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DISSERTATION

T-cell mediated suppression of neuroblastoma following fractalkine gene therapy is amplified by targeted IL-2

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Zusammenfassung

Das Induzieren und Aufrechterhalten einer tumor-protektiven Immunität sind wesentliche Ziele in der Immuntherapie des Neuroblastoms. Eine Erhöhung der Anzahl von tumor-infiltrierenden Leukozyten könnte ein Weg sein, um dieses Ziel zu erreichen. Fractalkine ist ein besonderes T_H1 CX3C Chemokin, welches sowohl Adhäsion und Migration von Leukozyten vermittelt. Gerichtetes IL-2 (ch14.18-IL-2) wurde durch eine genetische Fusion von anti-GD2 Antikörper mit IL-2 hergestellt, damit IL-2 spezifisch in das Mikromilieu von Neuroblastomen gebracht werden kann.

In dieser Arbeit habe ich die Hypothese getestet, dass Gentherapie mit dem Chemokin Fractalkine (FKN) eine wirksame Antineuroblastom-Immunantwort induziert, welche durch gerichtetes IL-2 amplifiziert wird. Zu diesem Zweck wurden NXS2-Zellen genetisch verändert, damit sie murines FKN produzieren (NXS2-FKN). Transkription und Expression des mFKN Gens konnte in NXS2-FKN Zellen und Tumorgewebe gezeigt werden. Die chemotaktische Eigenschaft von FKN wurde sowohl in vitro als auch in vivo gezeigt.

FKN zeigte eine Reduktion des Primärtumorwachstums, welches durch gerichtetes IL-2 mit nicht-kurativen Dosen von ch14.18-IL-2 deutlich verbessert wurde. Ferner wurden experimentelle Lebermetastasen nur in den Mäusen komplett eradiziert, welche die Kombinationstherapie erhalten haben. Die Mechanismen, welche an dieser Antitumorantwort beteiligt sind, schließen eine wirksame T-Zell-Aktivierung (Hochregulation von CD69, CD25, und von TNF-alpha und INF-gamma), sowie eine Erhöhung der tumorspezifischen CTL-Aktivität mitein. Die Depletion von $CD4^+$ und $CD8^+$ T-Zellen in vivo hat diesen therapeutischen Effekt aufgehoben, was die essentielle Rolle von T-Zellen in diesem immuntherapeutischen Ansatz unterstreicht. Zusammenfassend konnte ich zum ersten Mal zeigen, dass Chemokin-Gentherapie mit FKN durch gerichtetes IL-2 amplifiziert wird, was eine Kombination dieser beiden Strategien zur adjuvanten Therapie beim Neuroblastom nahe legt.

Abstract

Induction and maintenance of tumor-protective immunity are the major goals of neuroblastoma immunotherapy. Enhancing the amount of tumor infiltrating leukocytes might be a way to achieve these goals since they may be associated with residual evidence of the ineffective immune response. Fractalkine is a unique T_H1 CX3C chemokine known to induce both adhesion and migration of leukocytes mediated by a membrane-bound and a soluble form, respectively. Targeted IL-2 (ch14.18-IL-2) was constructed by anti-GD2 antibody fused with IL-2 so that IL-2 can be directed into the microenvironment of neuroblastoma tumor.

Here, I tested the hypothesis that chemokine gene therapy with fractalkine (FKN) induces an effective anti-neuroblastoma immune response amplified by targeted IL-2. NXS2 cells were engineered to stably produce murine FKN (NXS2-FKN). Transcription and expression of the mFKN gene in NXS2-FKN cells and tumor tissue were demonstrated. The chemotactic activity of FKN expressed by NXS2 cells was determined both in vitro and in vivo. Importantly, NXS2-FKN exhibited a reduction in primary tumor growth, which was boosted by targeted IL-2 using non-curative doses of ch14.18-IL-2. Furthermore, experimental liver metastases were completely eradicated in mice receiving the combination therapy, demonstrating the induction of a long-lived tumor protective response. The mechanisms involved in antitumor response included effective T cell activation as indicated by the up-regulation of T-cell activation markers (CD69, CD25) and proinflammatory cytokines (TNF-alpha, INF-gamma) as well as the enhancement of tumor specific CTL activity. The depletion of $CD4^+$ and $CD8^+$ T cells in vivo abrogated the therapeutic effect supporting the crucial role of T cells in this immunotherapeutic approach. In summary, I demonstrated for the first time

that chemokine gene therapy with FKN is amplified by targeted IL-2 suggesting a combination of both strategies as an adjuvant therapy for neuroblastoma.

Schlagwörter:
> Fractalkine, Neuroblastom, ch14.18-IL-2,GD2, Gentherapie, Immuntherapie, T-Zellen

Keywords:
> Fractalkine, Neuroblastoma, Ch14.18-IL-2, GD2, Gene therapy, Immunotherapy, T-cells

Index

Abkürzungsverzeichnis

ABC	Avidin/Biotinylated Enzyme Complex
AEC	3-amino-9-ethylcarbazole
bp	base pair
CD	cluster designation
CO_2	carbon dioxide
cpm	count per minute
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
FACS	Fluorescence Activated Cell Scanning
FCS	fetal calf serum
FKN	fractalkine
FITC	fluorescein
GAPDH	Glycerol-Aldehyd-Phosphat-Dehydrogenase
GD2	ganglioside 2
h	hour
IL	interleukin
IFN-γ	interferon-γ
IRES	internal ribosome entry site
i.v.	intra venous
kDa	Kilodalton
LPS	Lipopolysaccharides
mFKN	murine fractalkine
MHC	Major Histocompatibility Complex
min	minute
NK cell	natural killer cell
PBS	phosphate buffered saline
PE	phycoerythrin
PMA	phorbol-12-myristate-13-acetate
RNA	ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction

Preface

This thesis summarizes findings with a novel therapeutic approach to neuroblastoma, combining gene therapy using the chemokine fractalkine (FKN) with targeted IL-2 (ch14.18-IL-2). Own contributions to this topic are documented by four publications provided in the appendix. They are listed under 'Own publications' and are referred to in the text using square brackets ([]). These findings were supplemented with yet unpublished results summarized in 12 figures reporting novel findings with this concept. Publications of other authors are cited in numerical order in round brackets (()).

1   Introduction

The effective treatment of stage 4 neuroblastoma is one of the major challenges in pediatric oncology, since its outcome remains poor, even after high dose chemotherapy and autologous bone marrow or stem cell transplantation. The development of an effective adjuvant immunotherapeutic strategy appears to be an important option to further improve the outcome of this neoplasm. New approaches are under investigation including passive immunotherapy with monoclonal anti-GD2 antibody ch14.18 [1]. Active immunotherapy is also a promising approach, such as DNA vaccination using tyrosine hydroxylase or MHC class I peptide ligands derived from tyrosine hydroxylase as a tumor antigen to induce an active host-anti-tumor immune response (1), [2], [3].

Based on the consideration that tumor-associated leukocytes are residual evidence of the host's ineffective antitumor immune response, a major goal of immunotherapy may be further accumulation and activation of such immune cells in the tumor microenvironment. Given the chemoattractive and stimulative properties of chemokines on different leukocyte subpopulations, therapeutic manipulation of the chemokine environment constitutes one strategy to stimulate protective responses by delivering chemokines to the tumor microenvironment at more relevant concentrations than could be given systemically.

There are approximately 40 chemokines identified to date, which can be classified into four groups according to the number of NH2-terminal cysteine motif: C, CC, CXC, and CX3C. Furthermore, chemokines can be distinguished between 'inflammatory' (alternatively called inducible) chemokines, such as SLC (secondary lymphoid tissue chemokine), and 'homeostatic' (alternatively called constitutive, housekeeping or lymphoid) chemokines, such as IP10 (interferon-inducible protein 10), based on the pathophysiological condition and the location of chemokine production as well as the cellular distribution of chemokine receptors.

Fractalkine (FKN), which is also called neurotactin, is the sole member of CX3C chemokine subfamily consisting of a CX3C motif with three amino acids between the two terminal cysteines. It is expressed predominantly by endothelial cells and its expression is both constitutive and inducible upon stimulation with TPA, LPS, TNF-α or IL-1 (2) (3).

FKN is different from other chemokines, since it exists in both a soluble and a membrane-anchored form. Following the predicted signal peptide (Fig 1, blue), FKN contains an N-terminal chemokine domain (Fig. 1, red, residues 1 to 76) with the unique 3-residue insertion between cysteines. Its structure is also characterized by a mucin like stalk (Fig. 1, purple, residues 77 to 317) with predicted O-glycosylated serine and threonine residues, providing for a distance between the membrane anchor and the chemokine domain. The transmembrane domain (Fig. 1, green, residue 318 to 336) and the intracellular domain (Fig. 1, pink, residue 337 to 373) constitute the anchor in the cell membrane.
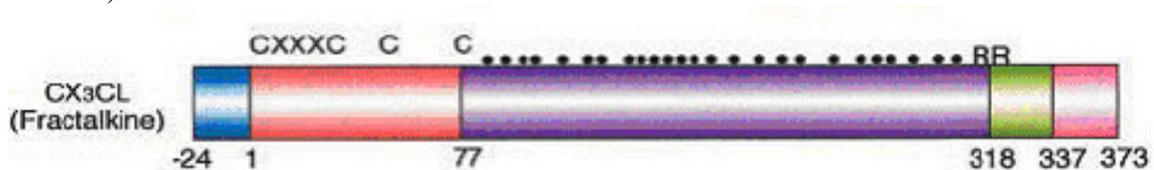

Fig 1. Schematic structure of FKN   (4)

Soluble FKN is released from the membrane bound version by proteolytic cleavage at a membrane-proximal region by the TNF-α-converting enzyme and exhibits the chemotactic activity to CX3C receptor positive cells in a way similar to other chemokines (5). In contrast to other chemokines, the membrane-bound FKN induces firm adhesion directly, rather than indirectly through selectins and integrins. In general, the transmigration of leukocytes from

the blood vessels into the surrounding tissue comprises several steps. It starts with the selectin-mediated interactions between leukocytes and the endothelium, which is followed by the activation of integrins on the leukocytes surface induced by chemokines, resulting in firm adhesion between leukocytes and the endothelium. Finally, leukocytes transmigrate through the endothelial layer in response to a chemokine gradient (6). As a result of the peculiar structure of FKN, it has a dual function of mediating both adhesion and migration and might directly mediate cell-cell interaction without involving selectins and integrins, which may represent a parsimonious solution to this complicated molecular event of leukocyte transmigration (7), (8). To date, FKN and the newly described CXCL16 are the only chemokines identified that have this kind of structure (9). This unique dual function may provide superior efficacy in therapeutic application of this chemokine in cancer immunotherapy.

Furthermore, FKN plays an exceptional role in polarized T_H1 type immune responses not only because of its unique structure but also on the basis of the following characteristics. First, it executes its multiple functions through the CX3CR1 receptor which is a seven-transmembrane protein containing several motifs conserved among the chemokine receptor superfamily. Importantly, CX3CR1 is expressed mainly on CD16$^+$ NK cells, CD8$^+$/CD3$^+$ T cells, CD4$^+$/CD3$^+$ T cells and CD14$^+$ monocytes (10). These cell populations are identical with cells migrating towards soluble FKN. Noteworthily, expression of CX3CR1 in both CD4$^+$ and CD8$^+$ T cells was strongly up-regulated by IL-2. Second, CX3CR1 is selectively expressed on various lineages of lymphocytes characterized by a high content of intracellular perforin and granzyme B including NK cells, γδT cells, and terminally differentiated CD8$^+$ T cells (11). These cytotoxic lymphocytes are the major effector cells against tumor cells. Third, T_H1 type cytokines and TH2 type cytokines have divergent effects on FKN expression. The FKN mRNA and protein expression can be induced by IL-1, TNF-α and INF-γ in human endothelial cells. Furthermore, INF-γ and TNF-α showed a synergistic effect in inducing FKN expression. In contrast, IL-4 and IL-13 do not induce FKN expression and even suppressed its induction by INF-γ and TNF-α (12). Moreover, the membrane-bound form of FKN can induce INF-γ produced by NK cells (13). In rheumatoid arthritis patients, peripheral CX3CR1$^+$/CD4$^+$ cells expressed INF-γ and TNF-α to a greater extend than CX3CR1$^-$/CD4$^+$, CX3CR$^+$/CD8$^+$ cells expressed INF-γ to a greater extend than CX3CR1$^-$/CD8$^+$ cells (14).

Based on these effects of FKN on providing for a T_H1 milieu, and the unique dual function of this chemokine, I selected this chemokine for immunogenetherapy of neuroblastoma.

However, the application of cytokine gene therapy used as a monotherapeutic approach has failed to translate the induction of tumor specific T-cells into objective clinical responses (15). Therefore, I expanded the efforts to combine FKN-gene therapy with a second immunotherapeutic strategy involving targeted IL-2, which was demonstrated to effectively amplify a suboptimal immune response following gene therapy with IL-12 (16).

The strategy of targeted IL-2 uses an antibody-cytokine fusion protein consisting of an anti-ganglioside GD2 antibody (ch14.18) [1] fused with interleukin-2 (ch14.18-IL-2), constructed by fusion of the synthetic sequence encoding for human IL-2 to the carboxy terminus of each IgG heavy chain of ch14.18. This construct can direct IL-2 specifically to ganglioside GD2, which is extensively expressed in neuroblastoma and melanoma. It was demonstrated in previous experiments that this immunocytokine, ch14.18-IL-2, is an effective agent in the treatment of murine melanoma through activating and expanding CD8$^+$ T cell (17). Furthermore, a long-lived protective immunity was demonstrated by a recombinant Ab-IL-2 fusion protein (huKS1/4-IL-2) in a murine carcinoma model (18). In addition, many investigators have clearly shown that IL-2 is of key importance for boosting the efficacy of anti-tumor immunity (19).

Here, the hypothesis was tested that the anti-tumor immune response induced by the increased production of FKN through transduction of the FKN gene, which may influence the

trafficking of resting and activated T cells, is amplified by ch14.18-IL-2 and subsequently, the development of tumor growth. I report a novel immunotheraputic strategy combining chemokine gene therapy with targeted IL-2 as a promising approach to neuroblastoma treatment. For this purpose, the murine FKN was cloned and expressed in the neuroblastoma cell line NXS2. Its chemotactic activity was determined both in vitro and in vivo. The antitumor effect of FKN combined with targeted IL-2 was demonstrated both for primary tumor growth and metastasis in syngeneic A/J mice. The main effector cells involved in induction of systemic immunity were indicated by the strongest T cell activation following the combination treatment. This was also demonstrated by up-regulation of T cell activation markers, T_H1 cytokines and CTL activity only in the combination group over all controls. In vivo depletion of $CD4^+$ and $CD8^+$ T cells abrogated the therapeutic effect, further supporting the pivotal role of these T-cell subpopulations in this antitumor immunity.

2 Material and Methods

Animals, cell lines, and the tumor model used for this study have been reported [2]. Furthermore, most of the methods used for the in vivo combination therapy strategy with FKN and targeted IL-2 including the construction of a plasmid encoding for mFKN and generation of a stable NXS2 cell clone expressing high levels of mFKN have been published [4]. New methods of unreported results are summarised in the following chapters.

2.1 FKN gene expression in NXS2 cells and neuroblastoma tissue was demonstrated by RT-PCR.

Briefly, total RNA from parental NXS2 cells, mock-transfected NXS2 cells (NXS2 cells transfected with pIRES empty vector) and FKN-transfected cells or primary neuroblastoma tumors formed by these three cell lines was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with SuperScript (Invitrogen, USA) and cDNA was then used for PCR. PCR amplification was accomplished by using Taq polymerase for 30-35 cycles (95°C for 1 min, 56°C for 1 min, 72°C for 90 s). Primers for FKN are: 5'- GCTAGCATGGCTCCCTCGCCGCTCGCG-3' (sense) and 3'- GAATTCTCACACTGGCACCAGGACGTA-5' (antisense). Primers for GAPDH which is used as an internal control are: 5'- CATTGACCTCAACTACATGG -3' (sense) and 5'- CACACCCATCACAAACATGG 3' (antisense). The PCR products were analyzed by agarose gel electrophoresis (1.2%).

2.2 FKN protein expression in vitro and in vivo

The secreted form of mFKN was measured by sandwich ELISA (R&D, USA) according to the protocol provided by the company. Cell culture supernatants were collected from 10^6 parental NXS2 cells, mock transfected cells and NXS2-FKN35 after 24h. The expression of membrane-bound mFKN protein was demonstrated by flow cytometry. 10^6 parental NXS2 cells, mock transfected cells and NXS2-FKN cells were incubated with goat anti-mouse FKN polyclonal antibody (M-18, Santa Cruz, CA) (1 µg/10^6 cells) primary antibody and FITC labeled anti-goat IgG (Calbiochem, San Diego, CA) secondary antibody (10 µg/ml).

In order to determine the FKN protein expression in vivo, primary neuroblastoma tumors were subjected to immunohistochemistry as previously reported [4].

2.3 Determination of the chemotactic activity of FKN expressed by NXS2 cells in vitro and in vivo

2.3.1 Migration assay

2×10^5 splenocytes were resuspended in 100µl of serum free RPMI medium and loaded on top of a 5-µm microporous transwell membrane in a 24-well plate (Boyden Chamber; Costar Corp, Cambridge, MA). The bottom of the chamber contained the supernatants collected from NXS2-FKN cells. The migration was compared to serum free medium and recombinant

mFKN (R&D, MN, USA) used as negative and positive control, respectively. After 6 hours incubation (37°C, 5% CO_2), transmigrated cells were manually counted in duplicate. In order to determine the specificity, functional blocking was performed by adding anti-FKN antibody (M-18, Santa Cruz, CA) into supernatants of FKN producing NXS2 cells at a final concentration of 2μg/ml and incubated for 1h at 37°C prior to the migration assay.

2.3.2 Immunohistochemistry

Tumor infiltrating leukocytes in primary tumors were determined 3 weeks after inoculation of 2×10^6 NXS2 parental cells, NXS2-mock cells, and NXS2-FKN cells in syngeneic A/J mice. Primary tumors were analyzed by immunohistochemistry as described [4]. Briefly, tumor tissues were cryosectioned into 5-μm slides and stored at –20°C. Slides were incubated with 2.5% blocking serum (goat serum, Vector, CA) and then were incubated with 1.25 μg/ml rat anti-mouse CD4 (RM4-5), CD8 (53-6.7) or CD45 (30-F11) (BDPharmingen, CA, USA), biotin labeled goat anti-rat IgG antibody (Calbiochem, San Diego, CA, USA) streptavidin-peroxidase (Elite ABC reagent, vector, CA). Slides were analyzed under light microscopy and quantification of infiltrating CD4^{+}, CD8^{+} and CD45^{+} cells was performed by counting 10 fields at a magnification of 400 x.

2.4 In vivo depletion of CD4^{+} and CD8^{+} T lymphocytes

In order to assess the role of CD4^{+} and CD8^{+} T cell subpopulation in the induction of a systemic tumor-protective immunity induced by FKN and ch14.18-IL-2, T cell subpopulations were depleted using anti-CD4 (Gk1.5) and anti-CD8 (53-6.7) antibodies in vivo. Depletion of CD4^{+} and CD8^{+} T-cells in these mice was accomplished by intraperitoneal injection of 200 μg of anti-CD4, anti-CD8 or PBS on the days –1, 7 and 14. Mice bearing NXS2-mock cells were used as negative control.

2.5 Cytotoxicity assay

Cytotoxicity was determined in a standard ^{51}Cr release assay. Briefly, 2×10^6 NXS2 target cells were labelled with 0.5 mCi sodium chromate 51 (PerkinElmer, MA, USA) for 2h at 37°C and seeded into flat bottom 96-well plates at a density of 5000 cells/100μl/well. Splenocytes isolated from each group were co-cultured with irradiated NXS2 (50 Gy, 15min) for 4 days and used as effector cells. Effector cells and target cells were added at various E:T ratios in triplicates to a final volume of 200 μl/well. Supernatants were collected after incubation (6h, 37°C 5% CO_2) and ^{51}Cr release was determined in a gamma counter (1470 WIZARD, PerkinElmer, MA, USA). Maximum release was induced with 10% SDS (10μl/well). MHC-class I restriction was determined by addition of anti-H-2K^K mAb (25 μg/ml, clone 36-7-5, BD PharMingen). Percent cytotoxicity was calculated using the following formular

$$\% \, lysis = \frac{experimental \; release\,[cpm] - maximum \; release\,[cpm]}{maximum \; release\,[cpm] - spontaneous \; release\,[cpm]} \times 100$$

2.6 Flow cytometry

Cell surface markers and intracellular cytokines expressed by splenocytes were examined by flow cytometry. Splenocytes were prepared as described for the cytotoxicity assay. Staining of surface activation markers was accomplished using 10^6 splenocytes, washed with FACS buffer (PBS, 0.1% BSA, 0.02% NaN3, PH 7.2) and incubated with 1 μg anti-CD3-FITC (145-2C11), anti-CD4-FITC (L3T4), anti-CD8-FITC (Ly-2), anti-CD4-PE (Gk1.5), anti-CD8-PE (53-6.7), anti-CD25-PE (3C7), and anti-CD69-PE (H1.2F3) (BDPharmingen, CA,USA) for 30 min at 4°C, respectively. Intracellular cytokines were analyzed after permeabilization of stimulated splenocytes. Briefly, cells (10^6) were stimulated in the presence of 50 ng/ml PMA, 1μg/ml ionomycin and 2μM monensine (Sigma, Munich, Germany) (6h, 37°C, 5% CO_2). After surface staining with 1 μg anti-CD4-FITC and anti-CD8-FITC (4°C, 30 min), cells were fixed with 1% paraformaldehyde in PBS at 4°C overnight, followed by intracellular staining

with anti-IFN-γ-PE (XMG1.2) and anti-TNF-α-PE (MP6-XT22) (BDPharmingen, CA,USA). Signals were measured with a FACS Calibur and analysed using CellQuest (Becton Dickinson, Mountain View, CA).

2.7 Statistics

The statistical significance of differential findings of in vitro assays and between liver weights of experimental groups of animals was determined by two-tailed Student's t test. The differential findings of hepatic metastasis scores of liver metastases between experimental groups was determined by the non-parametric Wilcoxon signed rank test. Findings were regarded as significant if two-tailed p values were <0.05.

3 Results

The following chapters summarize all the results obtained with this new immunotherapy concept. Novel, yet unpublished data are described in more detail (Fig. 3-12). The experimental design used throughout the manuscript is depicted in figure 2.
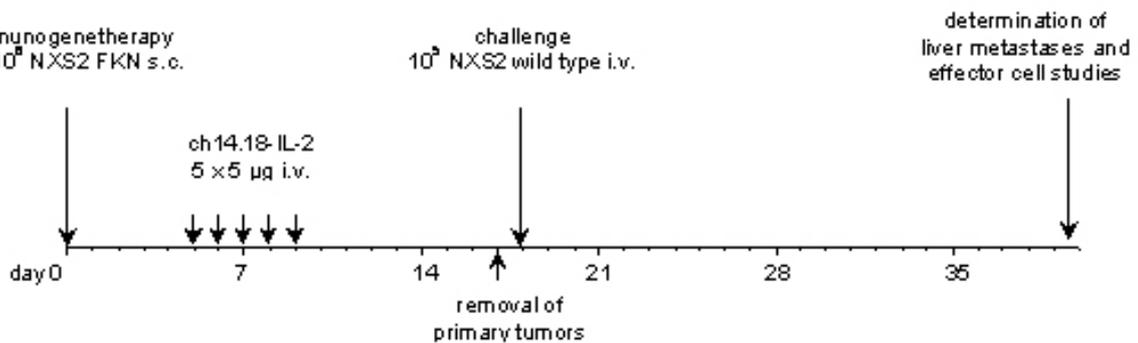

Fig. 2 Strategy of FKN gene therapy combined with targeted IL-2.

3.1 Construction of a mammalian expression vector encoding mFKN

The gene encoding for murine FKN was successfully cloned from the murine breast cancer cell line D2F2 by RT-PCR and subcloned into the mammalian expression vector pIRES, namely pIRES-FKN, using *NheI* and *EcoRI* restriction enzymes [4]. The mFKN sequence was verified by molecular sequencing.

3.2 Confirmation of the gene transcription and protein expression of mFKN in neuroblastoma cells and primary tumors

The successful transfection of pIRES-FKN into NXS2 cells and stable transcription of FKN in NXS2-FKN tumor tissue were confirmed by RT-PCR. The amplification of FKN cDNA (1118bp) was only found in NXS2-FKN cells in contrast to NXS2 parental and mock transfected controls (Fig. 3). Similar results were obtained from tumor tissue with NXS2-FKN cells in vivo [4]. GAPDH was used as a housekeeping gene (300 bp) to verify the integrity of RNA and cDNA preparations.
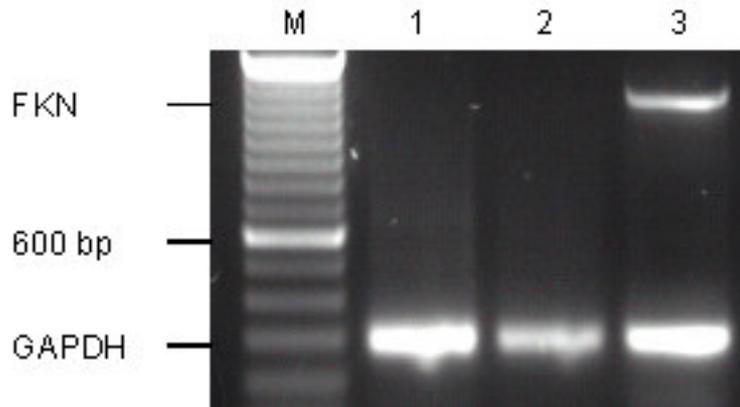
Fig. 3 Detection of FKN gene expression in NXS2 cell lines. NXS2 cells were stably transfected with a plasmid encoding for FKN (NXS2-FKN) as previously described [4] and subjected to gene expression analyzed by RT-PCR. Results were compared to NXS2 wildtype and NXS2 mock transfected control groups. The presence of a band at 1.1 kb indicates the expression of FKN. GAPDH was amplified as an internal control (0.3 kb). M: 100 bp ladder, Invitrogen. 1: NXS2 wildtype cells. 2: NXS2 mock transfected. 3: NXS2-FKN.

The expression of the FKN protein in the secreted and membrane bound form was assessed by ELISA (Fig. 4A) and FACS (Fig. 4B), respectively. In FKN-NXS2 bulk culture cells, the level of soluble form of FKN was quantified at a rate of 5.5±0.48 ng/ml/24h and flow cytometry demonstrated 31.32% of these cells express membrane bound form of FKN. After 2 rounds of subcloning, a NXS2-FKN subclone was obtained that showed a higher secretion rate of 14±1.04 ng/ml/24h and 75.32% cells expressed FKN bound to the cell surface. Furthermore, the continuous expression of the FKN protein was also determined by immunohistochemistry in NXS2-FKN tumor tissue in contrast to parental NXS2 and NXS2 mock-transfected controls [4]. These findings demonstrate efficient transcription and expression of FKN from my DNA construct in vitro and in vivo.
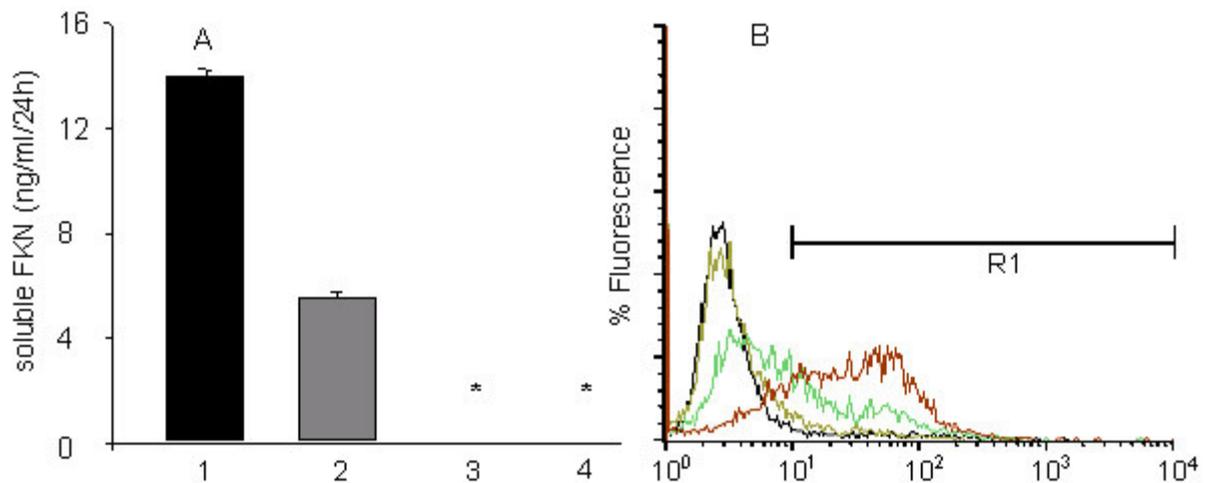

Fig. 4 Determination of the FKN protein expression by NXS2 cells. The FKN protein expression as a secreted and a membrane bound protein was determined by sandwich ELISA in the supernatant of cultured cells (A) and by flow cytometry on the cell surface (B). (A) The production of soluble FKN protein was quantified by a commercial ELISA in culture supernatants of 10^6 cells after 24h. Results indicate MV ± SD of FKN secretion rates in ng/ml/24h obtained from triplicate experiments. 1: NXS2-FKN 3rd generation subclone, 2: NXS2-FKN bulk culture, 3: NXS2 mock transfected cells, 4: NXS2 wildtype cells. Asterisks indicate non-detectable levels of FKN. (B) The presence of the membrane bound FKN protein was quantified by flow cytometry. Black: NXS2 wild type cells, yellow: NXS2 mock transfected cells, green: NXS2-FKN bulk culture, red: NXS2-FKN 3rd generation subclone.

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3.3 Determination of the chemotactic activity of FKN produced by NXS2 cells in vitro and in vivo

Chemotaxis mediated by supernatants from NXS2-FKN cells was tested in a boyden chamber assay in vitro, which revealed a maximum of 30% of migrated cells at a concentration of 47.6ng/ml (Fig. 5). This finding was in contrast to the negative control. This chemotactic activity can be partially blocked by adding anti-murine FKN Abs (2 µg/ml) to supernatants suggesting that it was specifically mediated by FKN in this assay. Recombinant FKN from R&D company served as a positive control. Interestingly, migration included recombinant FKN followed a 'bell-shaped' characteristics with a decrease at higher concentration, a phenomenon known, but not well understood in chemotaxis assay.
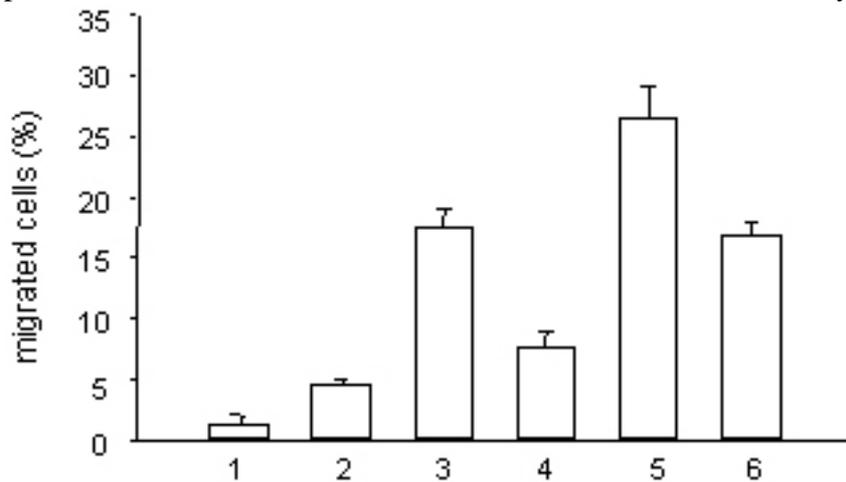

Fig. 5 FKN mediated chemotaxis in vitro. FKN mediated chemotaxis was determined in a boyden chamber assay using 2×10^5 splenocytes (37°C, 5% CO_2, 6h). The total number of transmigrated cells was determined microscopically. The data are expressed as percent of transmigrated cells and represent MV ± SD of triplicate experiments. 1: serum free NXS2-mock supernatant (negative control), 2: recombinant murine FKN 12,5 ng/ml, 3: 20 ng/ml, 4: 50 ng/ml, 5: serum free NXS2-FKN supernatant, 6: serum free NXS2-FKN supernatant plus 2 µg/ml anti-mFKN mAb (M18).

In order to determine FKN mediated chemotaxis in vivo, the migration of leukocytes into primary tumors was analyzed by immunohistochemistry. Primary tumors were induced by s.c. injection of 2×10^6 NXS2-FKN, NXS2-mock, NXS2 parental cells. Immunohistochemistry was performed 16 days after tumor cell inoculation. Effective migration of leukocytes into FKN producing primary tumors was clearly demonstrated, indicated by a factor 3 increase over the NXS2 wild type and mock transfected controls (Fig. 6A 6B). Interestingly, the highest increase in migration was observed in the CD8^{+} T-cell subpopulation (factor 7).
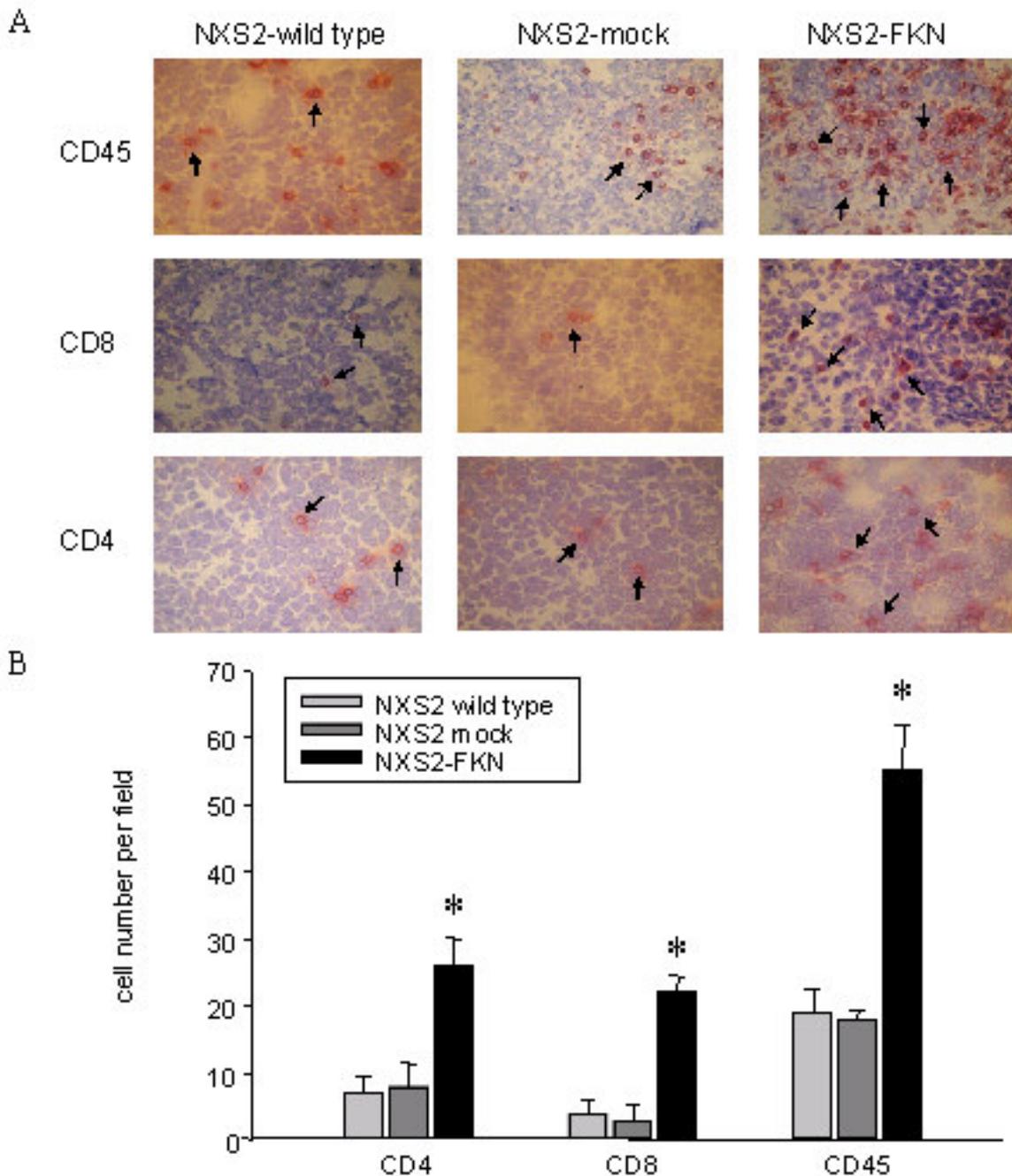
Fig. 6 Analysis of tumor infiltrating lymphocytes following FKN immunogenetherapy. The chemotactic activity of FKN produced locally in the tumor microenvironment for distinct lymphocyte subpopulations was determined by immunohistochemistry. (A) Cryosections of primary tumors induced with NXS2 wild type, NXS2 mock transfected and NXS2-FKN cells were stained with mAbs specific for CD45 (pan leukocyte marker), CD4 and CD8 (T-cell subpopulations). Each panel shows photographs taken at 400x of representative areas within distinct primary tumors. Black arrows indicate infiltrating cells with characterisitc red membrane staining. (B) The number of tumor infiltrating cells was quantified by counting the total number of infiltrating cells per high power field (HPF) at 400x. Bars represent MV ± SD of ten HPFs. The differences between mice receiving NXS2-FKN cells and all control groups were statistically significant (*p<0.01).

3.4   Effect of targeted IL-2 with ch14.18-IL-2 on FKN gene therapy

The effect of targeted IL-2 on FKN gene therapy was first evaluated on primary tumor growth as previously described [4]. Transfection of NXS2 cells with FKN induced a limited decrease of tumor growth rate (Fig. 7A) and tumor weight (Fig. 7B). This finding was in contrast to the simultaneous administration of ch14.18-IL-2, which resulted in a marked inhibition of primary tumor growth. Importantly, one third of mice showed a complete tumor rejection

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only in the FKN/ch14.18-IL-2 combination group. The effect of ch14.18-IL-2 was specific since a non-specific control using ch225-IL-2 had no additional effect on FKN gene therapy as indicated by an average tumor size of 297.7 ± 35.1 mm^3, which is not different from the average tumor size of FKN monotherapy at 315.3 ± 67.3 mm^3 ($p > 0.1$).
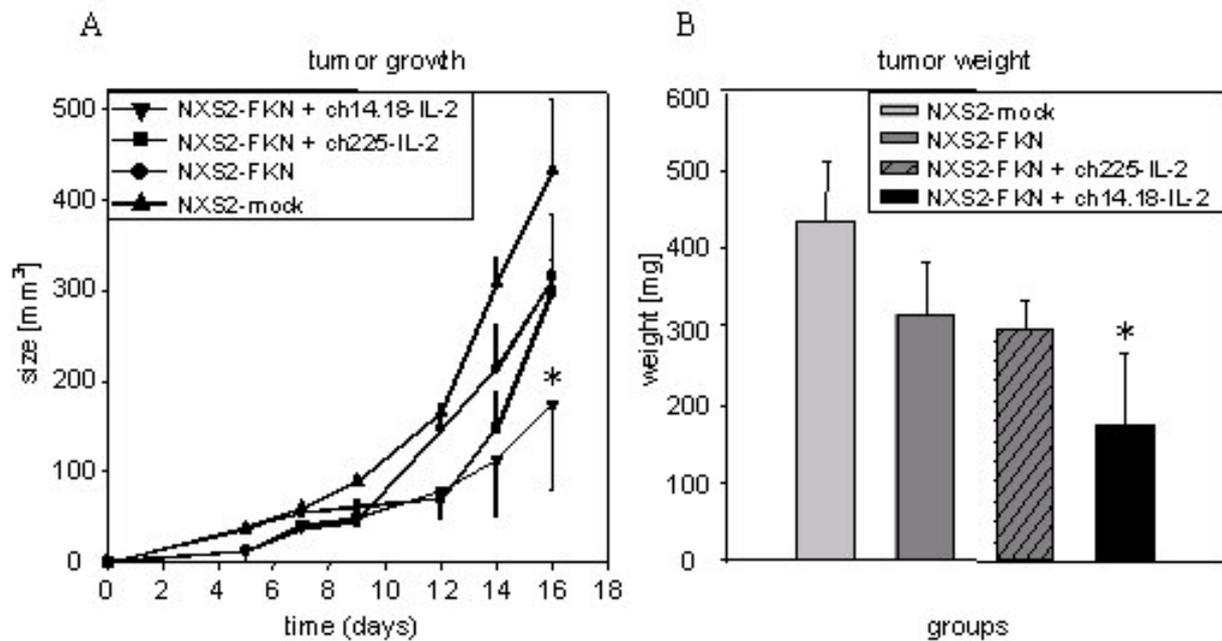

Fig. 7 Effect of FKN immunogenetherapy combined with targeted IL-2 on primary tumor growth. The anti-tumor immune response combining FKN immunogenetherapy with targeted IL-2 was determined following the experimental design as depicted in Figure 2. Experimental groups of mice (n=6) received s.c. injections with 2×10^6 NXS2-FKN cells and NXS2 mock transfected cells. 5 days after s.c. injection, mice (n=6) were treated with 5 daily injections of tumor specific anti-ganglioside GD2 antibody ch14.18-IL-2 fusion protein (5x5 µg). Mice (n=6) treated with a non-specific anti-human EGF receptor antibody ch225-IL-2 fusion protein (5x5 µg) were used as a control group. (A) Primary tumor growth was monitored over time by microcaliper measurements and the tumor size was calculated according to ½ x width^2 x length. Data points represent MV ± SD. (B) The primary tumor weight was determined following surgical removal 17 days after s.c. tumor inoculation. Bars represent MV ± SD. The difference between the FKN immunogenetherapy and targeted IL-2 combination group and mock control group was statistically significant (*p<0.01).

The efficacy of this combination therapy against liver metastasis was determined following a lethal challenge with 10^5 wild type NXS2 cells i.v. Importantly, 5/6 mice challenged with NXS2 wild type cells receiving the FKN and ch14.18-IL-2 combination therapy were free of the experimental liver metastasis (Fig. 8A) and the livers of mice in this group revealed a normal weight (Fig. 8B), which is around 1 gram. This finding was in contrast to the livers from naive control and unspecific control groups.
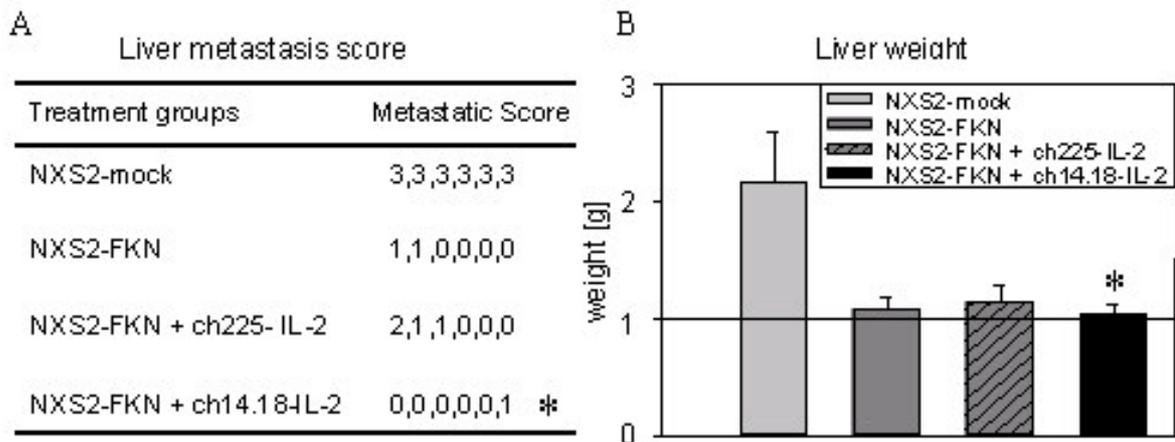
Fig. 8 Effect of FKN immunogenetherapy combined with targeted IL-2 on experimental liver metastasis. The anti-tumor immune response combining FKN immunogenetherapy with targeted IL-2 was determined following the experimental design as depicted in Figure 2 using the same experimental groups as described for results shown in Fig.7. All mice (n=6) received a lethal intravenous challenge with 10^5 NXS2 wild type cells one day after removal of the primary tumor. The level of experimental liver metastasis was determined 3 weeks after i.v. challenge. (A) Liver metastases were scored according to the coverage of the liver surface with neuroblastoma metastases as follows: 0% = 0, <20% = 1, 20 – 50% = 2, >50% = 3. (B) The level of liver metastasis was assessed by a determination of the wet liver weight. Bars represent MV ± SD, n=6. The difference between the FKN immunogenetherapy and targeted IL-2 combination group and all control groups was statistically significant (*p<0.05).

3.5 Tumor-specific CTL activity of mice following FKN and ch14.18-IL-2 combination therapy

Splenocytes receiving FKN and ch14.18-IL-2 revealed a 3-fold increase of the cytolytic response against NXS2 cells in contrast to the mock control group. Importantly, no lysis was observed against NXS2 cells in splenocytes from mice treated with NXS2-FKN alone or combined with ch225-IL-2 (Fig. 9). The lysis observed in the NXS2-FKN plus ch14.18-IL-2 group can be inhibited by adding anti-MHC-I (H-2K^k) antibody, indicating MHC class I restriction, characteristic for a CD8$^+$ T-cell response. These findings clearly correlate with in vivo findings and demonstrate the induction of a CTL response only following this combination treatment regimen. This contention is supported by the fact that neither NXS2-FKN nor ch14.18-IL-2 used as monotherapy could achieve such a CTL response.
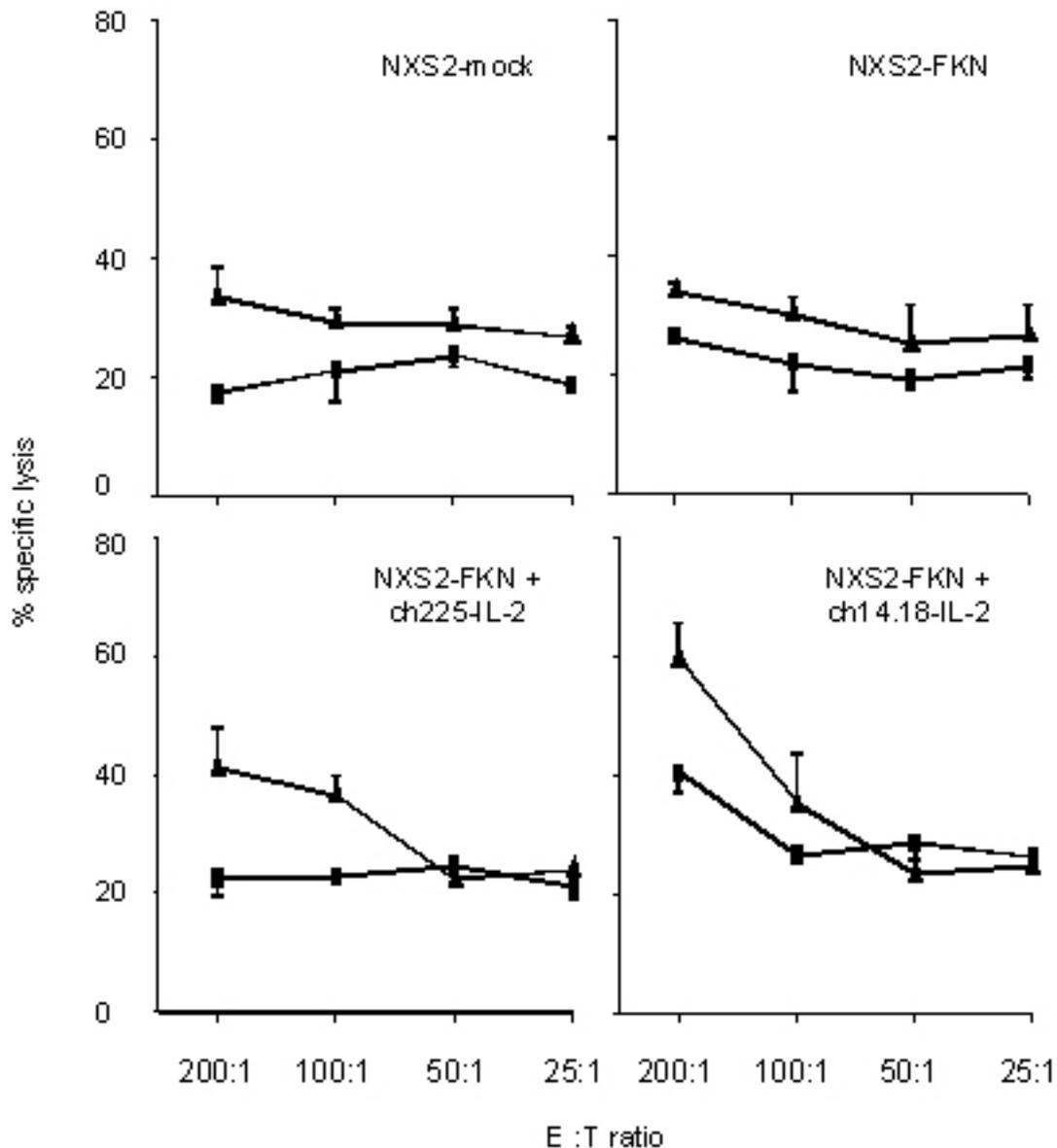
Fig. 9 Determination of the CTL response induced by FKN immunogenetherapy combined with targeted IL-2. The CTL response in mice receiving FKN immunogenetherapy combined with targeted IL-2 was determined by a standard ^{51}Cr release assay at varying effector to target cell (E/T) ratios. For this purpose, pooled splenocytes of all experimental groups of mice (n=6) were harvested at the end of the in vivo experiment (Fig. 2), and used after a 4-day in vitro stimulation phase as described in material and methods. CTL activity was determined in the absence (triangle) and presence (square) of anti-MHC class I antibody (anti-H-2K^k, 25µg/ml). Results show cytotoxicity in percent (MV ± SD) of experiments in triplicate.

3.6 Upregulation of T cell activation markers and pro-inflammatory cytokines following FKN gene therapy and targeted IL-2

In order to determine the level of T cell activation, the expression of T cell activation markers and the production of pro-inflammatory cytokines by distinct T cell populations were tested by flow cytometry.

Interestingly, it was observed that the highest increase of T cell activation markers (CD69, CD25) (Fig. 10A) and proinflammatory cytokines (TNF-α, INF-γ) (Fig. 10B) were in the NXS2-FKN and ch14.18-IL-2 combination group over all control groups including the NXS2-FKN group receiving non-specific ch225-IL-2 fusion protein. This finding clearly demonstrated superior efficacy and specificity of combining targeted IL-2 with FKN gene therapy in inducing activated T$_H$1 CD8$^+$-T-cells.
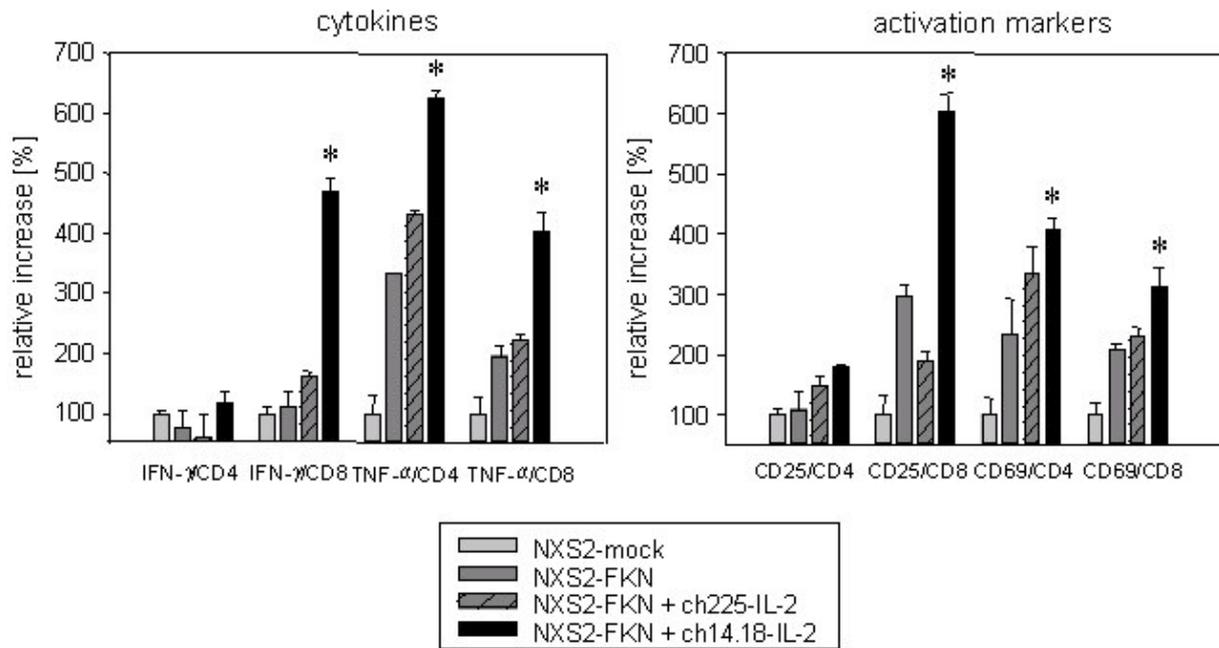
Fig. 10 Analysis of the T cell activation following FKN immunogenetherapy combined with targeted IL-2.
T-cell activation was determined in pooled splenocytes of experimental groups of mice (n=6) at the end of the in vivo experiment (Fig. 2), after the 4 day in vitro stimulation phase as described in material and methods. (A) The secretion of IFN-γ and TNF-α of distinct T cell subpopulations was determined by two-color flow cytometry. (B) T cell activation markers CD25 and CD69 of distinct T cell subpopulations were determined by two-color flow cytometry. Data represent the relative increase over naïve control splenocytes in percent (MV $\pm$ SD) of experiments in triplicate. The difference between the FKN immunogenetherapy and targeted IL-2 combination group and mock control group was statistically significant (*p<0.01).

3.7 Role of CD4^{+} and CD8^{+} T cells in tumor inhibition by FKN gene therapy and targeted IL-2

In order to further elucidate a role for T-cells in this treatment, the number of CD8^{+} and CD4^{+} T cells was determined in splenocytes harvested from treated animals by flow cytometry (Fig. 11). The number of CD3^{+}/CD4^{+} T cells rose 1.83 times in mice receiving FKN gene therapy and 2.46 times in the FKN gene therapy and ch14.18-IL2 combination group over the mock control group. Similarly, the number of CD8^{+}/CD3^{+} T cells was markedly increased in the combination therapy group, but only slightly increased in FKN gene therapy used as monotherapy or FKN gene therapy combined with ch225-IL-2 unspecific control. This result suggested that CD4^{+} and CD8^{+} T cells proliferated to the highest extent in the FKN genetherapy group combined with targeted IL-2.
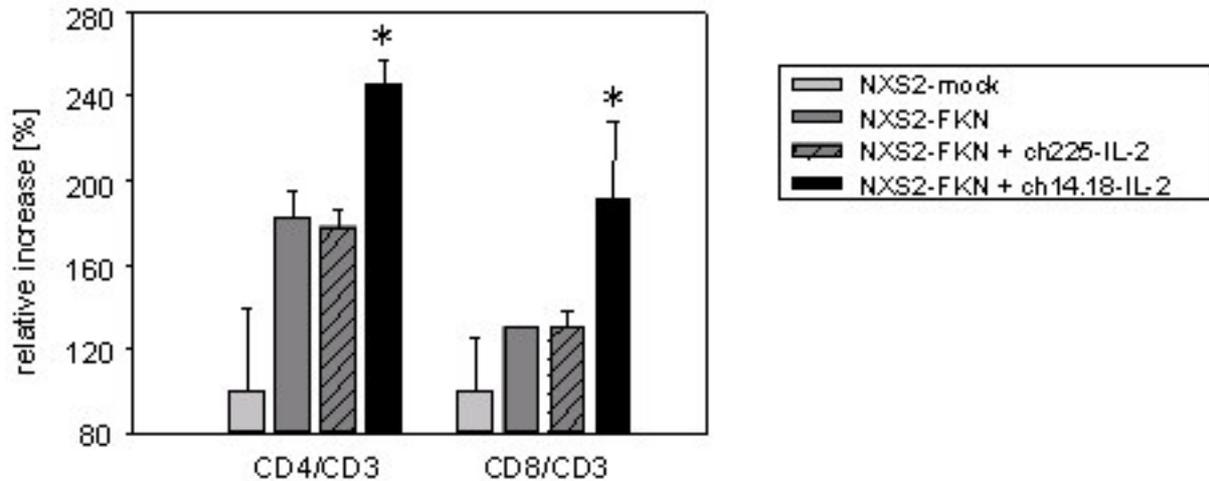
Fig. 11 Analysis of the number of CD4+ and CD8+ T-cells following FKN immunogenetherapy combined with targeted IL-2. The number of CD4+ and CD8+ T-cells was determined by two-color flow cytometry in pooled splenocytes of experimental groups of mice (n=6) at the end of the in vivo experiment (Fig. 2), prior to the 4-day in vitro stimulation phase. Data represent the relative increase over naïve control splenocytes in per cent (MV ± SD) of experiments in triplicate. The difference between the FKN immunogenetherapy and targeted IL-2 combination group and mock control group was statistically significant (*p<0.01).

Depletion assay further supported this conclusion (Fig. 12). The depletions of CD4+ T cells and CD8+ T cells were accomplished by injection of anti-CD4- and anti-CD8-antibody in vivo. As shown in Figure 12, FKN gene therapy and ch14.18-IL-2 induced significant inhibition of tumor growth in non-depleted mice. In contrast, the primary tumor growth rate increased in CD4- or CD8-depleted mice, indicating that the antitumor effect induced by NXS2-FKN and targeted IL-2 was mediated by both CD4+ T cells and CD8+ T cells.
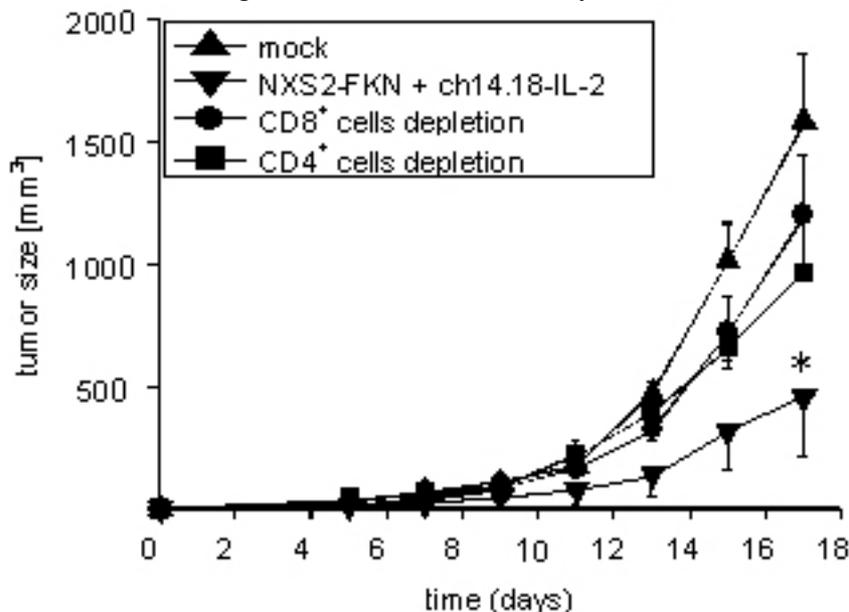

Fig. 12 Effect of T cell depletion on FKN immunogenetherapy combined with targeted IL-2 on primary tumor growth. The role of CD4+ or CD8+ T cells in mediating the anti-tumor immune response following a combination of FKN immunogenetherapy with targeted IL-2 was determined using the experimental design as depicted in Figure 2. Experimental groups of mice (n=6) received s.c. injections with 2x10^6 NXS2-FKN cells and NXS2 mock transfected cells. 5 days after s.c. injection, mice (n=6) were treated with 5 daily injections of tumor specific anti-ganglioside GD2 antibody ch14.18-IL-2 fusion protein (5x5 µg). Mice (n=6) were depleted of CD4+ or CD8+ T cells by intraperitoneal injection of 200 µg of anti-CD4 mAb (RM4-5) or anti-CD8 mAb (53-6.7) on days –1, 7 and 14. The efficacy of this depletion was previously described (20). Primary tumor growth was monitored over time by microcaliper measurements and the tumor size was calculated according to

(½ x width^2 x length). Data points represent MV ± SD. The difference between the non-depleted group and all control groups was statistically significant (*p<0.05).

4 Discussion

Effective amplification of immune responses induced by a cancer vaccine is an important strategy to achieve clinical responses in cancer patients receiving immunotherapeutic treatment. The important role of effective immune response amplifiers, called adjuvants, has captured the imagination of immunologists for decades and has led to important developments, such as the use of killed *Bordetella pertussis* for diphtheria and tetanus toxoids (21), muramyl dipeptide extracted form the mycobacterial cell wall (22) and immune stimulatory complexes, called ISCOMs, which simultaneously serve as antigen carriers and adjuvants (23). Additional adjuvants are cytokines and more recently also chemokines, such as IL-2 (24) or fractalkine, the former also used successfully as an adjuvant in a clinical trial of melanoma patients with a respectable response of 42% in patients receiving a gp-100 peptide vaccine. Preclinical data using fractalkine in cancer immunotherapy are limited (25) and results from clinical applications using this chemokine are not available at this time.

Here I demonstrate for the first time the efficacy of a fractalkine gene therapy approach in combination with targeted IL-2 in a neuroblastoma model using a genetically engineered fractalkine protein, constructed by PCR cloning. Expression of FKN and bioactivity in vitro and in vivo was demonstrated (Fig. 3-6) [4]. The efficacy for this type of combination gene therapy was established in an immunocompetent syngeneic model for murine neuroblastoma using NXS2 cells that naturally express the disialoganglioside GD2. This model also features experimental metastases to liver, expresses the neuroblastoma tumor marker tyrosine hydroxylase, and represents many pathophysiological similarities to human neuroblastoma (26).

The immune response induced by genetically engineered NXS2 cells which produce fractalkine is partially effective as indicated by a reduction of primary tumor growth by 30%. The effect was mediated by CD8$^+$ T cells as indicated by a strong infiltrate of primary tumors with CD8$^+$ T cells (Fig. 6). This finding is in contrast to NK-cell-mediated immune mechanism observed in the same animal model induced by a recombinant anti-disialoganglioside GD2-interleukin-2 fusion protein monotherapy that directs interleukin-2 (IL-2) into the tumor microenvironment (26) where infiltrating CD8$^+$ T cells were absent. Since such recombinant IL-2 fusion proteins were demonstrated to induce T cell mediated immune responses in other animal models, e.g. melanoma (27) and colon carcinoma (28), I concluded that poor immunogenicity and immunosuppressive factors secreted by NXS2 cells, e.g. IL-10 and TGF-β, could account for T-cell anergy in this model (26). However, this T-cell anergy is apparently overcome by the use of fractalkine gene therapy in combination with targeted IL-2.

One of the major advantages of fractalkine over other chemokines is its proinflammatory T$_H$1 potential capable of potentiating T- and NK- cell mediated cytotoxic responses. It is also a strong stimulator of T$_H$1 CD4$^+$ T cells. These cells elicit a helper function and are involved in the maturation of CD8$^+$ T cells, the main effector cells observed in this experimental system. In fact, it was possible to demonstrate the presence of CD4$^+$ T-cells in cellular infiltrates observed in primary tumors of mice previously vaccinated with fractalkine producing NXS2 cells (Fig. 6). Interestingly, this is again in contrast to previous studies with a recombinant antibody IL-2 fusion protein in this same tumor model, where such CD4$^+$ T-cells cells were completely absent (26).

Despite the presence of CD8$^+$ and CD4$^+$ T cells in the tumor microenvironment, fractalkine gene therapy used as a monotherapy showed limited efficacy in this neuroblastoma model as indicated by a reduction of primary tumor growth by only 30% (Fig. 7). This finding is in contrast to results reported in the literature on C26 colon carcinoma and B16F10 melanoma

models (25) with a reduction in primary tumor growth by almost 90% in both models. These differences are most likely a result of the tumor models used. A similar observation was made with exactly the same tumor models using tumor specific recombinant IL-2 fusion proteins (targeted IL-2). The induction of a T cell mediated memory immune response was demonstrated in melanoma (27) and colon carcinoma (28;29), but not in the neurobastoma model (20). The absence of an effective T cell response with targeted IL-2 or fractalkine gene therapy used as a monotherapy is a result of the poor immunogenicity of the NXS2 neuroblastoma model, thus reflecting immunologically the situation in man.

Based on these considerations, the efficacy of a novel approach was tested in this experiment, which specifically amplify a T-cell-mediated immune response initially induced with a cellular tumor vaccine genetically engineered to secrete fractalkine. Thus, tumor-specific targeting of the T cell growth factor IL-2 into the tumor microenvironment was shown to effectively boost a $CD8^+$ T-cell-mediated immune response, as indicated by increased $CD8^+$ T cell activation (Fig 10) and MHC class I restricted tumor cell killing (Fig. 9) in mice receiving both the fractalkine vaccine and tumor-specific IL-2 boost. The absence of such a response with a non-specific fusion protein clearly proves the concept of tumor-specific boost with ch14.18-IL-2 fusion protein specific for ganglioside GD2, which is highly expressed in the neuroblastoma tumor microenvironment (30). The primary effector cells involved in the anti-tumor immune response were $CD8^+$ T cells, since depletion of this T cell subpopulation abrogated the effect of this combination therapy (Fig. 11-12). Interestingly, depletion of $CD4^+$ T cells also abrogated the therapeutic effect indicating their important helper function.

The increase in effective concentrations of IL-2 in the tumor microenvironment appears to be the crucial step in effective $CD8^+$ T cell re-activation, which can be achieved with systemic injections of tumor-specific antibody-IL-2 fusion proteins (27;28;31-33). Thus, the presence of both tumor-associated T cell antigens and adequate co-stimulation in the tumor microenvironment provided by targeting of the tumor cell surface with IL-2, fulfills the necessary requirement for amplification of the $CD8^+$ T-cell-mediated immune response.

This principle of amplifying an immune response initially induced by a cancer vaccine with a tumor-specific antibody-cytokine fusion protein could find broad application in immunotherapeutic cancer treatments. Indeed, additional treatments with tumor-specific antibody-IL-2 fusion protein could benefit many clinical trials with cancer vaccines based on cytokine and chemokine gene therapy approaches, dendritic cells pulsed with immunogenic tumor-associated peptides or DNA vaccines encoding for tumor-associated peptide antigens. Such effective adjuvants are of major interest based on the clinical response rates observed thus far in vaccine trials (34).

In summary, I demonstrate that targeting of IL-2 into the tumor microenvironment with a ch14.18-IL-2 fusion protein effectively amplifies a $CD8^+$ T cell immune response induced by fractalkine gene therapy. This effect was specific, since non-specific ch225-IL-2 were ineffective in this regard. A mechanism for re-activated $CD8^+$ memory T cells was provided by increased $CD8^+$ T cell activation and MHC class I restricted tumor cell killing only in mice that received both the vaccine and the tumor-specific boost. Taken together, these data suggest that tumor targeted IL-2 may overcome weak immune responses induced by cancer vaccines and therefore lead to further improvement in the adjuvant treatment of patients with minimal residual disease.

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5.1 Own Publications

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Appendix

Anteilserklärung

Frau Yan Zeng hat ihre experimentelle kumulative Doktorarbeit mit dem Thema „T-cell mediated suppression of neuroblastoma following fractalkine gene therapy is amplified by targeted IL-2" angefertigt. Grundlage für die kumulative Doktorarbeit sind folgende 4 Publikationen:

[1]. Zeng Y; Fest S; Kunert R; Katinger H; Pistoia V; Michon J; Lewis G; Ladenstein R; Lode HN.: Anti-neuroblastoma effect of ch14.18 antibody produced in CHO cells is mediated by NK-cells in mice. Mol Immunol. *2005 Jul;42(11),*1311-9.

Diese Arbeit beschreibt die Charakterisierung eines neuen rekominanten anti-Gangliosid GD2 Antikörpers, der in CHO Zellen hergestellt wurde. Frau Zeng hat die Spezifität des Antikörpers für das Antigen GD2 durch Sandwich ELISA Untersuchungen vergleichend evaluiert. Die Wirksamkeit des Antikörpers hat sie in Zytolyseassays (CDC und ADCC) an GD2 positiven Targetzellen (Melanom und Neuroblastom) *in vitro* nachgewiesen. Darüber hinaus hat sie die Wirksamkeit des Antikörpers in einem syngenen Neuroblastommodell *in vivo* bewiesen und damit eine wichtige Grundlage für den Abschnitt über gerichtete IL-2 Therapie ihrer kumulativen Promotion gelegt. Darüber hinaus sind diese Daten die präklinische Grundlage für eine Phase I Studie zur Behandlung von Patienten mit Stadium 4 Neuroblastom mit dem unter GMP hergestellten ch14.18/CHO Antikörper. Diese Studie beginnt dieses Jahr an unserer Klinik. Aus diesem Grunde ist ihr Anteil an der dazu veröffentlichten Arbeit bei mindestens 90% einzustufen.

[2]. Huebener,N.; Lange,B.; Lemmel,C.; Rammensee,H.G.; Strandsby,A.; Wenkel,J.; Jikai,J.; Zeng,Y.; Gaedicke,G.; Lode,H.N.: Vaccination with minigenes encoding for novel 'self' antigens are effective in DNA-vaccination against neuroblastoma. Cancer Lett. *2003; Jul 18;197(1-2),*211-7.

Diese von Frau Huebener als Erstautorin veröffentlichte Arbeit beschreibt die Wirksamkeit eines DNA Impfstoffs zur aktiven Immunisierung beim Neuroblastom. Frau Zeng hat in dieser Arbeit bei der Klonierung und Herstellung des Impfstoffs wesentlich beigetragen. Deshalb ist ihr Anteil an der dazu veröffentlichten Arbeit bei 10% anzusiedeln.

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Diese Arbeit beschreibt den gegenwärtigen Kenntnisstand der aktiven Immunisierung gegen das Neuroblastom mit der Tyrosinhydroxylase als Antigen. Der Beitrag von Frau Zeng liegt hier ebenfalls in der Klonierung und Herstellung von Varianten den Tyrosinhydroxylase-DNA-Impfstoffs. Daher ist ihr Anteil an der dazu veröffentlichten Arbeit ebenfalls im Bereich von 10%.

[4]. Zeng Y; Jiang J; Huebener N; Wenkel J; Gaedicke G; Xiang R; Lode HN. Fractalkine gene therapy for neuroblastoma is more effective in combination with targeted IL-2. Cancer Lett. *2005 Jun 10.*

In dieser Publikation wird die Wirksamkeit einer neuartigen Immuntherapiestrategie aus Immunogentherapie mit Fractalkin in Kombination mit gerichteter IL-2 Therapie beschrieben. Frau Zeng hat in dieser Arbeit nicht nur selbständig das Fractalkin Gen kloniert sondern auch erfolgreich exprimiert sowie seine Biofunktion *in vitro* wie *in vivo* nachgewiesen. Darüber hinaus konnte sie zum ersten Mal in vivo zeigen, dass die suboptimal wirksame Imunogentherapie mit Fractalkin alleine durch die gerichtete Gabe von IL-2 mittels eines anti-GD2-IL-2 Fusionsproteins amplifiziert wird. Da sie diese Arbeiten selbständig durchgeführt hat liegt ihr Anteil an der dazu veröffentlichten Arbeit bei mindestens 90%.

Darüber hinaus ist eine 5. Publikation in Vorbereitung, deren Inhalt in der von Frau Zeng vorgelegten Arbeit zusammengefasst ist. Ihr Anteil an dieser Arbeit wird ebenfalls 90% betragen.

Mit freundlichen Grüßen,

Datum   Unterschrift

PD Dr. Holger N. Lode   *Yan Zeng*

Acknowledgment

Eidestattliche Erklärung

„Ich, Yan Zeng, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „T-cell mediated suppression of neuroblastoma following fractalkine gene therapy is amplified by targeted IL-2" selbst verfasst habe,
keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie ohne die
(unzulässige) Hilfe Dritter verfasst habe. und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

Datum   Unterschrift

                                                 Yan Zeng