tested, we did not perform a dual transfection but mixed the supernatant of individual transfected cells in equal amounts.

Determination of cytokine expression by IFN-γ bioassay. Since we were mainly interested in the bioactivity and not the absolute protein levels, the bioactivity of the various cytokines was measured indirectly by determination of the resulting IFN-γ expression according to the method primarily described by Okamura *et al.* (8). In brief, rat MN $(1x10^6)$ were co-cultured with Concanavalin A (Con A,1.25 μg/ml) (Boehringer) in 24-well plates. After replating $1.5x10^5$ Con A-primed mononuclear cells, 200 ml of supernatant collected from the different groups were added to cell suspensions and cultured in 96-well plates for 24 h. The conditioned supernatants were collected after 24 h and assayed by ELISA (R&D Systems, Wiesbaden, Germany) to determine the content of IFN-γ .

Cytotox assay. The cytotox assay was performed using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, Mannheim, Germany), which quantitatively measures lactate dehydrogenase that is released upon cell lysis. We therefore transfected UMR 108 cells with various plasmids which have been described earlier, isolated and stimulated MN cells with ConA in the way described above. The supernatant of pCMV (negative control) and pCMVIL-12 transfected cells were collected and added to the ConA preactivated MN cells. As a positive control, we supplemented recombinant IL-12 (200 pg/ml) protein to exactly the same medium as the transfected cells were cultured in and this was also added to the ConA-activated mononuclear cells. We then performed the Cytotox assay according to the instructions of the manufacturer, using freshly passaged UMR 108 osteosarcoma cells as target cells and the different stimulated MN cells as effector cells.

Statistical analysis. The Student's *t*-test was performed to analyze the significance of the differences between control and experimental groups. Differences were considered significant at *p*<0.01.

Results

Transfection efficiency. The transfection efficiency of various transfection reagents was assayed in preliminary experiments with the pEGFP vector and determined by FACS analyses. Using Fugene in a DNA to transfection reagent ratio of 1:3 we observed the highest transfection rates. The average transfection efficiency of repeated experiments was 44.27%±6.32% for the used cell line (data not shown). This obtained efficiency was regarded sufficient for further transfection of the interleukin expression vectors in bioactivity assays.

Cytotox assay. Stimulated MN cells taken from Wistar rats as effector cells and naïve UMR 108 osteosarcoma cells as target cells were used in various ratios in this cytotoxity assay. As shown in Figure 2, the ConA pre-stimulated MN cells were able to induce a baseline cell lysis in all effector to target cell ratios without additional activation by the different cytokines. This cytotoxic effect could be enhanced when the MN cells were additionally stimulated with the supernatant of pCMVIL-12 transfected tumor cells or the recombinat IL-12 protein (200 pg/ml).

The combined use of rIL-18 and the supernatant of pCMVIL-12 transduced UMR 108 cells showed a significant increase in cytotoxic activity, compared with the single application of rIL-12 or after transduction with the pCMVIL-12 plasmid (*p*<0.01).

Expression of the various cytokine genes 48 hours after transduction. The expression of the different cytokine genes by UMR 108 cell cultures was indirectly measured by an INF-γ bioassay after transduction with the above-described cytokine vectors.

The co-expression of pro-rat IL-18 with rat ICE, but not proIL-18 alone, in UMR 108 cells results in the secretion of bioactive IL-18 protein. The UMR 108 cell line did not show any ICE mRNA expression by RT-PCR (data not shown). The transfection of the pro form of IL-18(pCMVproIL-18) without ICE co-expression did not lead to significant induction of IFN-γ levels in MN cells compared with the control group (*p*>0.01)[shown in Figure 3].

After transfection of UMR 108 with the pCMVIL-18+ICE plasmid, which leads to co-expression of proIL-18 and ICE, the supernatant of these transfected UMR 108 cells induced a significant increase in IFN-γ production by the MN cells compared to the negative controls (*p*<0.01). Specifically, IFN-γ production for pCMV *versus* pCMVIL-18+ICE was 1439.67±65.03 *versus* 2367±24.98 pg/ml. As expected, the co-transfection of ICE and pro-IL-18 cDNA was superior to the pro-IL-18 alone and resulted in enhanced bioactivity of IL-18 in the ICE-negative cell line UMR 108.
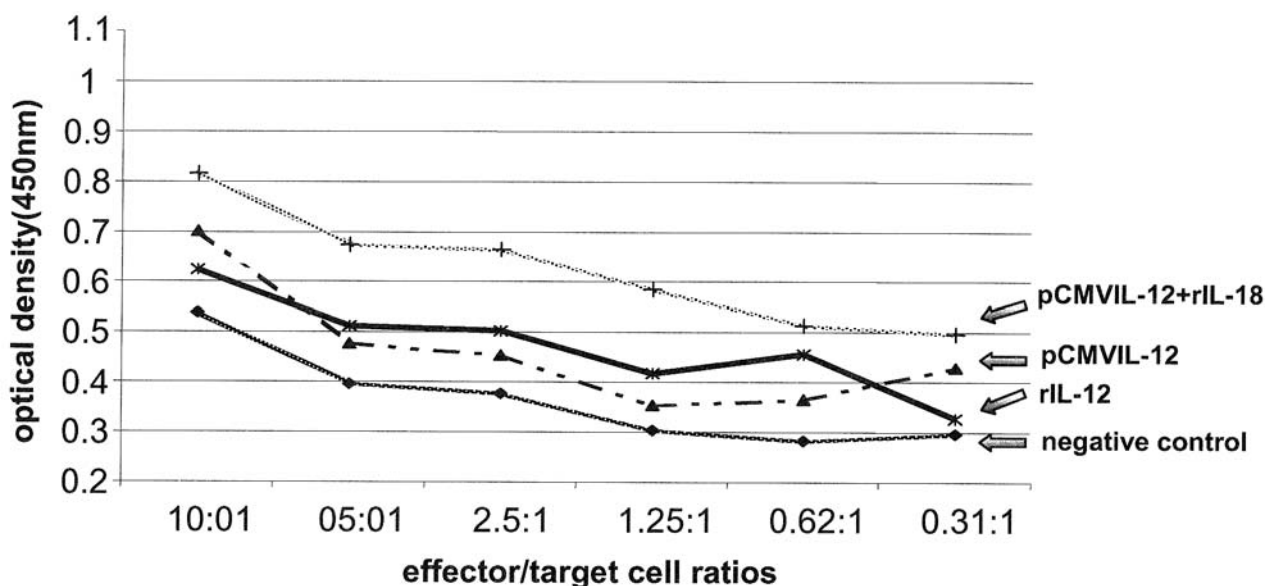
Figure 2. *The cytotoxic effects of the mononuclear cells after various cytokine stimulation towards the UMR 108 osteosarcoma cells are represented as optical density in different effector/target cell ratios.*
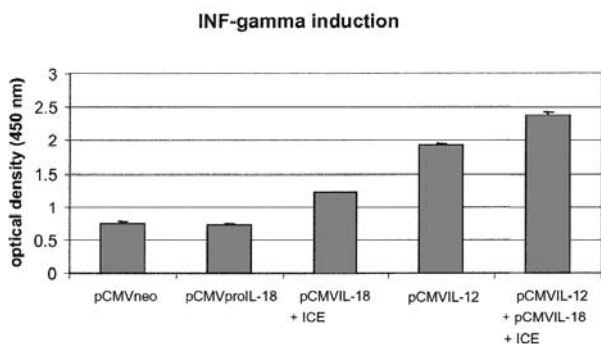

Figure 3. *The optical density in the bioassay represents the amount of IFN-γ being produced by MNCs in response to the IL-12 and IL-18 expression after transduction of the UMR 108 cells and to the combined application of both cytokines.*

The enhancement of IFN-γ induction that was achieved after transfection with pCMVIL-18+ICE was minor compared to the UMR 108 cells being transfected with the pCMVIL-12 vector (2367±24.98 pg/ml *versus* 3807±17.77 pg/ml). As expected, the combined use of both cytokines (IL-12 and IL-18+ICE) conditioned supernatants could further increase the IFN-γ production by the MN cells, resulting in a mean IFN-γ concentration of 4676.33±115.66 pg/ml. The difference compared to the single cytokine application was statistically significant ($p<0.01$).

As shown in Figure 4, the transfection of the osteosarcoma with the pCMVIL-23 vector gave only a moderate increase in IFN-γ expression by the ConA-activated MC cells and was less than the IFN-γ expression induced by pCMVIL-12. Compared to UMR 108 cells that have been transduced with pCMV, pCMVIL-23 transfection could raise the IFN-γ concentration in the supernatant of the MC cells from 1439.67±65.03 pg/ml in case of pCMV transfection to 2179.67±19.21 pg/ml for pCMVIL-23. In contrast to the combined application of IL-12 and IL-18, the IFN-γ induction of IL-12 alone could not significantly be enhanced by co-expression with IL-23 ($p>0.01$). We observed a slight increase in IFN-γ expression (3807± 17.77 IL-12 alone *versus* 3972.33±37.16 pg/ml IL-12+IL-23). The combination of IL-23 and IL-18+ICE showed that the IFN-γ production, which was observed in the single application of IL-23, could be enhanced by the co-expression of IL-18 ($p<0.01$), but was still less than the IFN-γ production after co-expression of IL-12 and IL-18+ICE (3474.33±206.11 pg/ml *versus* 4676.33±115.66 pg/ml). The measured IFN-γ concentrations from the IFN-γ bioassays are shown in Table I.

Discussion

We show that osteosarcoma cells that express bioactive IL-12 after a gene transfer with the IL-12 gene can enhance the cytotoxic activities of MN cells towards UMR 108 osteosarcoma cells. These cytotoxic effects were significantly higher after adding the recombinant IL-18 protein. It is
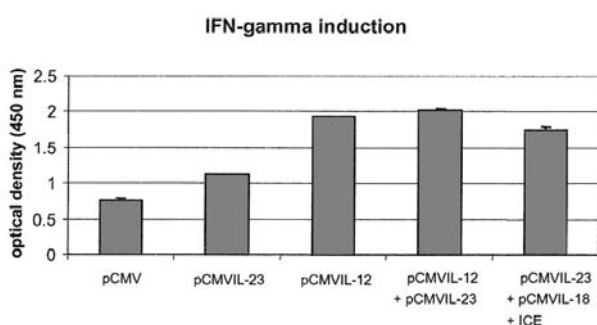
IFN-gamma induction

Figure 4. *IFN-γ induction measured in the IFN-γ ELISA is shown as optical density (450) after transfection of the UMR 108 osteosarcoma cells with pCMVIL-23, pCMVIL-12 and pCMVIL-18+ICE and activation of mononuclear cells.*

Table I. *All results obtained by ELISA from the IFN-γ bioassay are listed in Table I.*

Interferon-γ production by mononuclear cells.

Culture medium	IFN-gamma expression	Standard deviation
pCMV	1439.67	65.03
pCMVproIL-18	1401.67	24.03
pCMVIL-18 +ICE	2367	24.98
pCMVIL-12	3807	17.78
pCMVIL-12 + pCMVIL-18 +ICE	4676.33	115.66
pCMVIL-23	2179.67	19.22
pCMVIL-12 + pCMVIL-23	3972.33	37.17
pCMVIL-23 + pCMVIL-18 +ICE	3474.33	206.11

arguable that the MN cells, which are used as effector cells, are not derived from the same rat strain as the UMR 108 cells, so that there is a MHC mismatch which exhibits a lymphocyte reaction. But this reaction would be the same in all experiment at settings and is probably one reason for the baseline cell lysis seen in the negative control. The different ratios of cell lysis observed in the cytotoxity assay between the cytokine-stimulated effector cells and the negative control would not be affected by this MHC mismatch .

The data also presented in this study demonstrates that osteosarcoma cells (UMR 108) transfected with a plasmid encoding IL-23 could only slighty enhance IFN-γ expression by immuno-competent cells. In contrast to a combined approach with pCMVIL-12 and pCMVIL-18, addition of pCMVIL-23 resulted in almost no additional IFN-γ expression from MN cells to the combination with pCMVIL-12 or pCMVIL-18. We found, as in the case of IL-12, that the IFN-γ production of IL-23 could be increased when combined with IL-18. But this IFN-γ induction was minor compared to the combination of IL-12 and IL-18. The amount of IL-12 secreted within 48 hours by osteosarcoma cells transfected with pCMVIL-12 has been previously described (29).

IL-12 demonstrated significant antitumor activity in several animal models and therefore aroused interest as an antitumor therapeutic (12,31). The mechanisms underlying this antitumor activity are incompletely understood but may be related to the stimulation of T cells and NK cells, and be in association with IFN-γ production by these cells. IL-12 also shows anti-angiogenic properties and up-regulation of FAS, which is involved in cell mediated-apoptosis in different tumor cell lines (32). Some severe toxic effects from systemic rIL-12 therapy were reported when applied in a clinical trial for kidney carcinoma and other advanced malignancies (33). Even two deaths have been reported

after high dosage systemic therapy with rIL-12 (34). The two major drawbacks of the clinical use of rIL-12 are its short half-life, making a multiple-dose therapy necessary, and the high dosage of IL-12 needed to get an effective concentration at the tumor side.

IL-18 shows bioactivities that are similar to IL-12. It augments NK cell activity, induces IFN-γ production and also has an antitumor effect *in vivo* (27). IL-18 is able to enhance IFN-γ production even in the presence of saturating amounts of IL-12, which is due to the up-regulation of the IFN-γ-inducing factor receptor by IL-12 (23,35). The synergistic inhibition of tumor growth by a combined gene transfer of IL-12 and IL-18 cDNA has been described in several tumor models (14,36). We previously reported that administration of rIL-18 to genetically engineered IL-12-expressing osteosarcoma cells stimulates IFN-γ production to far higher levels than possibly achievable by IL-12 monotherapy. This synergistic action of IL-18 and IL-12 in IFN-γ production and NK cell activity has been previously described by other authors. IL-18 itself only slightly induces IFN-γ production by T cells because of their lack of IL-18 receptor expression, but IL-12 and IL-18 together exhibit a marked synergistic IFN-γ induction. This synergism is partially mediated by the induction of the IL-18 receptors by IL-12 and a reciprocal up-regulation of their receptors (34,37,38). Both cytokines also synergize at the transcriptional level of the IFN-γ gene expression by activating different transcriptional factors of the IFN-γ promoter.

Since IL-12 and IL-23 are closely related cytokines, we were interested whether IL-23 also has antitumor effects and if it shows the same synergistic effects in combination with

IL-18 on IFN-γ induction as IL-12 does. Oppman *et al.* showed that IL-23 could significantly enhance the production of IFN-γ by PHA blasts and that the maximal levels of IFN-γ production induced by saturating amounts of IL-23 were lower compared to those induced by saturating amounts of IL-12. According to these previous observations, we demonstrate that UMR 108 cells after transfection with the pCMVIL-23 plasmid show a lower IFN-γ expression from MNC cells than rat osteosarcoma cells after transfection with the pCMVIL-12 plasmid. We also tested the combined use of supernatant that has been conditioned by UMR 108 cells that were transfected with the pCMVIL-23 vector and supernatant of pCMVIL-12-transfected cells. In contrast to the results with the combined use of IL-12 and IL-18, we could not see a significant increase in IFN-γ induction with IL-12 plus IL-23. These observations may be due to the fact that IL-12 and IL-23 use, in some parts, the same signal transfection pathway.

We could also show that the moderate IFN-γ production which was induced by IL-23 could be increased when combined with the application of IL-18. However, still this effect was less compared to the combinatorial approach of IL-12 and IL-18. IL-12 and IL-18 show synergistic effects in the up-regulation of their receptors and in the transcriptional levels of IFN-γ expression.

Our results demonstrate that IL-23 can be regarded as a less effective antitumor therapeutic compared to IL-12, when taking only IFN-γ production into consideration. However, the mechanism underlying the antitumor activity of IL-12 includes not only IFN-γ induction of T and NK cells, but also the inhibition of angiogenesis and the up-regulation of FAS protein, which is related to cell-mediated apoptosis. So there is a need for further investigations of the new Interleukin-23 to learn more about its possible anti-tumor effects.

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