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ORIGINAL ARTICLE

Soluble TNF-α Receptor I Encoded on Plasmid Vector and Its Application in Experimental Gene Therapy of Radiation-Induced Lung Fibrosis

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Abstract Post-radiation inflammatory reaction leads to an irreversible pulmonary fibrosis which may cause lethal respiratory insufficiency. Pathological inflammatory and fibrotic changes might be attenuated by inhibiting tumour necrosis factor (TNF)-α activity using TNF-α soluble receptors. Thus, an experimental antifibrotic gene therapy with the plasmid vector encoding a mouse soluble receptor I for TNF-α (psTNFR-I) was assessed. Soluble TNFR-I encoding gene was cloned into pcDNA3.1 plasmid. The ability of psTNFR-I expressing vector to transfect cells, and its biological activity in vitro and in vivo were examined by PCR, RT-PCR, MTT assay and ELISA. The C57Bl/6J mice received single intramuscular injection of psTNFR-I, conjugated with polyetylenimine (PEI) 25 kDa, equally divided to both hind legs, 3 days before irradiation (20 Gy, Co60), and either a single injection or ten injections once a week after irradiation. The data proved the effectiveness of psTNFR-I product to neutralise TNF-α activity in vitro. The in vivo plasmid incorporation and maintenance was confirmed. Measurements of plasma soluble TNFR-I levels showed that the in vivo gene transfer was effective. PEI was found to enhance transfection efficiency in vivo. The psTNFR-I/PEI complexes caused no toxicity in the transfected mice. C57Bl/6J mice that received prolonged psTNFR-I/PEI injections developed lethal fibrotic syndrome and died 8 weeks later than the mice treated with a double plasmid injection and the control mice treated with a control plasmid. Sequential administration of soluble TNFR-I by a nonviral, intramuscular gene transduction in the early and late post-radiation inflammatory phase prolonged survival of irradiated mice and attenuated the symptoms of lung fibrosis. The psTNFR-I gene transduction may provide a safe and simple method to partially neutralise TNF-α activity and prevent radiation-induced lung injury.

Keywords Soluble TNFR-I · psTNFR-I · TNF-α · Post-radiation lung fibrosis · Plasmid expressing vector · Gene therapy

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Introduction

Late adverse effects of radiotherapy are important clinical complications of radiation exposure during cancer treatment (Anscher 2005; Coggle et al. 1986; Mehta 2005). Ionising irradiation can induce a cascade of events on tissue and cellular levels, which result in the early post-radiation inflammatory reactions and late post-radiation fibrosis (Marks et al. 2003; Trask et al. 1985). High risk of late pulmonary fibrosis development, the irreversible side-effect of radiotherapy, limits the dose of radiation which can be delivered to tumours in the thoracic region. An irreversible pulmonary fibrosis causes breathlessness, cachexy and patients' death. The pathophysiological tissue

response after lung irradiation implies the induction of numerous cytokines (Piguet 1993; Rube et al. 2005; Rubin et al. 1995) and results in multicellular interactions involved in inflammatory and fibrogenic processes. Deregulation of several cytokines, including interleukin (IL)-1, IL-2, IL-4, IL-6, interferon-γ, tumor necrosis factor α (TNF-α), fibroblast growth factor 2 (FGF 2) and transforming growth factor β (TGF-β) leads to the cytokine-mediated epithelial and endothelial cell damage, fibroblast and myofibroblast expansion, extracellular matrix deposition and lung architecture destruction (Dent et al. 2003; Morgan and Breit 1995). Drugs known to slow-down the clinical progression of fibrosis in vivo include antibodies against inflammatory cytokines, such as TNF-α and vascular–endothelial growth factor (VEGF) (Anscher et al. 2005; Antoniou et al. 2007).

A number of studies have documented the role of a proinflammatory cytokine, TNF-α, a 17 kDa polypeptide, in fibrotic disease (Miyazaki, et al. 1995; Oikonomou et al. 2006; Piguet et al. 1990). It is generally accepted now, that TNF-α may be a key mediator in the pathogenesis of post-radiation lung injury. TNF-α is known to play a role in tissue remodelling, to protect against infections and to induce an acute phase of inflammatory response (Wajant et al. 2003). TNF-α inhibits lipoprotein lipase activity, induces the secretion of other cytokines, e.g. IL-6, activates endothelial cell adhesion and stimulates fibroblast proliferation (Piguet 1993; Sime et al. 1998; Wolff and Crystal 1997). However, TNF-α may also cause cachexy, tissue damage and irreversible shock effects (Beyaert and Fiers 1994; Kalthoff et al. 1993; Wajant et al. 2003). In addition, TNF-α expression leads to the induction of TGF-β1 (Finkelstein et al. 1994; Rube et al. 2000; Sullivan et al. 2005). TGF-β is the major pro-fibrotic factor, which can "switch on" the process of epithelial-to-mesenchymal transition (Broekelmann et al. 1991; Finkelstein et al. 1994; Kalluri and Neilson 2003; Kasai et al. 2005). Thus, TNF-α can play a significant role in the fibroproliferative disorders of lungs by inducing fibroblast and myofibroblast proliferation and recruitment, and neutralisation of the TNF-α activity may have important clinical implications.

Various strategies involving sTNF-α inactivation have been designed to protect post-radiation damage of normal tissue. Anti-inflammatory and antifibrotic effects as well as a significant down-regulation of TNF-α expression have been reported following pentoxifiline treatment (Marques et al. 1999; Okunieff et al. 2004). A suppression of the biological activity of a soluble trimeric form of TNF-α has also be achieved by monoclonal anti-TNF antibody treatment (i.e. infliximab, adalimumab) (Antoniou et al. 2007; Bargagli et al. 2004) or by binding a soluble form of TNFR-I and TNFR-II. A potential to inhibit the TNF-α activity by systemic application of sTNFR-II receptors was evaluated in the treatment of chronic inflammatory diseases with the recombinant solTNFR-II:IgH fusion protein—etanercept (Miyazaki et al. 1995; Olsen and Stein 2004; Walter et al. 2006; Wolthuis et al. 2009).

Introduction of a gene encoding the soluble form of TNFR-I or TNFR-II by viral or non-viral transfection could be an alternative method to transfer of the recombinant protein to an organism. Gene therapy has many advantages compared with recombinant protein treatment, such as low production costs or the efficiency of protein production already after a single-dose application. In the past decade, there have been several attempts to apply anticytokine experimental gene therapy using local or intramuscular delivery of constructs encoding genes for the soluble VEGF receptors (Hamada et al. 2005), TGF-β (Haiping et al. 2006; Yamada et al. 2007) and TNF-α (Prosser et al. 2006; Sugano et al. 2002; Sugano et al. 2004) in animal models of inflammation/injury of different organs, including joint, liver, lung and heart. Moreover, local gene transduction by intratracheal gene transfer with adeno-associated virus (AAV) system has been shown to be a promising method to a prolonged enhancement of the levels of soluble TNF-α receptors in the lung and serum (Sandalon et al. 2004).

However, due to immunogenicity of viral vectors, re-administration may be hindered by the inflammatory disease threat. This creates additional obstacles for gene therapy of chronic diseases associated with inflammatory processes, such as lung fibrosis which is radiation side-effect and results from long-lasting chronically increased levels of TNF-α. Thus, the therapeutic gene administration should be extended to a few months of pre-fibrotic inflammatory phase; however, the repeated application requires a safety system of gene delivery.

Therefore, to prevent/attenuate post-radiation lung injury, we constructed a plasmid vector encoding the soluble TNFR-I protein and took advantage to use it in an anti-TNF-α therapy in a fibrosis-sensitive mouse model. The obtained gene construct was used to compare the effects of short and prolonged plasmid administration on survival of the thorax-irradiated mice.

The C57/Bl/6J inbred mouse strain model has been commonly employed to study late post-radiation fibrosis development. Recent data have shown that the C57Bl/6J mice develop late irreversible effects—lethal late fibrosis without any evidence of acute pneumonitis. In the standard, single-dose thoracic irradiation of the C57Bl/6J mice, the timing of pneumonitis and occurrence of changes of the TNF-α mRNA levels in the irradiated lung depended on the irradiation dose (Johnston et al. 1998). Also lung fibrosis has been shown to depend on the radiation dose and to occur at 4, 6, or 8 months after treatment with 20, 15 or 12 Gy, respectively (Chiang et al. 2005).

In this study, a mouse skeletal muscle was considered an attractive target tissue for gene therapy, being a potential "biofactory" to produce the therapeutic, non-immunogenic proteins (Manthorpe et al. 1993; Prud'homme et al. 2001). Therefore, we performed the intramuscular gene transduction by repeated plasmid injection with polyetylenimine (PEI) as the transfection enhancer. The nonviral polycationic carriers, potent transfection mediators, is a widely used tool for transferring transgenes both in vitro and in vivo. It allows of a simple, non-immunogenic transfer of small amounts of plasmid DNA to a given organism. (Bragonzi et al. 1999; Erbachere et al. 2004).

Materials and Methods

Construction of the psTNFR-I Expressing Vector

Total RNA was isolated from the mouse spleen using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. cDNA was transcribed from 1 μg of total RNA at a final volume of 20 μl, using advantage RT-for-PCR Kit (CLONTECH, Palo Alto, CA). MMLV reverse transcript polymerase and oligo-dT were used. The gene encoding full-length mouse sTNFR-I was amplified using the following primers: F-5′GTTGTCAATTGCTGCCCTGT3′; R-5′ATTTGCAA GCGGAGGAGGTAG3′. The product of the PCR reaction was isolated from agarose gel and reamplificated with the following primers: F-5′ATGCGGAAGCTTGTTGTCAA TTGCTGCC3′; R-5′TAGTAT GGATCCTAATTTGCA AGCGGAGGAG3′. The second PCR reaction was performed to add restriction sites for HindIII 5′and BamHI 3′ and six nucleotide flanking sequences. The sTNFR-I encoding gene was ligated with the plasmid expressing vector containing neomycin-resistance gene, pcDNA3.1 (Invitrogen), using T4 ligase (USB Corporation, Cleveland, OH) overnight, at 16°C. The product of ligation was transferred into the competent *E. coli* DH5α cells, selected on LB Broth (Sigma-Aldrich, Inc.) bacterial culture medium containing ampicillin. The clone with an insert was propagated and isolated with Endofree Giga kit (Qiagene, Valencia, CA, USA).

Cells, Transient Transfection Conditions and Stable Clone Selection

To evaluate the psTNFR-I vector efficiency in vitro, human and mouse cancer cells were transiently and/or stably transfected. Human, TNF-insensitive, prostate carcinoma cells DU-145 (ATCC: American Tissue and Cell Collection), were transiently transfected to obtain conditioned media for sTNFR-I detection by ELISA (enzyme-linked immunosorbent assay). Transfections were performed on 24-well plates (5×10^4 cells/well) using PolyFect Transfection Reagent (Qiagene, Valencia, CA, USA). Shortly, for each well, 1 μg of plasmid diluted in a serum-free medium was complexed with 8 μl of the reagent, and incubated for 10 min at room temperature. Next, the transfection mix was added to the cells growing in the medium (RPMI-1640, Sigma-Aldrich Inc), 1 ml/well, containing 100 U/ml penicillin, 50 μg/ml streptomycin and 10% foetal bovine serum (FBS; Gibco). After transfection, cells were propagated for 48 h in the media with FBS for molecular investigations, or without FBS to obtain conditioned media. Mouse, TNF-sensitive, sarcoma cell line L1 (Miłoszewska et al. 2010), stably transfected with psTNFR-I was also derived. Clones were selected in the presence of 750 μg/ml G-418 (Geneticin, Sigma-Aldrich Inc.). The ability of the plasmid to produce soluble TNFR-I mRNA and protein was examined by RT-PCR and ELISA methods in transiently transfected L1 and DU-145, or stably transfected L1 cells.

Detection of Plasmid-derived sTNFR-I mRNA Expression

To distinguish the exogenous soluble TNFR-I gene transcript from the endogenous form, primers complementary to given region of sTNFR-I gene along with the flanking fragments of pcDNA plasmid were designed. Total mRNA was isolated from stably transfected L1 cells with TRIzol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription reaction was performed using Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA). PCR was performed with the following primers: F-5′GCCAAGCTGACAAGGACA CG3′; R-5′TGGCAACTAGAAGG CACAGTCG3′. The PCR conditions were as follows: 95°C for 5 min; 35 cycles 95°C for 30 s, 56.3°C for 30 s, 72°C for 30 s; 72°C for 10 min. Amplified gene products were analysed using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualised under UV light.

Effectiveness of psTNFR-I Transfection in Vitro

The presence of soluble TNFR-I in the culture media of the transfected cells was also tested indirectly, by exposure of stable L1/sTNFR-I clones, number 5 and 6 and control L1 cells to the 2, 5, 10 and 50 ng/ml of recombinant TNF-α (Sigma-Aldrich, Inc.). The cytotoxic effect of TNFα on transfected and non-transfected cells was examined by the MTT assay.

Cell Viability Assay (MTT)

The cytotoxic effect of TNF-α on cell clones stably transfected with psTNFR-I was evaluated by MTT assay.

Dimethyl thiazolyl diphenyl tetrazolium salt (Sigma-Aldrich, Inc.) was added to the cells at a concentration of 0.5 mg/ml in RPMI medium and cells were incubated for 3 h at 37°C. Purple formazan crystals were dissolved with isopropanol, added at 1 to 1 ratio. The results were read at 540 nm, and corrected for the value of reading at a wavelength of 690 nm (background). Each assay was performed in duplicate.

Animal Model and Post-radiation Fibrosis Induction

The radiation fibrosis-prone C57BL/6J mice were purchased from the Department of Genetic and Animal Breeding of the M. Skłodowska-Curie Memorial Cancer Centre (Warsaw, Poland). Sixty-four adult, 3 month-old female mice, approximately 22 g in weight, were anaesthetised and irradiated with 20 Gy ($LD_{50/150}$), in a single fraction applied selectively to the thorax, by a Cobalt 60 irradiator. Lead blocking was used to shield the remaining parts of the mouse body. The lung fibrosis-inducing dose is 12.5 or 15 Gy, but post-radiation reaction is then relatively slow. Higher radiation dose, 20 Gy, was used to accelerate the occurrence of lung fibrosis, and shorten the observation time. The irradiated mice were divided into two groups: pDNA and psTNFR-I recipients. Three days before irradiation all mice were treated with the first plasmid injection. Next, pDNA- or psTNFR-I- transfected mice were divided into two subgroups, one of which received a single plasmid injection 1 week after irradiation. The mice from the other subgroup received a prolonged treatment of 10 injections within 10 weeks. The therapeutic plasmid was applied once a week. Ten animals from each experimental subgroup were followed until death. These mice were clinically assessed, weighed, and the time of death was noted. The remaining mice were killed for histopathological and molecular studies 2 weeks and 1, 2, 3 and 4 months after irradiation.

Confirmation of Plasmid Transduction

Neomycin-resistance gene, presented in the therapeutic vector was chosen to confirm the transfection effectiveness. DNA from transfected muscles were extracted using SherlockAX Kit (A&A Biotechnology, Gdynia, Poland). Specific fragments were amplified with primers for the neomycin resistance gene, F: 5-AGGCTATTCGGCTATG-3 and R: 5-TT CCACCATGATATTC-3. Specific PCR products for neomycin confirmed that the muscle cells carried the psTNFR-I plasmid.

In Vivo Transfection Efficiency

Two groups of 12 C57Bl/6J mice were treated with the first injection of either pDNA or psTNFR-I plasmid. For in vivo transfection the pDNA/PEI 25 kDa (1:3 ratio) mix per one mouse was obtained as follows: 30 μg DNA was added to 70 μl NaCl and 90 μl (10 mM) PEI was supplemented with 10 μl NaCl, and the DNA-mix was slowly dropped to the PEI solution. After 30 min of incubation, the transfection mix was ready to use. Plasmids were introduced by intramuscular injection of 200 μl of transfection mix, equally divided to both hind legs. Three days later, six mice of each group were irradiated with 20 Gy by Co60 exposure. Transfection procedure was repeated after 1 week. Samples of muscles for plasmid detection and blood samples for protein sTNFR-I detection were collected from the transfected and six control, non-transfected mice.

sTNFR-I Level Assessment in Mice Plasma (ELISA)

In the non-irradiated and irradiated C57Bl/6J mice, two transfections were conducted, with 1 week interval, as described above. The sTNFR-I levels at 7th and 14th day following plasmid delivery were determined by using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instruction (R&D Systems, USA). Optical density was determined using a microplate reader at 450 nm wavelength. Soluble TNFR-I level was shown in pg/ml of plasma. All samples were examined in duplicates.

Total Soluble Collagen Determination

For total collagen measurement, lungs were collected from animals that received prolonged, 10 week therapy. Lung tissue samples were obtained from therapeutic and control plasmid recipients 2 weeks and 1–4 months after 20 Gy irradiation (3 mice per each point of time) and stored at −70°C for further analysis. The frozen lung tissue samples were weighed and homogenised in 0.05 M Tris buffer, pH 7.5, containing protease inhibitor cocktail (Sigma). Ten volumes of solvent-to-wet tissue weight ratio were used. Crude homogenates were centrifuged at $5{,}000\times g$ for 15 min. To obtain a transparent solution the samples were centrifuged at $15{,}000\times g$ for 10 min. The supernatants were used for cytokine analysis. Protein content was assessed by the Bradford method. The total collagen in mice lung homogenates was measured by Sircol™ Soluble Collagen Assay (Biocolor Ltd., UK), according to the manufacturer's instructions.

Histology

Mice were euthanised by intraperitoneal injection of sodium pentobarbital. The lungs of the same mice that were used for total collagen assay were 4% formaldehyde fixed and paraffin-embedded, sliced and processed for

histology. Tissue sections were stained with hematoxylin and eosin and by Masson trichrome methods.

Results

In Vitro Effectiveness of psTNFR-I Plasmid Transfection and Expression

The expressing vector activity was confirmed by the results of RT-PCR and ELISA assay. The presence of plasmid derived sTNFR-I transcript was found in the L1 cells transiently transfected with the psTNFR-I expressing vector and in the stably transfected clones derived from L1/psTNFR-I cells. Biological activity of the psTNFR-I plasmid was confirmed by ELISA and the data showed protein overexpression in conditioned medium samples (Table 1). We obtained stable transfectants presenting an overexpressed mRNA for soluble TNFR-I compared with base expression level in control L1 cells. (Fig. 1a). The presence of soluble TNFR-I protein in the media from stably transfected cells was also confirmed in the selected clones L1/psTNFR-I, number 5 and 6, exposed to increasing concentrations of TNF-α. The presence of TNF receptor I was found to protect cells against the toxicity of TNF-α (Fig. 1b). The in vitro results show that plasmid-derived soluble TNFR-I is a potent inhibitor of TNF-α activity. Previous studies on mouse and human cell lines demonstrated that psTNFR-I plasmid causes no toxic effect in transfection doses used (data not shown).

In Vivo Effects of the psTNFR-I/PEI Vector Incorporation and Its Capability to Produce the Therapeutic Protein

Plasmid transduction into the muscle cells in the site of transfection was determined. As shown by densitometry, higher levels of psTNFR-I incorporation were observed for 15 μg of plasmid/PEI than for 100 μg of a naked plasmid.

Table 1 sTNFR-I level in the conditioned media

Conditioned media from cells	sTNFR-I (pg/ml)	Increase	±SD
DU-145, control cells	5.1		1.5
DU-145, transient transfectants	47.7	×9.3	8.2
L-1, control cells	21.3		6.0
L-1/clone 5, stable transfectants	45.0	×2.1	4.5

Conditioned media (24 h, without FBS) from control L1 cells or stably transfected L1/psTNFR-I cells, clone 5, and control DU-145 or transiently tranfected DU-145 cells were obtained. Overexpression of sTNFR-I protein in the media was confirmed by the ELISA assay. Values were calculated in pg/ml of medium. Data are shown as mean ± SD, $n = 4$

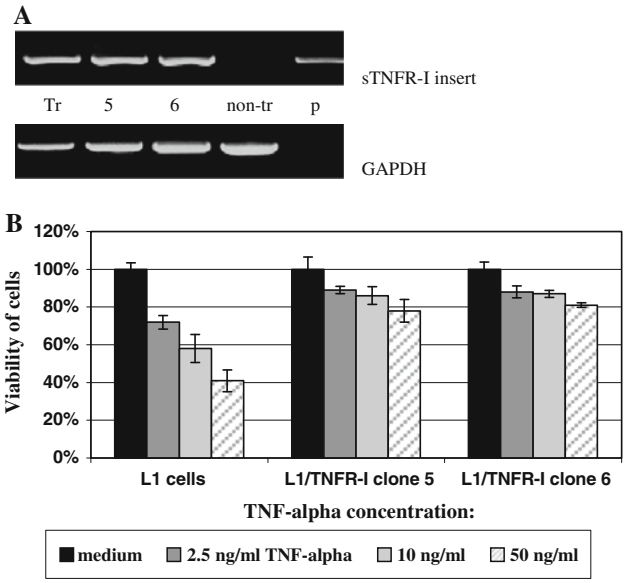

Fig. 1 RT PCR for the TNFR-Is insert expression and TNF-α neutralisation assay. The TNF-sensitive L1, mouse sarcoma cell line, was transiently or stably transfected. Transfections were performed using PolyFect Transfection Reagent. Stable clones were selected in a medium with G-418. The ability of the plasmid to produce sTNFR-I mRNA was confirmed using RT PCR method. **a** Representative results of stably transfected clones number *5* and *6*, and transient transfectants (tr) are shown on the photograph; *Non-tr* Non-transfected L1 cells as a control, *p* plasmid psTNFR-I. **b** The TNF-α neutralisation assay. Control L1 cells and stably transfected L1/psTNFR-I-clones *5* and *6*, were seeded on 96-well plate (2.5×10^3 per well) and after 1 day cells were treated with 2.5, 10 or 50 ng/ml recombinant TNF-α for 24 h. The cytotoxic effect of TNFα- was determined using MTT assay. L1/psTNFR-I-transfectants were protected against toxic effect of TNF-α

The results of psTNFR-I and pTNFR-I/PEI detection by PCR after the first and second transfection are shown in Fig. 2.

Elevation of soluble TNFR-I levels in plasma of irradiated and non-irradiated mice following repetitive psTNFR-I plasmid application was observed. After the first and second transfection (with 1 week interval), plasma TNF-α receptor I levels in the non-irradiated mice increased for about 30 and 60% of control sTNFR-I levels, respectively. The data showed that in the irradiated mice, 7 days after primary transfection and 3 days after Co60 exposure, plasmid-induced sTNFR-I levels summed-up with sTNFR-I induced by radiation, peaking to almost 60%. After a second transfection, on the 14th day, both sTNFR-I levels, if added, exceed 60%.

Intramuscular Administration of the psTNFR-I/PEI for Two and Half Months Following Thoracic Irradiation Prolongs Survival of Mice

The obtained data show that transfection with psTNFR-I/PEI 3 days before and 7 days after 20 Gy exposure had no

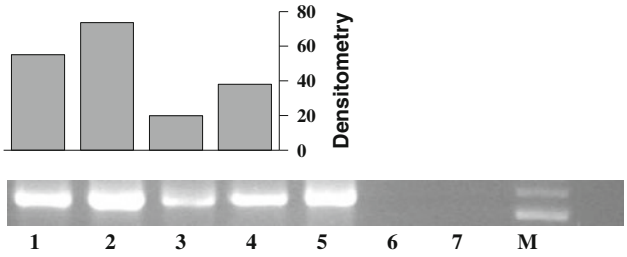

Fig. 2 Detection of psTNFR-I plasmid in the muscles. Muscle DNA was extracted with Sherlock AX kit. The specific fragment of DNA, selected for plasmid detection, was amplified with primers for the neomycin-resistance gene. The PCR product confirmed the maintenance of psTNFR-I plasmid in the mouse muscles 7 days after a single or double transfection (with 1 week interval) by naked plasmid or plasmid complexed with PEI, administered as follow: *1* single, psTNFR-I/PEI; *2* double, psTNFR-I/PEI; *3* single, psTNFR-I naked; *4* double, psTNFR-I naked; *5* control plasmid samples, next, plasmid detection following 2 months after double transfection; *6* psTNFR-I/PEI; *7*. psTNFR-I naked; *M*. marker. Similar levels of psTNFR-I incorporation were observed with 100 μg of naked plasmid and with 15 μg of plasmid with transfection enhancer, PEI. Repetition of plasmid transfection caused intramuscular psTNFR-I plasmid cumulation. Therapeutical plasmid, both naked and complexed with PEI, was cleared from the site of injection during 2 months following transfection

influence on survival. In consequence, the control mice and the psTNFR-I treated group died at the same time after irradiation (Fig. 3a). Mice that received a prolonged, sequential intramuscular injections of psTNFR-I/PEI polyplexes (1 before and 10 times after thorax irradiation) (Fig. 3b) in the early and late radiation induced inflammation phases, presented longer survival time. The supralethal radiation dose of 20 Gy killed 50% of control mice during 21–22 weeks (20 Gy = $LD_{50/150}$). At the same time, 90% of psTNFR-I/PEI recipient animals survived ($LD_{10/150}$). In the group of animals that received the psTNFR-I/PEI injections, life span was prolonged about 7–8 weeks compared with pDNA recipients, but within 29th to the 37th week of observation time all psTNFR-I treated mice were dead (*p* value: $\leq$0.028). No toxic effect or changes of mice body weight after psTNFR-I treatment were observed (Fig. 4b). Thus, injections of PEI complexed with plasmid encoding soluble TNFR-I were safe for the recipients, partly prevented fibrosis and delayed mouse death.

Histological Assessment of Post-radiation Lung Fibrosis

In the irradiated animals that received prolonged, 10 week plasmids injections, the development of lung fibrosis area and progressive collagen deposition was shown by the hematoxyline–eosine and Masson's trichrome staining. The fibrogenesis phase was characterised by the development of typical fibroblast foci and deposition of extracellular matrix in the irradiated lungs. The psTNFR-I plasmid administration slightly reduced collagen deposition, alveolar wall thickness and all other histologically visible signs of fibrosis in the lung samples obtained from mice 4 months after irradiation. Histopathological data demonstrated that also focal and perivascular fibrotic lesions with consolidation of lung parenchyma and the loss of alveolar architecture were less developed in psTNFR-I-treated group compared with the control mice. The representative histological sections of the lungs of the control plasmid- and psTNFR-I-treated mice, examined 4 months after irradiation are shown in Fig. 5. To present the treatment effectiveness the histology images of lungs of non-irradiated and of irradiated mice obtained 6 months after exposure are exemplified. Histopathological fibrotic injury detection performed 2 and 4 weeks after irradiation showed no visible differences between control mice and the psTNFR-I recipients (data not shown).

Total Soluble Collagen Contents in Lung Tissue during Post-Radiation Period

To illustrate the degree of fibrotic process total collagen was measured. During 4 month observation a tendency for a decreased total collagen level in the psTNFR-I treated mice was noted, but the difference was not significant (Fig. 6).

Discussion

TNF-α is apparently an important pulmonary fibrosis mediator, since radiation- or bleomycin-induced lung injury in mice is associated with a marked up-regulation of the TNF-α at mRNA and protein levels (Dent et al. 2003; Distler et al. 2008). Thus, fibrotic injury could be prevented by anti-TNF antibodies (Antoniou et al. 2007; Bargagli et al. 2004; Olsen and Stein 2004; Zhang et al. 2008). Within past two decades there is a growing interest in the potential of the TNF-α soluble receptors I and II as inhibitors of normal tissue injury caused by radiation or chemotherapy (Nakamura et al. 1996; Sandalon et al. 2004; Walter et al. 2006; Wolthuis et al. 2009). The role of sTNFR-I and II as regulators the TNF-α-induced inflammation and fibrosis was further confirmed by Huaux et al. (1999). The authors suggested that the soluble TNFR-I and TNFR-II overproduction observed in the resolving and fibrosing alveolitis mouse models represents an attempt of the host to limit the extension of pulmonary inflammation and fibrosis. At the same time the data on the effect of systemic TNF-α blocking were obtained in a different study which examined the effect of etanercept—solTNFR-II:Fc-Ig

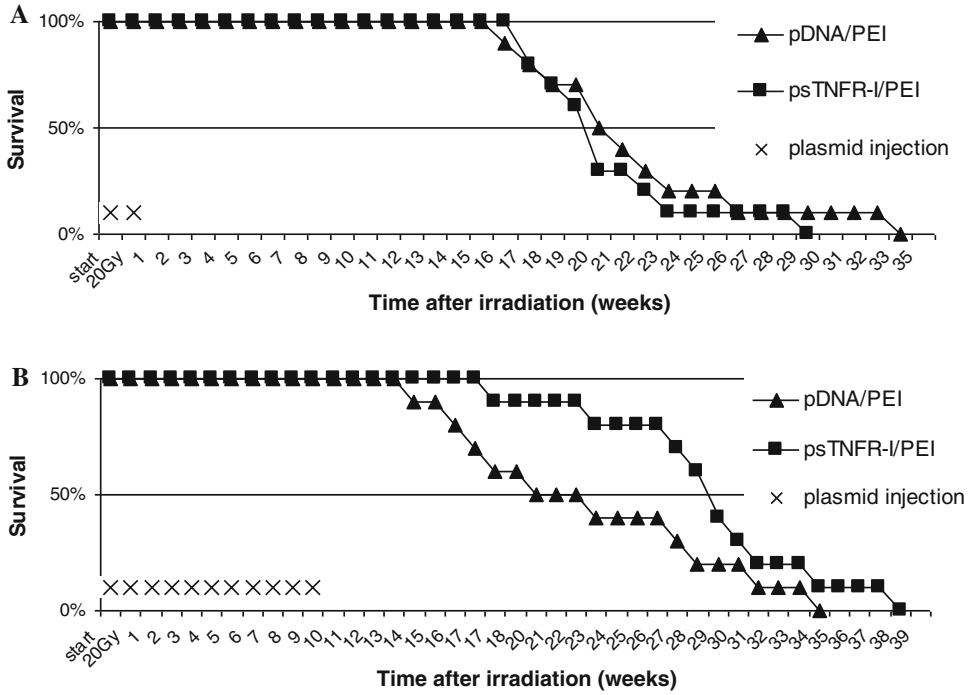

Fig. 3 The survival of irradiated C57Bl/6J mice with psTNFR-I/PEI polyplexes treatment. The C57Bl/6J female mice were irradiated with 20 Gy in a single dose selective to the thorax with Co60. The animals were divided into two groups: mice, which received pDNA only and psTNFR-I recipients. **a** The mice injected pDNA or psTNFR-I 3 days before and 7 days after irradiation. No effect on survival time was observed. **b** The mice treated with plasmid for prolonged period of time—the first, 3 days before and next, 10 plasmid transfections, weekly. In subgroup of mice that received therapeutic, psTNFR-I plasmid, life span was prolonged about 7–8 weeks compared with pDNA recipients. Irradiation of 20 Gy killed 50% of control mice during 21–22 weeks (20 Gy = $LD_{50/150}$). At the same time, 90% of psTNFR-I/PEI recipient animals survived, but within 29th to the 37th week of experiment all psTNFR-I treated mice were dead (p value: $\leq$0.028)

fusion protein in experimental arthritis and next—in lung injury (Loetscher et al. 1991; Piguet et al. 1993; Smith et al. 1998; Wolthuis et al. 2009). Also antifibrotic activity of gene constructs encoding soluble TNFR-II and TNFR-I in lung, heart and hepatic injuries (Li et al. 2000; Prosser et al. 2006), was tested in a few in vivo experiments involving local gene delivery (Sandalon et al. 2004; Walter et al. 2006).

In our Gene Therapy Laboratory, despite the promising results in in vivo transgene delivery shown with viral vectors (i.e. AAV), we still rely on administration of plasmid DNA into skeletal muscle (Małecki et al. 2003; Małecki et al. 2005; Proczka et al. 2006). These vectors are low immunogenic as against viral vectors, and long expressed in the transfected nondividing striated myocytes. Plasmid DNA expressing vectors are relatively safe, can be repeatedly administered to animals and are preferred for the therapy of chronic inflammatory lesions. However, naked DNA injection usually generates relatively low levels of circulating protein, i.e. cytokines and/or their soluble receptors. The augmentation of plasmid dose is not recommended because of possible intensification of anti-CpG immunological response. Presence of unmethylated CpG motifs (of bacterial origin) and its immunogenicity could limit the expression of introduced gene (Mitsui et al. 2009; Reyes-Sandoval and Eartl 2004). Thus, when higher expression levels are desired, such as those necessary to achieve the soluble TNFR-I levels optimal for TNF-α inhibition, applying transfection enhancer methods is needed.

One of the most non-invasive, safe and effective methods for a single or multiplicated transgene administration is to transfer small amounts of plasmid DNA with synthetic polycationic DNA carriers, PEI. The efficiency seems to be comparable with electrotransfer method. These two methods, or their modifications, enhance the delivery of molecules to animal and human tissues 10–100 times (Heller et al. 2008; Smolarczyk et al. 2005). Conjugation of plasmid DNA with the cationic polymers at optimised ratios results in pDNA/PEI polyplex formation, which are known to display high transfection efficiency in cell culture and have a potential for gene delivery in vivo (Erbachere et al. 2004; Ogris et al. 2003). It allows significant reduction of the amount of psTNFR-I plasmid, which is necessary for therapeutic gene application and minimises the number of immunogenic CpG motifs.

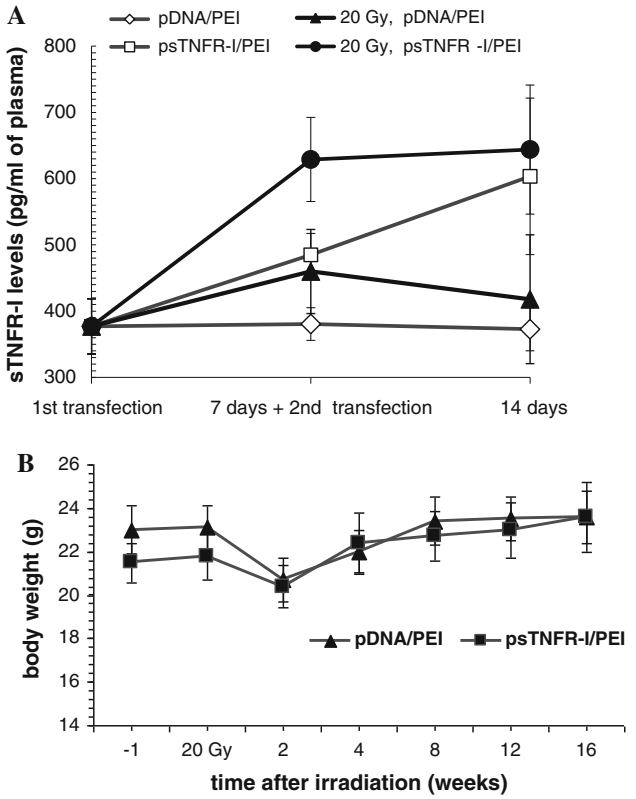

Fig. 4 In vivo biological effects of psTNFR-I transfection. The irradiated (20 Gy) and non-irradiated C57Bl/6J mice were divided into two groups, pDNA and psTNFR-I plasmid recipients. Mice received one or two transfections, in 1 week interval. The first plasmid injection took place 3 days before Co60 exposure. The soluble TNFR-I levels in the plasma of irradiated and non-irradiated C57Bl/6J mice at the 7th or 14th day after initial plasmid injection were determined using ELISA assay. **a** Compared with control mice and pDNA recipients, plasma soluble TNFR-I levels in non-irradiated mice increased about 30 and 60% following single and double of psTNFR-I transfection, respectively, In the irradiated mice (20 Gy pDNA and 20 Gy psTNFR-I), plasmid-induced sTNFR-I levels summed-up with sTNFR-I induced by radiation, peaking to almost 60% on the 7th day. After a second transfection, on the 14th day, both endogenous and exogenous sTNFR-I levels, if summarised, exceed 60%. **b** Changes of mice body weight after irradiation and during psTNFR-I treatment was also measured. No toxic effect and differences in the body weight between the irradiated mice treated with pDNA plasmid and psTNFR-I were observed

In our study, prior to the preparation of optimal transfection mix to in vivo therapy, higher effectiveness of transfer of the psTNFR-I with PEI compared with the naked psTNFR-I was confirmed. After DNA/PEI complexation, compared with naked DNA, nearly ten times smaller amount of psTNFR-I administered in one injection was needed to obtain the same level of plasmid incorporation. On this basis, the therapeutic dose of psTNRF-I/PEI was determined. Next, our data showed a cumulative, dose-dependent effect of intramuscular psTNFR-I/PEI transfer on the increased plasma level of soluble TNF-α receptor in transfected mice.

To investigate antifibrotic effects of systemic gene therapy, we chose a special, fibrosis-prone C57Bl/6J mice strain, with a well-documented lung TNF-α level alteration following irradiation (Ao et al. 2009; Rube et al. 2004). After a single-dose thoracic irradiation, the time of pneumonitis occurrence and changes of the TNF-α mRNA and protein levels in the irradiated lung have been shown to depend on the irradiation dose (Johnston et al. 1998). As shown by Rube et al. (2004); Ao et al. (2009), following 20 Gy irradiation, the dose used also in our study, the expression of mRNA for TNF-α in the whole lung tissue increases with time in a bimodal fashion, peaking at 1 h and at the 2nd month. In addition, markedly increased TNF-α mRNA levels observed 1 h after irradiation decline within 48 h.

We have previously confirmed the increase of soluble TNFR-I levels in parallel with the TNF-α levels in plasma of irradiated C57Bl/6J mice (Przybyszewska et al. 2009; Przybyszewska et al. 2010). Elevated TNF levels are considered to switch the fibrotic mechanisms on. Therefore, in the present study, the period of therapeutic plasmid injection comprised the timing of the two peaks of TNF-α (mRNA in lung and serum protein) expression and the interval between them. Based on the data described above we determined the start and the end-point of gene therapy application (first weekly dose 3 days before and the last dose 10 weeks after irradiation, respectively). It was an optimal timing for therapeutic intervention to target the two known peaks of the radiation-induced elevated TNF-α levels in the lungs and serum (24 h and 8 weeks after irradiation). Considering the transgene activity that lasts for 2 weeks following injection, we managed to cover the 3 month period of elevated TNF levels with the anti-TNF therapy.

To differentiate two radiation-induced lung reactions, pneumonitis and fibrosis, we applied the criterion of time. In the model of irradiated C57Bl/6J mice, the biphasic increase of serum and lung tissue TNF levels occurs during first 1–2 months in pre-symptomatic phase. It is closely linked to the development of pneumonitis after 3–4 months. TNF-α declines after the second peak of TNF level, and after 5–6 months, at the time of the fibrosis onset, TNF is not markedly increased (Chiang et al. 2005). On the fourth month, the first symptoms of fibrotic disease occur in mice lungs and can be identified by histological examinations.

Our data suggest that in irradiated C57Bl/6J mice, antifibrotic effect of psTNFR-I/PEI administration depends on the length of application and that there is time-correlation with the levels of TNF-α mRNA and protein in lung tissue. We showed that a two-dose anti-TNF-α transfection: first, before and second, shortly after irradiation, does not prevent lung injury. However, long-term plasmid

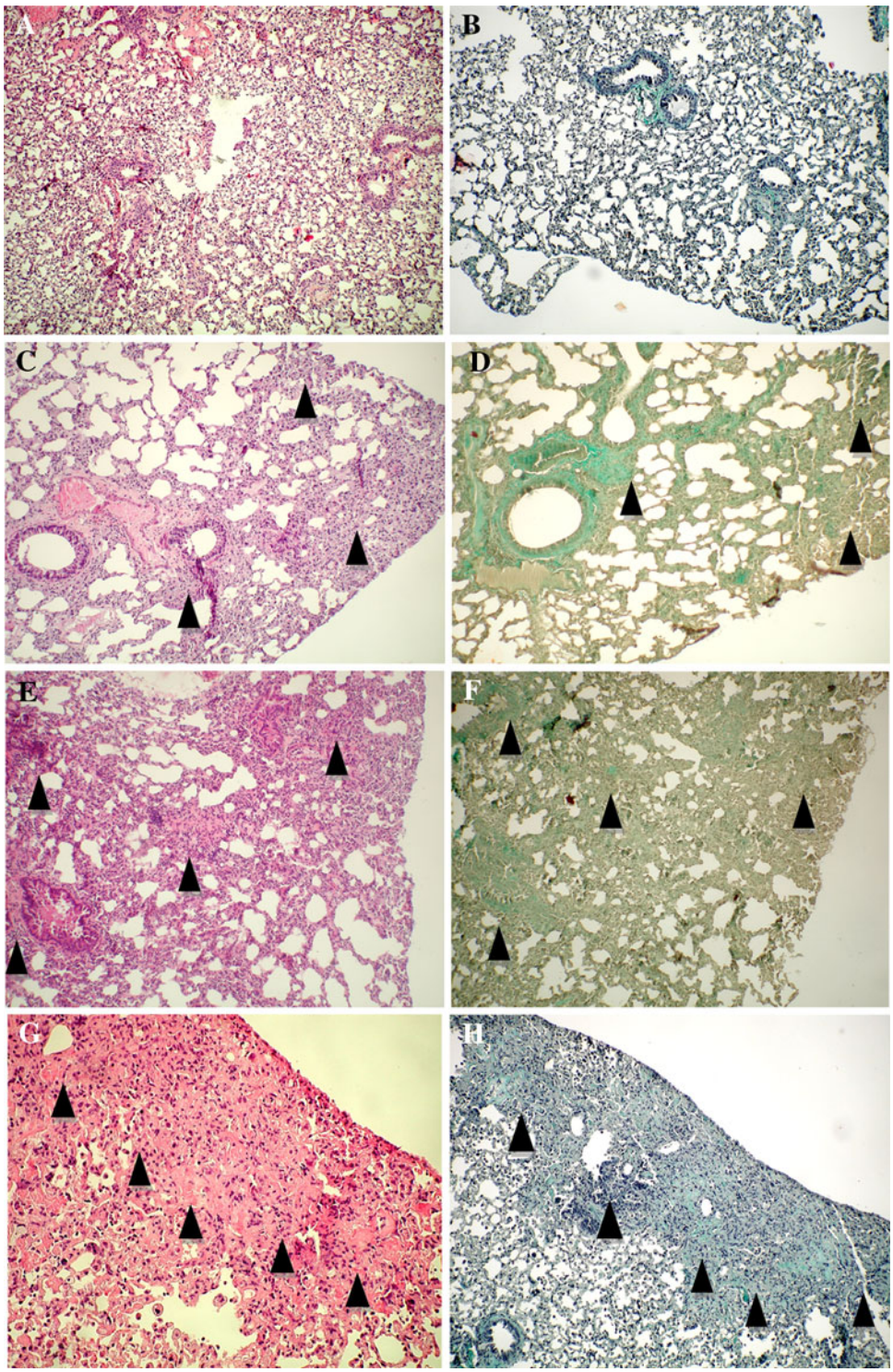

Fig. 5 Histology of the irradiated lungs of C57Bl/6J mice. Three days before to 20 Gy (Co60) exposition animals were transfected with the first dose of therapeutical or control plasmid. After irradiation the mice were treated with psTNFR-I (**c**, **d**) or pDNA plasmid (**e**, **f**) for 10 weeks. Animals received 10 plasmid injections, weekly. Representative images taken from after 4 months show the lungs stained with hematoxylin-eosin (**c**, **e**) and Masson tricolour (**d**, **f**). The psTNFR-I plasmid administration slightly reduced collagen deposition and alveolar wall thickness in the lung. The signs of fibrosis (focal and perivascular fibrotic lesions—shown by *arrows*) were less developed in psTNFR-I recipients compared with the control mice. Lungs section from healthy, non-irradiated mouse (**a**, **b**) and from irradiated mouse—6 months after exposure (**g**, **h**), are presented as an example. Magnification ×10

administration yields promising results. We found that to obtain a preventive/therapeutic effect, it was necessary to repeat the sTNFR-I gene transfer at least ten times. Ten-week administration scheme prolonged elevated sTNFR-I levels and resulted in the prolonged survival of the irradiated mice for about 8 weeks. This effect was confirmed by the total collagen accumulation and differences observed between control and psTNFR-I recipient mice in lung parenchymal lesions in 4 months after irradiation. It should be underlined that, in spite of high, supralethal radiation dose (20 Gy) applied, 50% post-radiation survival in the psTNFR-I-treated group occurred on the 29th week, and not on the 21st week, as in the non-treated mice and we consider that this observation seems promising.

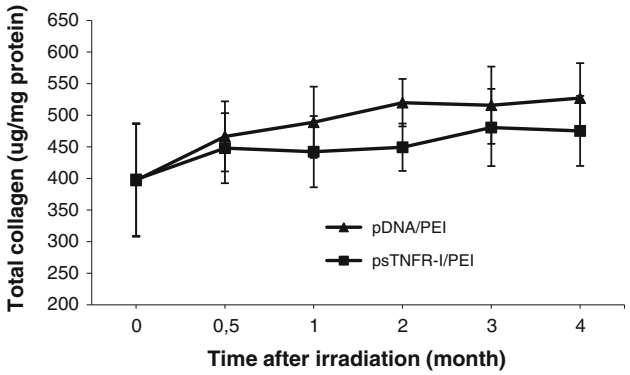

Fig. 6 Total collagen deposition in the lungs of irradiated mice. Total collagen was measured by Sircol Solouble Collagen Assay in the lungs, 2 weeks and 1, 2, 3 and 4 months after irradiation of mice transfected with control plasmid or with psTNFR-I. The tendency for a decrease in total collagen level in mice treated with psTNFR-I construct was observed. The data received in three independent measurements are expressed as the mean ± SD for each time-point

However, after the 27th week following irradiation, the mortality rate in the psTNFR-I recipients suddenly increased. As a result, total survival time of psTNFR-I-treated animals was equal to that of the last control mice that survived the whole observation time. We suppose that it happened because since the last, 10th plasmid injection there has been a gradual decrease of therapeutic soluble TNFR-I protein production by the "biofactory" of the transfected muscles. Furthermore, as we and other authors examined (Ao et al. 2009; Wolff and Budker 2005), in the case of intramuscular pDNA introduction, the plasmid was cleared from the site of injection during 2 months after transfection. Inflammatory condition also lowers expression of introduced gene in the later period. It is assumable that local immunological reaction anti-CpG is possible in the site of plasmid injection (and decreasing transgene expression), but we did not investigate it. However, psTNFR-I product by association with its ligand, TNF-α, one of the cytokines involved in anti-CpG inflammatory reaction (Smolarczyk et al. 2005) could inhibit TNF-α activity in transfection area. Then, psTNFR-I plasmid could also have a self-protecting activity but this hypothesis should be confirmed.

The protein soluble TNFR-I, non-stabilised, has a short lifetime in circulation (Bazzoni and Beutler 1996; Olsen and Stein 2004), thus, part of soluble TNFR-I molecules could had been destroyed and never reached lung tissue. Therefore, the unrelenting plasmid-derived therapeutic protein delivery is necessary to maintain of antifibrotic effect.

The delayed increase in mortality rate in the psTNFR-I-treated group can also be explained by a reversible binding of soluble TNF receptors, which stabilises the trimeric TNF molecules. Thereby, paradoxically, the half-life of TNF-α is prolonged and the reversible-stabilised TNF molecules serve as a reservoir of TNF-α when its levels are low (Aderka et al. 1992). We suppose that in order to obtain better results of gene therapy, plasmid administration for more than 10 weeks may be necessary. As we mentioned above, following 20 Gy irradiation, lung fibrosis in experimental animals begins not earlier than after the 4th month and progressively increases in severity at the 5th and 6th month. To verify this hypothesis we consider a late administration of psTNFR-I in the next step of investigations. To improve the antifibrotic effect of gene therapy not only a prolonged time of transfection, beyond the inflammatory phase, but also more efficient PEI carrier selection and duration of transgene expression should be considered. The latter is possible by methylation of cytosine residues of CpG sequences before therapeutic plasmid applications in vivo. This method can improve plasmid sustainability and extend the transgen expression (Reyes-Sandoval and Eartl 2004; Smolarczyk et al. 2005).

We conclude that modified, improved intramuscular soluble TNFR-I gene delivery with PEI polyplexes may be a simple, safe and effective lung radioprotection treatment. However, due to the more complexed role of TNF and TNFRs in fibrosis development (Distler et al. 2008; Khasnis and Calabrese 2010), than it had been assumed, any anti-TNF therapy, including gene therapy, must be applied with caution and requires preclinical studies in different animal models.

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