Research Article

A Human Recombinant Autoantibody-Based Immunotoxin Specific for the Fetal Acetylcholine Receptor Inhibits Rhabdomyosarcoma Growth In Vitro and in a Murine Transplantation Model

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Rhabdomyosarcoma (RMS) is the most common malignant soft tissue tumor in children and is highly resistant to all forms of treatment currently available once metastasis or relapse has commenced. As it has recently been determined that the acetylcholine receptor (AChR) γ-subunit, which defines the fetal AChR (fAChR) isoform, is almost exclusively expressed in RMS *post partum*, we recombinantly fused a single chain variable fragment (scFv) derived from a fully human anti-fAChR Fab-fragment to Pseudomonas exotoxin A to generate an anti-fAChR immunotoxin (scFv35-ETA). While scFv35-ETA had no damaging effect on fAChR-negative control cell lines, it killed human embryonic and alveolar RMS cell lines in vitro and delayed RMS development in a murine transplantation model. These results indicate that scFv35-ETA may be a valuable new therapeutic tool as well as a relevant step towards the development of a fully human immunotoxin directed against RMS. Moreover, as approximately 20% of metastatic malignant melanomas (MMs) display rhabdoid features including the expression of fAChR, the immunotoxin we developed may also prove to be of significant use in the treatment of these more common and most often fatal neoplasms.

1. Introduction

Three forms of RMS are recognized, each of which shows skeletal muscle differentiation, but are distinguished by other genetic and biological characteristics: (1) embryonal RMS, characterized by loss of heterozygosity at 11p15; (2) the more aggressive alveolar RMS, often accompanied by Pax3 or Pax7-FKHR gene fusions resulting from translocations t(2;13) (q35; q14) and t(1; 13) (p36; q14), respectively; and (3) the rare pleomorphic RMS, which is genetically complex and tends to occur in adults [1]. As a group, RMS is the most frequent soft tissue tumor in children [2]. Metastatic

alveolar RMS in children older than 10 years as well as relapse of disease tends to be refractory to all current forms of therapy including adjuvant bone marrow transplantation [3], resulting in 5-year survival rates of no more than 5–20% [2–4]. Therefore, novel first line or adjuvant treatment strategies are urgently needed for high-risk RMS patients [5]. As we have previously shown that, after birth, the fetal AChR (fAChR) is almost exclusively expressed in RMS [6–8] and that anti-fAChR chimeric T-cells could kill fAChR-positive RMS cell lines in vitro [9], this target seemed promising for the development of an immunotoxin, a molecule consisting of a toxin coupled to an antibody fragment designed to destroy a specific type of cell [10]. Therefore, we generated an immunotoxin targeting fAChR in order to exploit its expression pattern for the treatment of RMS.

The nicotinic Acetylcholine Receptor (AChR) is a pentameric ion channel which appears in an embryonic (fAChR) and an adult isoform (AChR). Both isoforms comprise two α, one β, and one δ subunits, while the γ-subunit is specific for fAChR. In the process of muscle innervation during embryonic development, the γ-subunit is replaced by an ε-subunit, leading to the replacement of fAChR by AChR. After birth, expression of fAChR is restricted to few tissue forms including extraocular muscle fibers of unknown function [11–13], thymus myoid cells [14–16], and skeletal muscles following denervation [17, 18]. In contrast, the vast majority of human embryonic and alveolar rhabdomyosarcomas strongly express fAChR on the tumor cell surface [6–8], making this receptor ideal for immune-targeting by an immunotoxin.

Several disorders involving the immunization against fAChR shed light on the molecular mechanisms of anti-fAChR antibodies. In myasthenia gravis (MG), an autoimmune disease most commonly caused by the appearance of anti-AChR autoantibodies [19], the majority of patients develop autoantibodies against AChR as well as fAChR [19, 20]. In studies of MG, it has been shown that anti-AChR autoantibodies are internalized along with AChR after binding, which is a necessary mechanism for an immunotoxin against RMS to exert its effect on target cells [19]. The unborn children of parous pregnant women who developed autoantibodies against fAChR can develop arthrogryposis multiplex following diaplacentar passage of these antibodies, while their mothers remain free of symptoms. This not only shows that the γ-subunit of fAChR harbors immunologically specific epitopes that are potentially suitable as target for an immunotoxin in the therapy of RMS but also suggests that the use of an anti-fAChR immunotoxin in RMS treatment is unlikely to induce serious side effects [21]. Finally, cDNA libraries derived from MG patients and mothers of children suffering from arthrogryposis multiplex should be ideal sources for the generation of fAChR-antibodies in vitro.

This report is the first description of the cloning, expression and functional testing of an immunotoxin targeting the AChR γ-subunit. The immunotoxin consists of an scFv antibody fragment derived from a human combinatorial thymus cDNA library prepared from a woman producing autoantibodies against fetal AchR [21] and a truncated version of the pseudomonas exotoxin A. This toxin killed

RMS cells in vitro and delayed tumor growth in a murine transplantation model.

2. Results

2.1. An Immunotoxin Targeting the Main Immunogenic Region (MIR) of the AChR α-Subunit Kills RMS Cells In Vitro.
Initially, we generated an immunotoxin containing ETA based on a recombinant single chain Fv fragment derived from the rat monoclonal antibody mAb192 against the main immunogenic region (MIR) of the human AChR α-subunit [22, 23]. This model immunotoxin (scFv192-ETA) bound to several RMS cell lines and killed the RMS cell lines TE-671, FL-OH-1 and RD in a dose-dependent manner, while fAChR-negative RMS and non-RMS hematopoietic control cell lines (A-204, U937) were killed only at much higher immunotoxin concentrations (not shown). These experiments showed that an immunotoxin targeting AChR is able to kill AChR-positive RMS cells in vitro while leaving AChR-negative cell lines relatively unharmed.

2.2. Construction and In Vitro Characterization of Immunotoxins Targeting the γ-Subunit of Human Fetal AChR.
Subsequently, we generated an ETA-based immunotoxin targeting the AChRγ-subunit, which is specific for the fetal form of the AChR. Two recombinant human Fab fragments (Fab35 and Fab38) were isolated from a combinatorial cDNA library derived from the thymus of a seropositive MG patient with high titers of anti-AChR γ-subunit antibodies [21]. Although the two Fab fragment cDNAs were inserted in-frame into the ETA-vector, they were translated inefficiently in *E. coli* BL21DE3, resulting in several abnormal protein fragments of 70–80 kDa instead of the expected 120 kDa full-length product (not shown). Therefore, we used the Fab35 and Fab38 cDNAs to produce single-chain Fv (scFv) fragments by overlap extension PCR (Figure 1). VH and VL fragments were linked by a 36-nucleotide (glycine/serine) spacer to produce scFv fragments with a VH-VL and a VL-VH orientation from both Fabs, yielding four different scFvs.

The four scFv cDNAs were inserted into the ETA vector pBM-1.0, checked for proper ligation by sequencing and expressed in *E. coli* BL21DE3. The resulting immunotoxins were purified and the binding capacity to RMS cells was determined by flow cytometry. Of the four molecules examined, only the Fab35 derivative scFv35/VL-VH-ETA specifically bound to cells of the fAChR-positive embryonal RMS cell lines TE-671, RD, and FL-OH-1 as well as to the alveolar RMS cell lines RH-30 and Ax-OH-1 and while showing virtually no reactivity with the fAChR-negative control RMS line A-204 or the hematopoietic cell line U937 (Figure 2).

This immunotoxin, termed scFv35-ETA, killed fAChR-positiveRMS cell lines in a dose-dependent manner, while it had almost no effect on the control cell lines A-204 and U937 (Figures 3(a) and 3(b)). Specificity of the growth inhibitory effect was demonstrated using a nonbinding control composed of a non-binding scFv fused to ETA (mock-ETA). The IC_{50} values of scFv35-ETA and the control

TABLE 1: Comparison of IC_{50} values of scFv35-ETA and mock-ETA control on various cell lines.

	Mean IC_{50} (nM)						
	TE-671	RD	FL-OH-1	Ax-OH-1	RH-30	U937	A-204
scFv35-ETA	34	1.5	0.13	14	113	>500	>500
mock-ETA	113	22	12	90	>500	>500	>500

IC_{50} values indicating concentrations required to achieve a 50% reduction of protein synthesis relative to untreated control cells as derived from colorimetric XTT assay in Figure 3.
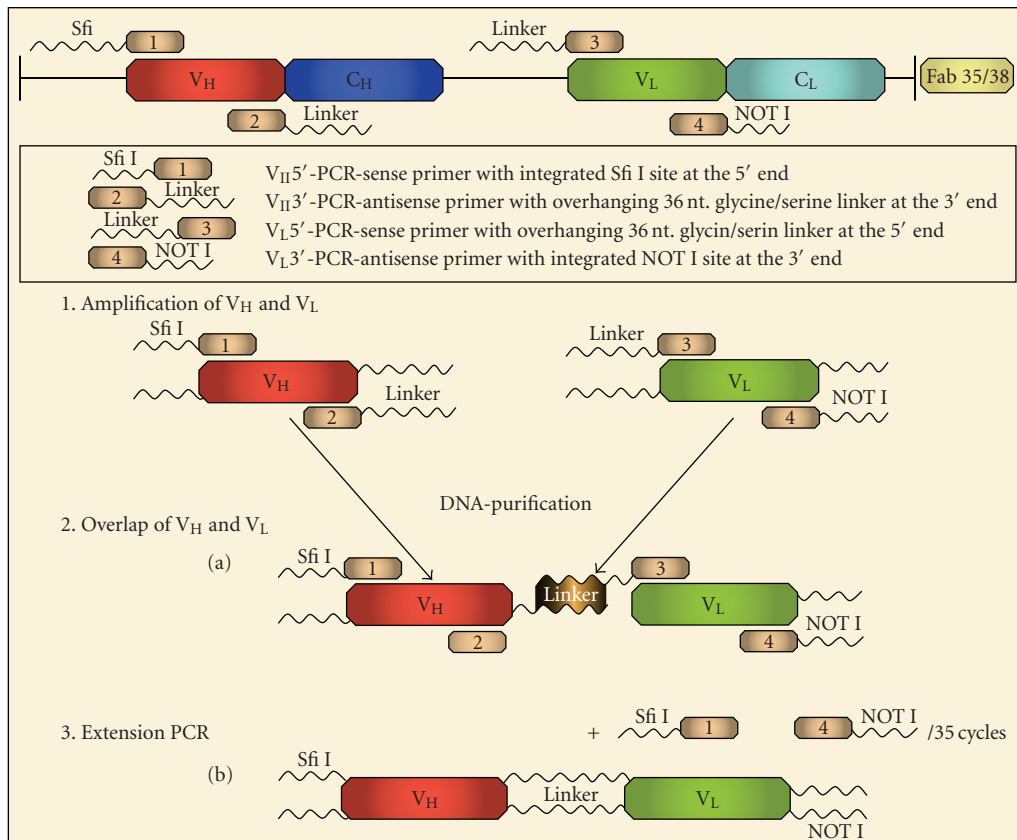

FIGURE 1: Schematic presentation of the synthesis of scFv fragments based on the recombinant Fab fragments Fab35 and Fab38 [21] using overlap extension PCR.

immunotoxin on the tested cell lines are summarized in Table 1. The inhibitory effect could be significantly enhanced using the fAChR-specific scFv35-ETA as shown by a decrease of IC_{50} values ranging from 3 (TE-671) to 100-fold (FL-OH-1). Furthermore, addition of 100-fold molar excess of free scFv35 in a competitive approach could completely knockdown the toxic effect of scFv35-ETA fusion protein emphazising its specificity (Figure 3(c)). To further characterize the toxic effect of scFv35-ETA on RMS cells, an annexin V-based apoptosis assay was performed on embryonal RMS cell line RD. The amount of cells staining positive for annexin V, an early apoptosis marker, clearly increased during the time points of 12, 14, and 48 hours of incubation. Finally, after 48 hours a total of 51,7 % of cells incubated with scFv35-ETA stained positive for annexin V compared to 9.28 in the PBS control (Figure 4).

2.3. The γ-Subunit-Specific Immunotoxin Delays Tumor Development in a Mouse RMS Transplantation Model. The in vivo toxicity of scFv35-ETA against RD RMS cells was tested in a murine RMS transplantation model [24]. Initial tests on healthy SCID mice revealed that intraperitoneal injection of $10\,\mu$g scFv35-ETA per mouse ($n = 10$) had no apparent adverse effects on mobility, weight gain, or survival (up to 60 days). Therefore, this dose was used in concert with the subcutaneous injection of 5×10^6 RD cells per mouse ($n = 10$), with saline injections as controls ($n = 4$). Twice daily injections of $10\,\mu$g scFv35-ETA per mouse were repeated up to day 10. Tumor development was monitored by daily inspection and palpation. As shown in Figure 4, 100% of mock injected mice developed palpable tumors by day 7 of the experiment and clearly visible subcutaneous tumors by day 10 (volume: 59 +/− 9 mm³).

(a)

(b)

(c)

Secondary antibody control
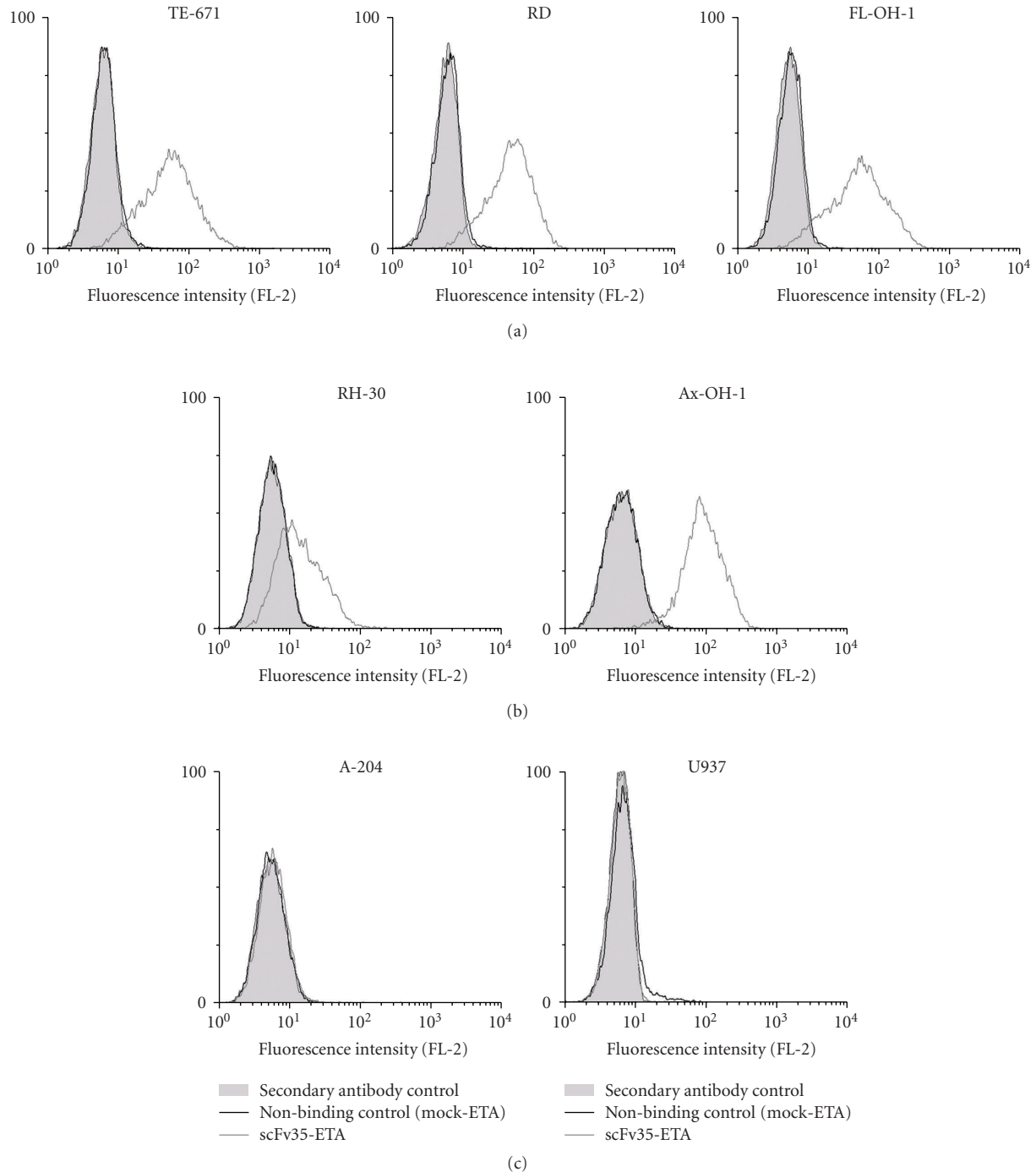Non-binding control (mock-ETA)
scFv35-ETA

FIGURE 2: FACS Analysis of scFv35-ETA.

In contrast, the mice injected with the immunotoxin showed little evidence of tumor development by day 7 and no visible sign of subcutaneous tumor development by day 10, although smaller tumors could be palpated (volume: $8 +/-27 \, mm^3$). Until day 12, the tumors in the treated group were significantly smaller than those in the controls. After finishing the course of daily immunotoxin injections, however, subcutaneous tumors began to enlarge rapidly in the test group and reached the same size as the tumors in the control group by day 14 (Figure 4).

3. Discussion

In our study, which involved the development of an immunotoxin against RMS, we report three major new
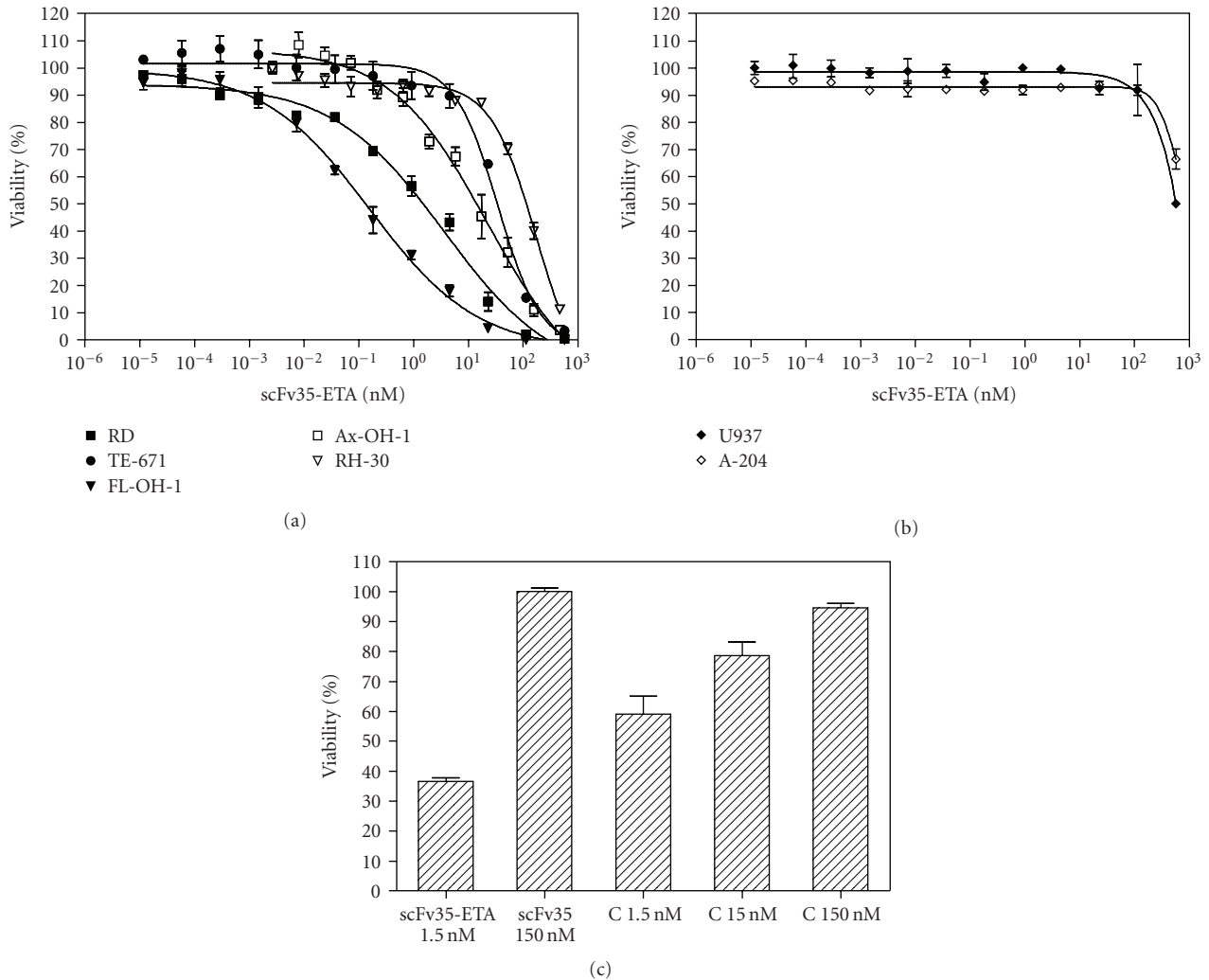
(a)

(b)

(c)

FIGURE 3: Colorimetric XTT cytotoxicity assays with various immunotoxin concentrations ($n = 3$ parallel cell cultures per dilution) showing strong dose-dependent toxicity of the immunotoxin scFv35-ETA directed against the acetylcholine receptor (AChR) γ-subunit on positive RMS cell lines TE671, RD, and FL-OH-1, RH-30 and Ax-OH-1 even at higher dilutions (a). Lower to no toxicity of the immunotoxin were observed towards the control cell lines A-204 and U937 not expressing fAChR (b). Competition of toxicity could be achieved with 1–100-fold molar excess (C 1.5–150 nM) of scFv35 added to cells incubated with 1.5 nM scFv35-ETA, whereas scFv35 alone does not inhibit cell growth even at 100 fold higher concentration (c). Cell viability is expressed as percentage inhibition of cell proliferation compared to non-treated cells.

findings: (1) we showed that the major immunogenic region of the nicotinic AChR α-subunit can be used as an immunotoxin target to kill RMS cells in vitro, (2) we were able to generate a human scFv autoantibody fragment that binds to the AChR γ-subunit of human RMS cell lines, and (3) a recombinant ETA-based immunotoxin derived from this antibody fragment killed human RMS cells in vitro and significantly delayed tumor development in a mouse RMS transplantation model, although it alone was insufficient to cure the disease.

Antibody-based immunotherapy is a well-established method in the treatment of various tumor types, such as ErbB2-positive breast cancer [25], Hodgkin lymphoma [26–30], acute myeloid leukemia [31–33], colon cancer [34, 35], lung cancer [36], melanoma [37], and neuroblastoma

[38–40]. However, immunotoxin therapy has not yet been applied to RMS, as an antigen expressed specifically and at high levels on the surface of these tumor cells was not known until recently [9]. This obstacle was overcome when we found that the fetal AChR isoform (fAChR) is expressed on the surface of the vast majority of RMS cells, while it is absent from normal innervated muscles [6, 9, 18]. Recently, we showed that anti-fAChR chimeric T-cells could kill fAChR-positive RMS cell lines in vitro while fAChR-negative cells remained unharmed [9]. However, chimeric T-cells based on antibody fragments linked to T-cell receptor subunits (such as the ζ-chain) are notoriously susceptible to deletion and anergy in vivo [41], suggesting that an immunotoxin specific for the same epitope would be a more promising approach.
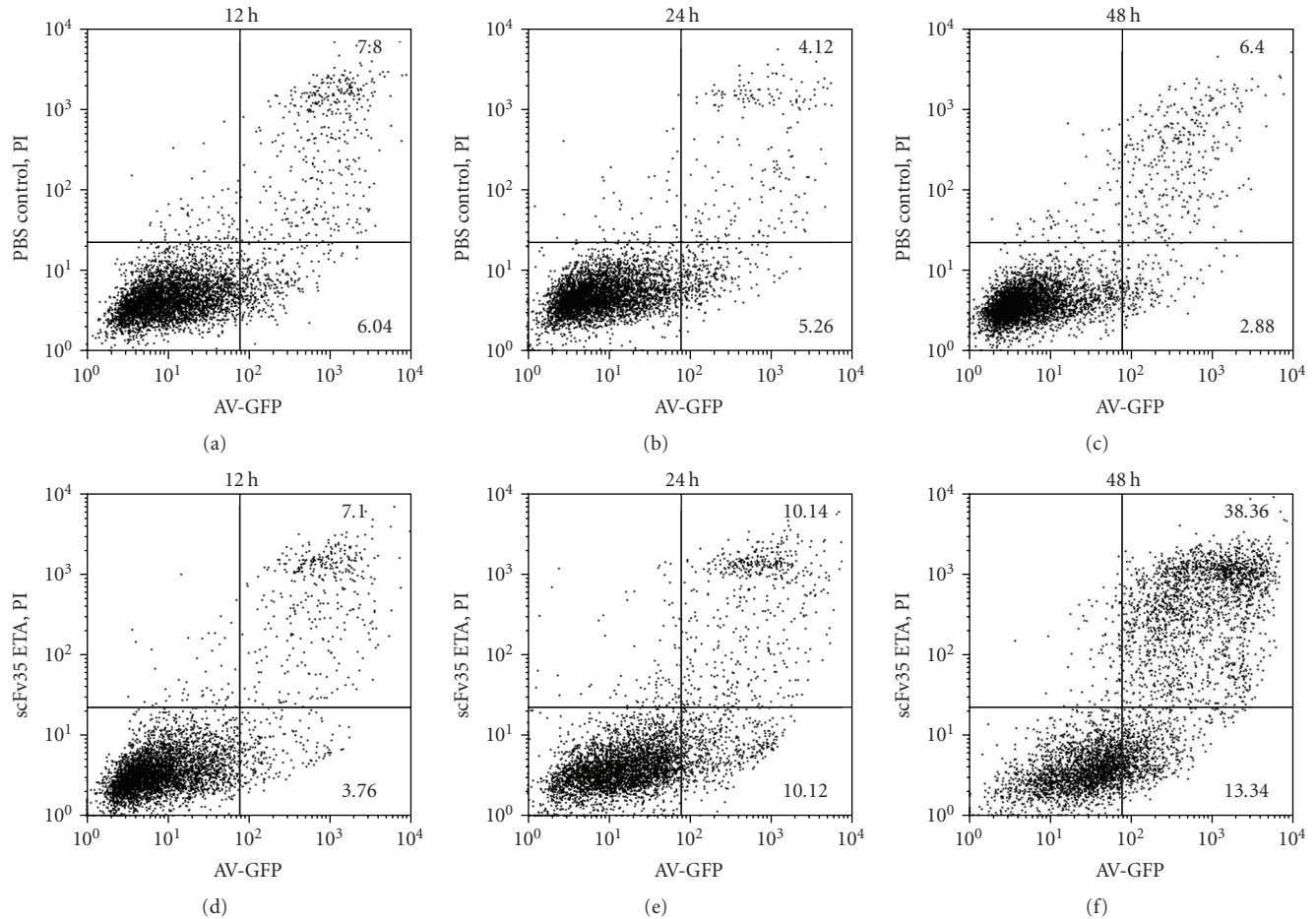
FIGURE 4: Annexin V apoptosis assay on RD cells incubated with either scFv35-ETA or PBScontrol for 12, 14 and 48 hours. Quadrants distinguish viable cells (lower left), early apoptotic cells (lower right), late apoptotic cells (upper right) and necrotic cells (upper left). Numbers indicate percentage of early and late apoptotic cells, respectively.

Therefore, we set out to generate an immunotoxin to target fAChR by first expressing a fusion protein between an anti-AChR antibody fragment and the Pseudomonas exotoxin A (scFv192-ETA). In these experiments, we found proof that scFv192-ETA kills RMS cells in vitro, while leaving AChR-negative control cell lines relatively unharmed. These data also show that fusion of scFv192 to a truncated Pseudomonas exotoxin A and expression in bacteria has no detrimental effect on the binding specificity of the rat antibody fragment [22]. Subsequently, we constructed immunotoxins specific for the AChR γ-subunit by using two recombinant human Fab fragments (scFv35 and scFv38) known for their specificity and strong affinity for the γ-subunit of the AchR [21] and generated four different scFv derivatives. A human antibody fragment was chosen firstly as there are no easily available well-characterized mouse or rat monoclonal antibodies with γ-subunit specificity and secondly because human anti-AChR autoantibodies from MG patients are known for their high affinity and specificity, properties that are often conserved in recombinant Fab or single chain Fv derivatives [22, 42, 43]. Furthermore, human monoclonal autoantibody fragments are preferable

as immunotoxin components as they are less likely to be immunogenic than their rodent counterparts and could pave the way towards the development of a fully human immunotoxin using a human toxin component such as a ribonuclease [28] or an effector such as an interleukin [44]. Surprisingly, only one of the immunotoxins generated (scFv35-ETA) showed significant binding to cell lines with abundant, surface-displayed fetal AChR. As myasthenia gravis autoantibodies critically depend on the 3-dimensional conformation of their binding sites on several AChR subunits [45], it is likely that steric hindrance prevented efficient binding of the three unsuccessful variants. ScFv35-ETA was cytotoxic to RMS cells both in vitro (Figures 3 and 4) and in a pilot in vivo mouse model (Figure 5). For the efficacy studies cell lines from both embryonal and alveolar subtype were chosen that have all previously been shown to express fAChR on the cell surface, indicating the responsiveness of both subtypes to scFv35-ETAe. The specificity of scFv35-ETA was confirmed in vitro by the lack of toxicity in control cells not expressing fAChR and by competition of toxicity with scFv35 alone as well as in vivo in the 10 mice administered 20 μg immunotoxin per day. This was not surprising, as carriers
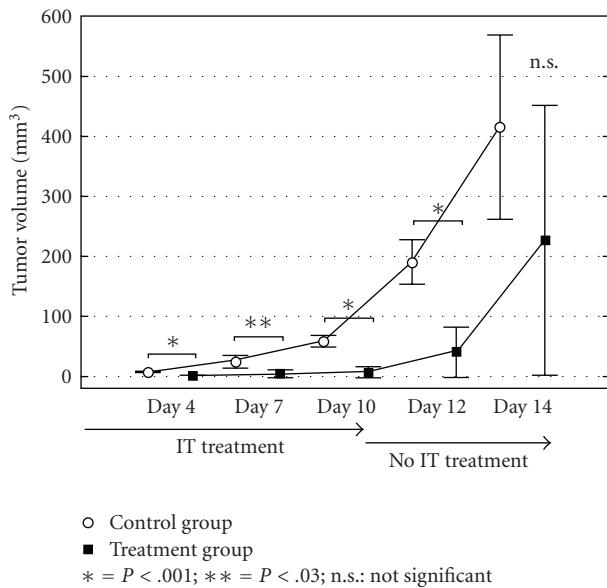
FIGURE 5: In vivo effect of scFv35-ETA on RD embryonal RMS cells (5×10^6 RD cells per mouse) transplanted subcutaneously into SCID mice. The first intraperitoneal immunotoxin injection ($10\,\mu g$ twice daily) was carried out 2 days after the injection of tumor cells and repeated daily until day 10.

of autoantibodies against fAChR are generally completely asymptomatic [21], although it is known that, after birth, fAChR is expressed in certain extraocular muscle fibers [12], thymic myoid cells [14], and skeletal muscles following denervation [17, 18].

However, the "single agent" in vivo treatment of mice with scFv35-ETA for 10 days did not cure the animals, as tumor growth was significantly delayed but not totally blocked as long as the immunotoxin was administered and progressed rapidly after the end of treatment. Among the known mechanisms leading to immunotoxin resistance, the poor vascularization of sarcomas [37, 46] is the most likely reason for this shortfall in therapeutic efficacy. However, this could be addressed by dose elevation as well as prolonged administration, an approach in which the use of a fully human immunotoxin may be necessary to circumvent sensitization against a mouse, rat, or humanized antibody component [37, 46].

In addition, other strategies to improve the treatment of RMS using scFv35-based immunotoxins can be envisaged. First, there are well-established techniques to stabilize immunotoxins [47], increase their affinity [47], and improve their uptake into tumor cells [48]. Second, as shown in leukemia [49], the therapeutic effect of immunotoxins can be improved when the expression level of the target antigen and the percentage of target-positive tumor cells are elevated pharmacologically. Third, single-agent antibody-based therapies can be made more efficient when combined with traditional chemotherapies or the targeted application of cytokines, chemokines, antiangiogenic agents, or modifiers of degradation pathways that inactivate toxins [44, 50–59]. Any or all of these strategies could be applied to

the treatment of RMS using scFv35-ETA. For example, it has previously been shown that chemotherapy can induce the differentiation of immature, AChR^{low} RMS cells to more mature rhabdomyoblasts with increased expression of muscle specific proteins including fetal AChRs [9, 60]. Thus, the synchronous or metachronous application of chemotherapy and scFv35-ETA could be more successful in treating RMS than scFv35-ETA alone. However, further preclinical trials are needed to substantiate whether any of the strategies described above actually have a positive impact on the efficacy of RMS treatment using scFv35-ETA.

In summary, we have shown that the fetal AChR is a specific marker of human RMS that can be recognized by a scFv-based immunotoxin (scFv35-ETA). Our experiments showed that the immunotoxin, the first sarcoma-directed agent based on a fully human autoantibody fragment described so far, was effective both in vitro and in vivo against RMS cells and is therefore a promising candidate for further preclinical development. This would include the use of more refined murine RMS transplantation models using a broad spectrum of RMS cell lines as well as molecular modification of the immunotoxin for better stability and higher binding affinity in vivo as well as the coupling of the immunotoxin to human toxins or alternative effectors. Finally, our recent observation that a subgroup of metastatic malignant melanomas (MMs) can transdifferentiate into fAChR-expressing RMS-like cells [61] suggests that a refined fAChR-directed immunotoxin may also be of significant use in the treatment of these tumors and may therefore have a broader spectrum of therapeutic indications than anticipated so far.

4. Methods

4.1. Generation of Anti-AChR Fab and scFv Fragments. The cloning and expression of the high-affinity rat scFv192 fragment derived from the rat anti-AChR antibody mAb192 specific for the $\alpha67$–76 sequence (main immunogenic region, MIR) has been described previously [22]. The generation of the recombinant human Fab fragments Fab35 and Fab38 using a combinatorial cDNA thymus library from a patient with thymic lymphoid hyperplasia and production of autoantibodies specific for the anti-AChR γ-subunit has also been described [21]. Fab35 and Fab38 were used to produce scFvs by overlap extension PCR as shown in Figure 1. In detail for the production of V_H-V_L scFvs from Fab35 and Fab38 primers were used as indicated in Figure 1. By contrast for V_L-V_H orientation of the scFvs the V_L primers harboured the SfiI site at the 5′ end and the glycin/serin linker at the 3′ end, whereas the V_H primers had the glycin/serin linker at the 5′ end and the NotI site at 3′ end. The primers are listed in Table 2.

4.2. Production and Purification of the Immunotoxins. The protocol for the production and purification of recombinant immunotoxins was published previously [62]. Briefly, *E. coli* strain BL21DE3 pLysS (Stratagene, Heidelberg, Germany) was transformed with the corresponding plasmids and single

Table 2

Primers for scFv from Fab35/38 in orientation V_H-V_L
scFv/Fab35 V_H5$'$ SfiI: 5$'$-AGTCTAACGTTCGGCCCAGCCGGCCTCGGGGGGCGACTTGGTCCAGCCGGGGGGG-3$'$
scFv/Fab38 V_H5$'$ Sfi I:5$'$-AGTCTAACGTTCGGCCCAGCCGGCCTCGGGGGGAGGCGTGGTCCAGCCGGGGGGG-3$'$
scFv/Fab35/38 V_H3$'$ linker:5$'$-GCCACCCGACCCACCACCGCCCGAGCCACCGCCACCTGGAGAGACGGTGACCGTTGTCCCTTG GCC-3$'$
scFv/Fab35/38 V_L5$'$ linker:5$'$-GGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCAGTGATGACCCAGTCTCCA-3$'$
scFv/Fab35 V_L3$'$ Not I:5$'$-TGCTGCTGCGGCCGCTTTGATCTCCAGCTTGGTCCC-3$'$
scFv/Fab38 V_L3$'$ Not I: 5$'$-TGCTGCTGCGGCCGCCGTGATCTCCAGCTTGGTCCC-3$'$
Primers for scFv from Fab35/38 in orientation V_L-V_H
scFv/Fab35/38 V_L5$'$ Sfi I: 5$'$-ATGGCTCAGGGTTCGGCCCAGCCGGCCGTGATGACCCAGTCTCCA-3$'$
scFv/Fab35 V_L3$'$ linker: 5$'$-GCCACCCGACCCACCACCGCCCGAGCCACCGCCACCTTTGATCTCCAGCTTGGTCCC-3$'$
scFv/Fab38 V_L3$'$ linker:5$'$-GCCACCCGACCCACCACCGCCCGAGCCACCGCCACCCGTGATCTCCAGCTTGGTCCC-3$'$
scFv/Fab35 V_H5$'$ linker: 5$'$-GGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCATCGGGGGGCGACTTGGTCCAGCCGG GGGGG-3$'$
scFv/Fab38 V_H5$'$ linker: 5$'$-GGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCATCGGGGGGAGGCGTGGTCCAGCCGG GGGGG-3$'$
scFv/Fab35/38 V_H3$'$ Not I: 5$'$-TGCTGCTGCGGCCGCTGGAGAGACGGTGACCGTTGTCCCTTGGCC-3$'$

colonies used to inoculate 10 mL LB medium containing 50 μg/μL kanamycin. After 6–8 hours incubation at 26°C, 5 mL was added to 50 mL Terrific Broth containing 0.5 mM ZnCl, 50 μg/μL kanamycin, and shaken overnight at 26°C. After 14–16 hours, the 50 mL culture was used to inoculate 5 $\times$ 200 mL Terrific Broth supplemented as above and incubated for further 3–5 hours at 26°C. The broth was supplemented to final concentrations of 0.5 M sorbitol, 4% NaCl, and 40 mM betain and allowed to stand for 30 minutes before protein synthesis was induced by IPTG (final concentration 2 mM) and overnight shaking. After 20 hours, the bacteria were harvested by centrifugation (3700 g, 4°C), lysed in 75 mM Tris.HCl (pH 9), 300 mM NaCl, 10% glycerol, 5 mM DTT, 5 mM EDTA, sonicated and centrifuged at 25000 g, 4°C for 60 minutes. The supernatant was filtered, desalted on an FPLC column at a flow rate of 6 mL/minute (Amersham Biosciences, Heidelberg, Germany), and pooled protein fractions were purified and eluted by nickel-NTA-agarose chromatography (Qiagen, Hilden, Germany) using 75 mM Tris-HCl (pH 8.8), 10% glycerol, 1 M NaCl, and 0.5 M imidazol, at a flow rate of 2 mL/minute. Aliquots of each fraction were separated by 10% SDS PAGE and proteins were blotted onto nitrocellulose membranes as described [63]. Fractions containing scFv35-ETA were pooled, concentrated by ultrafiltration, and purified by size exclusion chromatography using a Bio-Prep SE-100/17 column (Bio-Rad) on the Aekta purifier workstation (GE). Protein was eluted with PBS (pH 7.4) and 1 M NaCl, analyzed by SDS-PAGE, quantified by densitometry (GS-700 Imaging Densitometer, Bio-Rad) after Coomassie staining in comparison with BSA standards, and verified by the Bradford assay (Bio-Rad).

4.3. Cell Lines and Cell Culture Reagents. Cell lines were obtained from American Type Culture Collection (Embryonal rhabdomyosarcoma: TE-671, RD, A-204, histiocytic lymphoma: U937), German Collection of Microorganisms, and Cell Cultures (Alveolar rhabdomyosarcoma: RH-30) or were provided by Ewa Koscielniak (Olgahospital, Stuttgart, Germany; embryonal rhabdomyosarcoma: FL-OH-1, alveolar rhabdomyosarcoma: Ax-OH-1). All cell lines were cultured in RPMI 1640 (Gibco Invitrogen, Carlsbad, USA) supplemented with 10% bovine calf serum (Biochrom AG, Berlin, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco Invitrogen).

4.4. Flow Cytometry. The cell-binding activity of scFv35-ETA was evaluated by flow cytometry. Cell suspensions containing 5 $\times$ 10^5 cells per mL were washed in PBS incubated on ice with 1 μg scFv35-ETA protein diluted in 100 μL PBS with 0.5% BSA. The cells were washed again three times and treated with 100 μL of the anti-ETA monoclonal antibody TC-1 [63] (1 : 100) for 30 minutes. After three further washes, the cells were incubated with PE-labeled antimouse immunoglobulin (1 : 50; Jackson ImmunoReseacrch Europe Ltd, Suffolk, GB) for 30 minutes at 4°C. Finally, cells were washed three further times and analyzed on a FACScalibur (Becton Dickinson, Heidelberg, Germany).

4.5. Colorimetric XTT Cytotoxicity Assay. The cytotoxicity assay was performed as described [64]. Briefly, 5 $\times$ 10^3 cell per well was plated in 50 μL on 96-well plates and let it attach for 3 hours at 37°C. A serial dilution of scFv35-ETA immunotoxin was applied in 50 μL aliquot resulting in a final volume of 100 μL. Plates were checked for chromogen development 72 hours after immunotoxin application by adding 50 μL XTT/phenanzine methosulfate and further incubation at 37°C for 4 hours. Absorbance was measured at 450 nm–630 nm as reference wave length. Colorimetric XTT conversion was also used in a competitive approach. Therefore, scFv35-ETA was added with a final constant

concentration of 1.5 nM to varying concentrations (1–100 molar excess) of scFv35.

4.6. Determination of IC$_{50}$ Values. The concentration required to achieve a 50% reduction of protein synthesis (IC$_{50}$) relative to untreated control cells was calculated using GraphPad Prism 4. All measurements were performed in triplicates.

4.7. Annexin V Apoptosis Assay. Induction of apoptosis was measured by analysis of Ca^{2+}-dependent binding of Annexin V to phosphatidylserine on the cell surface of apoptotic cells as described previously [33]. Briefly, 5×10^5 RD cells were seeded in 12-well plates in 1 mL medium containing either 1 μg scFv35-ETA or phosphate buffered saline as control in triplicates. Cells were harvested after 12, 14, and 48 hours of incubation at 37°C and incubated for 30 minutes on ice with 500 μL supernatant of Annexin V-eGFP-expressing HEK293T cells supplemented with 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, and 2 mM CaCl$_2$. After washing in PBS twice, final measurements were performed under the same buffer conditions with addition of 1 : 300 dilution of Propidium Iodide (1 mg/mL) on a FACScalibur (Becton Dickinson, Heidelberg, Germany).

4.8. Generation and Treatment of RMS in an In Vivo SCID Mouse Model. SCID mice were injected subcutaneously with 5×10^6 RD cells suspended in DMEM serum-free culture medium on day 1 of the experiment. On days 2–10, the mice received twice daily intraperioneal injections of either 10 μg scFv35-ETA ($n = 6$) or saline ($n = 4$), as described for other toxins [62]. Tumor size was monitored transcutaneously on days 4, 7, 10, 12, and 14 after inoculation of RD tumor cells using a caliper. Tumor size was calculated according to the formula: (length $\times$ width $\times$ height)/2.

Acknowledgment

References

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