An antibody fusion protein for cancer immunotherapy mimicking IL-15 trans-presentation at the tumor site

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6 figures and tables
Abstract

Cytokines driving the immune response are powerful tools for cancer immunotherapy, but their application is generally limited by severe systemic toxicity. Targeted approaches by means of antibody-cytokine fusion proteins might enable to focus the cytokine activity to the tumor site, thereby reducing unwanted side effects. Here we investigated the possibility to improve the efficiency of IL-15 presentation in a targeted approach by the incorporation of an IL-15Rα chain fragment, mimicking physiological trans-presentation. Therefore, an antibody cytokine fusion protein (scFv_RD_IL-15) composed of an antibody moiety targeting the tumor stromal fibroblast activation protein (FAP), an extended IL-15Rαsushi domain (RD) and IL-15 was generated, exhibiting antibody-mediated specific binding and cytokine activity in soluble and targeted form. Comparative analysis with a corresponding antibody fusion protein devoid of RD (scFv_IL-15) showed for scFv_RD_IL-15 in solution enhanced stimulatory activity on Mo7e (IL-15Rβγ) cells and reduced proliferation response on CTLL-2 (IL-15Rαβγ) cells, while in FAP-targeted i.e. membrane-bound form, comparable proliferation of CTLL-2 (IL-15Rαβγ) cells was obtained. In addition, scFv_RD_IL-15 achieved in its soluble and target-bound form stronger proliferation and cytotoxicity on unstimulated and activated T cells, respectively. Furthermore, in vivo analysis in a lung metastasis tumor mouse model revealed a superior antitumor effect for scFv_RD_IL-15 in comparison to that obtained by an untargeted or RD missing version of IL-15 fusion protein. Thus, tumor-directed trans-presentation of IL-15 in association with RD in form of an antibody fusion protein appears as a promising approach to further improve the antitumor effect of IL-15.
Introduction

The application of cytokines to induce or promote the generation of an effective antitumor immune response is an attractive approach in cancer immunotherapy. Among a continuous growing list of cytokines with antitumor potential (1), IL-15 appears as a candidate with especially promising features (2, 3).

IL-15 belongs to the 4 α-helix bundle cytokine family and was initially described in relation to IL-2, with whom it shares several biological functions, e.g. the induction of proliferation and differentiation of NK, T, and B cells (4, 5, 6). Currently, the major function of IL-15 appears to focus on the generation, proliferation and activation of NK cells and the support of the survival of CD8+ memory T cells (7, 8). Unlike IL-2, IL-15 rather inhibits activation-induced cell death (AICD) (9) and seems not to exert crucial influence on regulatory T cells (10), important properties in view of an immunotherapeutic application.

IL-15 and IL-2 have two receptor units (IL-2/15Rβ and γc) of their heterotrimeric receptors in common, while the third receptor unit (IL-2Rα and IL-15Rα) is specific for each cytokine. Functional differences observed between IL-2 and IL-15 presumably ascribe mainly to the interaction with their respective alpha receptor unit. While IL-2 is secreted by activated T cells and binds in a soluble form to its high affinity heterotrimeric receptor (IL-2Rαβγ) on activated cells, IL-15 acts mainly in a membrane-bound form, presented in trans in association with its specific IL-15Rα subunit by monocytes and dendritic cells (APCs) to NK cells or CD8+ T cells, expressing the heterodimeric intermediate affinity receptor IL-2/15Rβγ (11) (Fig. 1A). For the process of trans-presentation intracellular IL-15 binds to the IL-15Rα chain that shuttles to the cell surface where it presents IL-15 to neighboring cells expressing the receptor IL-2/15Rβγ. Thus, a more directed and controlled action of the cytokine is
achieved (12). The sushi domain of the IL-15Rα chain has been identified as the structural region involved in IL-15 binding (13). There is also the possibility that IL-15 binds to soluble versions of IL-15Rα forming soluble complexes with stimulatory or inhibitory activity. In this case, soluble IL-15Rα might arise from proteolytical cleavage of the membrane form by metalloproteinases (14, 15).

The antitumor potential of IL-15 was observed in mouse models employing recombinant IL-15 (16, 17), antibody-IL-15 fusion protein (18), IL-15 gene-modified tumor cells (19, 20) and IL-15 transgenic mice (21). The relevance of IL-15 trans-presentation for an efficient antitumor response was shown in a mouse model with IL-15Rα transfected MC38 tumor cells. Here, the expression of IL-15Rα dose-dependently delayed the formation of fatal pulmonary metastases through the activation of NK cells (22). Subsequently, it was shown in vitro and in vivo that soluble complexes of recombinant IL-15 and IL-15Rα were much more stimulatory than soluble IL-15 alone (23). Treatment of mice with IL-15xIL15RαFc complexes enhanced the proliferation of CD8+ memory T cells and NK cells in vivo. Furthermore, tumor burden could be reduced and survival extended in a systemic B16 melanoma mouse model (24, 25). Similar results were obtained by an IL-15/IL-15Rα domain fusion protein (26, 27).

We hypothesize that targeting a fusion protein consisting of IL-15 and an extended IL-15Rαsushi domain by an antibody moiety to the tumor site will mimic locally the trans-presentation of IL-15 by the IL-15Rα, enhancing its stimulatory potential, thus further improving the antitumor effect of IL-15 (Fig. 1A). In this study we report the generation of the antibody-cytokine fusion protein scFv_RD_IL-15, composed of the fibroblast activation protein (FAP)-specific scFv (scFv36), an IL-15Rα fragment (RD) and IL-15. We show antibody-mediated binding and cytokine activity for this construct in solution or targeted form
and describe the influence of the RD on the receptor activation properties of the fusion protein. Proliferation of an IL-15Rβγ receptor-expressing cell line (Mo7e) was enhanced by the RD-containing fusion protein in solution, whereas proliferation of a cell line expressing the IL-15Rαβγ receptor (CTLL-2) was reduced. Nevertheless, in target bound form, the proliferation effect on CTLL-2 was mainly RD-independent. Also, PBMC could be effectively stimulated by scFv_RD_IL-15 in targeted and soluble form. Here, scFv_RD_IL-15 was more efficient than scFv_IL-15 enhancing the proliferation and cytotoxicity response of unstimulated and activated T cells, respectively. Furthermore, scFv_RD_IL-15 was more effective than scFv_IL-15 or RD_IL-15 in the treatment of a lung metastasis mouse model. Thus, the combination of antibody-mediated tumor targeting and IL-15Rα domain associated trans-presentation of IL-15 in form of a fusion protein appears as a promising approach for cancer immunotherapy.

Materials and Methods

Materials

Antibodies were purchased from Biolegend [αCD3-PerCP, αCD8-PE], Immunotools [αCD4-PE, αCD56-PE], Miltenyi Biotec [αhexahistidyl-tag-PE] and Santa Cruz [αIL-15, αhexahistidyl-tag-HRP, αCD107a-FITC]. Recombinant human IL-2 and GM-CSF were obtained from Immunotools and recombinant fibroblast activation protein (FAP) from R&D Systems. B16-FAP and B16 cells (K. Pfizenmaier, IZI, Stuttgart) were cultured in RPMI 1640, 5% FBS. Mo7e (H. van der Kruip, IKP, Stuttgart) and CTLL-2 (P. Scheurich, IZI, Stuttgart) were cultured in RPMI 1640, supplemented with 20% FBS, 10mM HEPES, 2mM L-Glutamine, 0,13mM L-asparagine, 0,05mM beta-mercaptoethanol, 1mM natriumpyruvate, non essential amino acids and 10ng/ml rhGM-CSF (Mo7e) or 400IU/ml rhIL-2 (CTLL-2),
respectively. Cells were tested for mycoplasms and their morphologic appearance monitored by microscopic means. Antigen (FAP) expression and cytokine growth-dependence of the respective cell lines was verified by flow cytometry and proliferation-assays, respectively. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat of healthy donors (blood bank, University Medical Center Ulm, Germany) by ficoll gradient and were cultivated in RPMI 1640, 10% FBS. C57BL/6Jrj mice were purchased from Elevage Janvier. Animal care and all experiments performed were in accordance with federal guidelines and have been approved by university and state authorities.

**Generation of the recombinant fusion proteins**

The procedure is described in the Supplementary Data (S1).

**Binding analysis**

2.5x10^5 cells (B16-FAP/B16) were incubated with fusion protein (200nM or titrated, accordingly) for 2 hours at 4°C. After washing, cells were incubated with a hexahistidyl-tag directed PE-conjugated mouse monoclonal antibody, for 1 hour at 4°C. Washing and incubation steps were carried out in PBS, 2% FBS and 0.02% sodium azide. Finally, cells were analyzed in a Cytomics FC500 (Beckman Coulter).

**Proliferation assays with cell lines**

Cytokine growth-dependent cells (CTLL-2/Mo7e) were washed and suspended in culture medium without neither IL-2 nor GM-CSF. 20,000 cells/well were seeded in 96-well-plates and starved for 4 hours. Then the fusion proteins were added to the cells. After 3 (CTLL-2) or 4 (Mo7e) days, proliferation of the cells was measured by MTT-assay (28). The cytokine activity of the fusion proteins presented in an antibody-mediated membrane-bound form was analyzed in the following setting: 10,000 cells/well (B16-FAP/B16) were seeded in a 96-well-
tissue culture plate. Next day, cell growth was arrested by treatment with 10µg/ml mitomycin C (Sigma), for 2 hours at 37°C. Cells were washed twice with medium before addition of the fusion proteins. After 1 hour incubation at room temperature, the supernatant was discarded and adherent cells washed twice with medium. Then previously starved CTLL-2 (20,000 cells/well) were added and incubated for 3 days. Finally, the cells in suspension (CTLL-2) were carefully transferred to a new 96-well-plate, followed by the MTT-assay (28).

Proliferation assays with PBMC

PBMC were stained at a concentration of 1x10^6 cells/ml with 625nM CFSE (carboxyfluorescein diacetate succinimidyl ester) using the CellTrace™ CFSE cell proliferation kit (Life Technologies), following the instructions of the manufacturer. Proliferation induced by the fusion proteins in solution was assessed by incubating 2x10^5 PBMC/well with the corresponding fusion protein for 5 days, followed by flow cytometry analysis. Additional antibody-mediated staining was performed to identify T cells (CD3-PerCP), CD4^-T cells (CD3-PerCP/CD4-PE), CD8^-T cells (CD3-PerCP/CD8-PE) and NK cells (CD3-PerCP/CD56-PE).

In order to determine PBMC proliferation induced by targeted fusion proteins, recombinant FAP (3µg/ml) was coated over night at 4°C on an ELISA-plate. Fusion proteins (1µM) were added and incubated for 1h at RT. After washing with PBS, 2x10^5 PBMC/well were added. Alternatively, the fusion proteins (50, 100, 200nM) were incubated for 1h with 2x10^5/well B16-FAP cells. Then, B16-FAP cells were washed and 2x10^5 preactivated PBMC/well added. After 5 days proliferation of PBMC or T cells (additional CD3-PerCP staining) was determined by flow cytometry. Preactivated T cells were used in the targeted assay, as coculture with B16-FAP cells inhibited the responsiveness of unstimulated T cells. Preactivation of PBMC was achieved by coculture with adherent B16-FAP cells in presence
of 2,5pM bispecific antibody scDbFAPCD3 for 24 h. Subsequently, PBMC were harvested and washed before their addition to the assay as indicated above.

**Cytotoxicity assay**

Cytotoxic potential of T cells was assessed in terms of degranulation and determined by measuring CD107a exposure on the cell surface by flow cytometry analysis. In the first step 2x10^5 unstimulated PBMC/well were incubated with the fusion proteins in solution or alternatively 2x10^5 preactivated PBMC (as indicated above) were incubated with the fusion proteins targeted to B16-FAP cells (as indicated above). After 5 days, cells were washed and cytotoxic T cell degranulation triggered. 2x10^4 B16-FAP cells/well were seeded the day before and incubated with 30pM bispecific antibody scDbFAPCD3 for 1h, before the addition of pretreated PBMC for additional 6h in presence of 1,4µl/well BD GolgiStop™ (monensin) (BD Biosciences). Degranulation of T cells was monitored by CD107a-FITC/CD3-PerCP staining and flow cytometry analysis.

**Pharmacokinetics**

Groups of five C57BL/6JRj mice (4-6 month) were injected i.v. with 1nmol fusion protein/mouse. Blood samples were taken at different time intervals (3, 30, 60min, 2, 6, 24h) from the tail and serum concentration of the fusion proteins analyzed by sandwich ELISA. In brief, anti-IL-15 monoclonal antibody (3µg/ml) was coated. After blocking, serum and purified fusion protein were titrated and bound fusion protein detected via HRP-conjugated mouse hexahistidyl-tag-specific antibody as described elsewhere (29). Serum concentration of the fusion proteins was determined by interpolating the corresponding standard curve. For comparison, the first value (3min) was set to 100%. Pharmacokinetic parameters (t_{1/2α}, t_{1/2β}, AUC) were calculated via Excel using the first 3-4 time points to calculate t_{1/2α} and the last
three measurable time points to calculate $t_{1/2}\beta$. AUC was determined for the time interval of the first six hours.

**B16-FAP lung metastasis model**

C57BL/6JRj mice (4 month) were injected i.v. with $8.5 \times 10^5$ B16-FAP cells/animal. Groups of 5-6 animals were treated with PBS, scFv_IL-15, scFv_RD_IL-15 or RD_IL-15, respectively. Treatment of 0.2 nmol fusion protein was applied i.p. on day 1, 2 and 10, after cell injection. Mice were sacrificed on day 20. Lungs were removed and metastasis counted.

**Statistical Analysis**

For comparison of multiple groups in the *in vitro* experiments, the one-way analysis of variance (ANOVA) followed by the Tukey post test was applied. Statistical analysis of the *in vivo* experiments was performed by Student’s *t* test. (GraphPad Prism software)

**Results**

**Generation of the fusion proteins**

The fusion protein scFv_RD_IL15 is composed of an antibody moiety, a receptor domain and a cytokine component. The antibody component corresponds to a scFv that consists of the variable regions of the heavy and light chain of the antibody mo36 (30) (scFv36) directed against the tumor stromal fibroblast activation protein (FAP). This antibody is cross-reactive in mice and humans and had been previously generated at the institute (30). It was used in our study in order to target a stably FAP-transfected B16 melanoma cell line, suitable for in vitro and in vivo studies. Thus, FAP is used here as a model antigen to evaluate the feasibility of our approach, which might be extended later on to other tumor targets. The receptor domain (RD) comprises the amino acids 31-107 of the human IL-15Rα (sushi domain plus the
subsequent 12 amino acids). The cytokine component corresponds to human IL-15. In the scFv_RD_IL-15 construct, the scFv36 is located at the N-terminus, followed by the RD and the IL-15 domain. Furthermore, scFv_IL-15, an antibody fusion protein without RD was generated as well as the control fusion proteins scFv_RD and RD_IL-15, missing IL-15 and scFv, respectively. All constructs are provided with a hexahistidyl-tag at the C-terminus (fig. 1B).

The fusion proteins were produced in stably transfected HEK293 cells and purified by immobilized metal ion affinity chromatography with yields of 2.6 mg/l (scFv_RD_IL-15), 0.26 mg/l (scFv_IL-15), 0.66 mg/l (scFv_RD) and 0.88 mg/l (RD_IL-15), respectively. Analysis of the purified proteins by SDS-PAGE (Coomassie staining) under reducing conditions (fig. 1C) revealed high degree of purity and a single band for the scFv_RD construct (36 kDa), pronounced double bands for the fusion proteins scFv_RD_IL-15 (48 and 53 kDa) and RD_IL-15 (24 and 28 kDa), and three bands for scFv_IL-15 (42, 46 and 50 kDa). The IL-15 sequence comprises three potential N-linked glycosylation sites. The N-glycosylated nature of the fusion proteins with IL-15 component was demonstrated by PNGase F digestion of the proteins, resulting in a reduction of the slower migrating bands in SDS-PAGE (data not shown). The bands correlated with the predicted molecular mass of the fusion proteins: scFv_RD_IL15 (51.4 kDa), scFv_IL-15 (41.5 kDa), scFv_RD (37.2 kDa) and RD_IL-15 (25 kDa). The identity of the fusion proteins was further confirmed by immunoblot analysis with an anti-hexahistidyl-tag antibody (fig. 1D). HPLC-analysis of scFv_RD_IL-15 and scFv_IL-15 revealed a mainly homogeneous population, taking into account the glycosylation status of these proteins. A second minor peak probably corresponds to a dimeric form, as scFvs might dimerize to a certain degree. No higher aggregate formation was observed (supplementary data, S2).

**Binding properties**
Specific binding of the antibody fusion proteins to FAP was demonstrated by flow cytometry (fig. 1E). Stably FAP-transfected B16 cells (B16-FAP) were used as FAP-positive cell line and B16 wild-type cells (B16) were used as FAP-negative control cell line. ScFv_RD_IL-15, scFv_RD and scFv_IL-15 showed binding to FAP-positive B16-FAP cells but not to FAP-negative B16 cells. No binding was detected for RD_IL-15 neither on B16-FAP nor on B16 cells. Furthermore, titration of the antibody fusion proteins revealed similar binding affinity of scFv_IL-15 and scFv_RD_IL-15 that was approximately 7- and 5-fold higher than the binding affinity shown by the scFv and scFv_RD, respectively (fig. 1F).

Cytokine activity of the fusion proteins

The cytokine activity of the fusion proteins was analyzed in proliferation assays with the cytokine growth-dependent cell lines CTLL-2 and Mo7e. Human IL-15 induced the proliferation of both cell lines, allowing the analysis of stimulatory properties in the context of different IL-15 receptor chain compositions. CTLL-2, a murine cytotoxic T lymphocytic cell line, expresses IL-15Rα and IL-15Rβγ, while Mo7e, a human megakaryocytic leukemic cell line, expresses IL-15Rβγ chains only. Proliferation assays with the fusion proteins in solution revealed cytokine activity on both cell lines for all fusion proteins containing IL-15 (fig. 2A/B). On CTLL-2 cells, scFv_IL-15 showed high activity at picomolar concentrations, while fusion proteins comprising the RD fragment (scFv_RD_IL-15, RD_IL-15) were clearly less active (approximately 50-fold, comparing constructs with and without scFv, respectively) (fig. 2A). In contrast, on Mo7e cells, proliferation in response to scFv_RD_IL-15 and RD_IL-15 was also comparable but significantly more active (20- to 30-fold) than scFv_IL-15 (fig. 2B). The scFv and the scFv_RD did not show any stimulatory effect neither on CTLL-2 nor on Mo7e cells.

Next, the cytokine activity of the constructs was investigated in their membrane-bound form, targeting the FAP antigen on the cell surface, thereby mimicking a trans-presentation of IL-15.
either in presence (scFv_RD_IL-15) or in absence (scFv_IL-15) of the receptor alpha chain domain (RD). The fusion proteins were incubated on B16-FAP or B16 cells and the IL-15 activity of bound constructs was determined by the proliferation of cocultured CTLL-2 cells. Here, proliferation was observed in response to the antigen-dependent membrane-bound presentation of both, scFv_RD_IL-15 and scFv_IL-15 (fig. 2C). Unlike in solution, both constructs displayed a similar proliferation effect. As expected, no stimulation of CTLL-2 was observed for the experimental setting with FAP-negative B16 cells (fig. 2D).

Then, we analyzed the proliferation effect of the fusion proteins on PBMC. Similar to the proliferation pattern observed for Mo7e cells, fusion proteins in solution presenting IL-15 and RD (RD_IL-15 and scFv_RD_IL-15) achieved a stronger proliferation response (11- to 15-fold) than the fusion protein with IL-15 only (scFv_IL-15). Also in this case, scFv_RD_IL-15 and RD_IL-15 were equally effective, while scFv_RD did not induce proliferation at all (fig. 3A). In addition, scFv_RD_IL-15 and scFv_IL-15 were also shown to be active in their target-bound form (fig. 3B). Furthermore, we determined the proliferation effect of scFv_RD_IL-15 and scFv_IL-15 in solution in regard to different immune cell populations (fig. 3C). Here, NK cells and CD8+ T cells were more responsive to IL-15 stimulation than CD4+ T cells. Irrespective of the cell type, scFv_RD_IL-15 was significantly more effective than scFv_IL-15 at low concentration (1 nM). Improved proliferation of T cells in response to IL-15 presented in combination with RD was demonstrated for the fusion proteins in solution (fig. 4A) as well as for the fusion proteins in target-bound state (fig. 4B). For the latter, scFv_IL-15 and scFv_RD_IL-15 were bound via the antibody moiety to FAP-expressing cells (B16-FAP), presenting IL-15 or RD_IL-15, respectively on the cell surface. Thus, by mimicking IL-15 \textit{trans}-presentation, the proliferation effect on preactivated T cells was determined, indicating an advantage for the membrane presentation of IL-15 in an additional RD-bound form. Furthermore, the influence of the fusion proteins on the cytotoxic potential of T cells was analyzed (fig. 4C/D). In solution, scFv_RD_IL-15 as well as scFv_IL-15 were
able to enhance the percentage of degranulating T cells, whereby at low concentrations (0.1 and 1 nM) scFv_RD_IL-15 showed a stronger effect than scFv_IL-15 (fig. 4C). Also in the target-bound form, scFv_RD_IL-15 was at lower concentration more effective than scFv_IL-15, increasing the cytotoxic potential of preactivated T cells (Fig. 4D). Therefore, the fusion of RD to IL-15, presented either in soluble or targeted form, improves significantly the response of T cells in terms of proliferation and cytotoxicity.

Pharmacokinetics and therapeutic potential in a tumor mouse model

Pharmacokinetic analysis in mice revealed some differences in the half life of the fusion proteins. ScFv_RD_IL-15 showed an approximately 2-fold longer $t_{1/2\alpha}$ than RD_IL-15 and scFv_IL-15 ($p<0.05$). $T_{1/2\beta}$ was similar for scFv_RD_IL-15 and RD_IL-15, but 6-fold ($p<0.01$) and 3.5-fold ($p<0.001$) increased in comparison to scFv_IL-15. The AUC calculated for scFv_RD_IL-15 was approximately 1.7- and 2.5-fold higher than the AUC determined for RD_IL-15 and scFv_IL-15, respectively ($p<0.01$) (table 1) (fig. 5A).

Finally, the therapeutic potential of the fusion proteins was investigated in a B16-FAP lung metastasis mouse model, whereat dosing and treatment schedule was chosen based on data of the IL-15-IL-15Rαdomain fusion protein (RLI) in the literature (26). B16-FAP cells were injected i.v. into C57BL/6JRj mice on day 0 and treated on day 1, 2 and 10 with a single dose of 0.2 nmol fusion protein (scFv_RD_IL-15, scFv_IL-15, RD_IL-15), respectively. After 20 days, lungs were removed and the number of tumor cell foci counted (fig. 5B). In comparison with the PBS control group, all groups treated with IL-15 fusion proteins showed significantly less metastasis. Strongest effect was achieved by treatment with scFv_RD_IL-15 (approx. 86% metastasis reduction), followed by application of RD_IL-15 and scFv_IL-15 with similar outcome (approx. 46% and 53% metastasis reduction, respectively). Thus, comparing the targeted versions of the IL-15 fusion proteins (scFv_IL-15 and scFv_RD_IL-15), the presence
of RD significantly enhanced the therapeutic potential. On the other hand, comparing the targeted and non-targeted versions of RD bearing IL-15 fusion proteins (scFv_RD_IL-15 and RD_IL-15) showed that the targeted form was more effective. Thus, scFv_RD_IL-15, combining tumor targeting and IL-15 presentation in the RD context, revealed the best therapeutic potential. No signs of toxicity (e.g. weight loss) were observed during the therapeutic experiment.

**Discussion**

Initially, soluble IL-15Rα, generated by shedding from human cell lines was observed to exert an antagonistic effect. Binding of soluble IL-15Rα to soluble IL-15 led to competition with cell surface receptors inhibiting IL-15-mediated function (14). Later, complex formation of recombinant soluble IL-15 and IL-15Rα-Fc molecules was reported to enhance the bioactivity of IL-15, inducing proliferation of NK cells and memory T cells *in vitro* and *in vivo* (23, 25). Structural studies identified the “sushi” region in the IL-15Rα as the main responsible element for IL-15 binding (31). *In vitro*, soluble complexes of recombinant IL-15 and the IL-15Rα sushi domain were described to induce agonistic effects on the intermediate affinity receptor (IL-15Rβγ), inducing stronger proliferation than IL-15 alone (32). This effect could be even enhanced when the sushi domain of IL-15Rα was extended by 13 amino acids of the exon 3 (IL-15αRsushi+), thereby increasing the binding affinity for IL-15 (33). Although this complex showed reduced bioactivity on high affinity receptor IL-15Rαβγ expressing cell lines, the combination of IL-15 and IL-15αRsushi+ into a fusion protein (RLI) retrieved a molecule that presented maximal enhancement of IL-15Rβγ stimulation without losing stimulatory capacity for IL-15Rαβγ (32, 33). Furthermore, the fusion protein (RLI) showed efficiency *in vivo* by inducing antitumor effects in diverse mouse models (26). Therefore, for
the generation of the targeted scFv36_RD_IL-15 fusion protein, we decided to relay on the RLI model with the IL-15Rαsushi+ variant building the IL-15Rα domain (RD). We could show that the fusion to the scFv component did not interfere with the cytokine activity of IL-15 in combination with the RD fragment, as our untargeted control construct RD_IL-15 displayed similar activity than scFv_RD_IL-15 in solution. On the other hand, fusion of the scFv to the cytokine component and or the receptor unit did not impede specific binding of the construct to the tumor target. Thus, scFv_RD_IL-15 and scFv_IL-15 bound with similar affinity to FAP-expressing cells. Furthermore, we analyzed the cytokine activity of the antibody-fusion proteins in the context of intermediate (IL-15Rβγ) and high (IL-15Rαβγ) affinity receptor expressing cell lines. In solution, constructs presenting IL-15 in combination with the IL-15Rα domain (RD) stimulated more efficiently the proliferation of IL-15Rβγ expressing Mo7e cells. This observation was in accordance with the data reported for the (RLI) fusion protein and the IL-15/IL-15Rαsushi+ complex, showing enhanced cytokine effect on Mo7e cells in comparison with IL-15 alone (32, 33). In regard to the stimulation of the high affinity receptor IL-15Rαβγ, the presence of the RD fragment in the fusion proteins decreased the cytokine activity of the constructs in solution, independently of the presence of the scFv. Thus, proliferation of CTLL-2 (IL-15Rαβγ) cells was induced with picomolar concentrations of scFv_IL-15, but rather nanomolar concentrations of scFv_RD_IL-15 and RD_IL-15. This resembles the results described for soluble complexes of IL-15 and IL-15Rαsushi+ on high affinity receptor expressing cells, where the extension of the sushi domain interferes with IL-15 receptor stimulation, but differs from the stimulatory effect described for the RLI fusion protein that showed to be equivalent or even better than IL-15 in terms of IL-15Rαβγ activation (33, 26). The latter might be due to the particular cell system used (Kit225 expressing intermediate and high affinity receptors, where intermediate receptor activation is specifically blocked by an antibody), although differences in the sterical
presentation inherent to the configuration of the particular recombinant fusion protein cannot be excluded. Nevertheless, the inhibitory effect in cytokine activity observed for scFv_RD_IL-15 in solution was abolished in the targeted form, i.e. bound to B16-FAP cells, presenting RD_IL-15 in a membrane-bound form. Here, scFv_RD_IL-15 as well as scFv_IL-15 showed equivalent stimulatory potential on CTLL-2 cells. Thus, for targeted constructs, effective trans-presentation of IL-15 to IL-15Rαβγ expressing cells appeared independent of the association to RD.

Resting NK cells and naïve T cells express the intermediate affinity IL-15Rβγ phenotype (10, 22, 34). Thus, proliferation assays with unstimulated PBMC retrieved similar results than those obtained with the Mo7e (IL-15Rβγ) cell line in response to the fusion proteins in solution, achieving stronger effects with scFv_RD_IL-15 than with scFv_IL-15. NK cells as well as T cells (CD8+ T cells and CD4+ T cells) were identified to be responsive, following this activation pattern. Overall higher proliferation response of CD8+ T cells and NK cells in comparison to CD4+ T cells was observed, correlating with the report of very low IL-15Rβ expression levels on naïve CD4+ T cells (35). In addition, better performance of scFv_RD_IL-15 in comparison to scFv_IL-15 was also determined for the constructs in targeted form, i.e. bound to FAP-expressing tumor cells, enhancing proliferation and cytotoxicity of activated T cells. Considering that for effector T cells high expression levels of IL-15Rα and therefore potential expression of high and intermediate affinity IL-15 receptors have been described (10), our results likely reflect the target-directed trans-presentation of RD_IL-15 to both receptor types. Even in this case, better performance of scFv_RD_IL-15 in comparison to scFv_IL-15 appears reasonable, considering that in regard to IL-15Rαβγ activation, both constructs showed in targeted form comparable cytokine activity on CTLL-2 cells (IL-15Rαβγ), while stimulation of IL-15Rβγ is clearly favoured by the presence of RD in the construct.
In vivo analysis of scFv_RD_IL-15, scFv_IL-15 and RD_IL-15 in the B16-FAP lung metastasis mouse model resulted in clear antitumor effects for all three constructs, corroborating the antitumor potential of this cytokine. The best result was achieved with scFv_RD_IL-15, followed by scFv_IL-15 and RD_IL-15 to similar extent. Pharmacokinetic studies in mice indicated small differences between the constructs in half life and AUC, favouring scFv_RD_IL-15. According to the in vitro data obtained with these constructs, cytokine activity could be remarkable enhanced by presenting IL-15 in association with the receptor alpha domain to IL-15Rβγ expressing cells. This effect was also reported for the RLI fusion protein in comparison with recombinant IL-15 (approximately factor 500) (26). Also here, in vivo analysis in a B16F10/lung metastasis model revealed a stronger antitumor effect for RLI than for IL-15. Nevertheless, pharmacokinetic analysis of RLI and IL-15 indicated a 6-fold and 34-fold increase in half life and AUC for RLI, respectively, suggesting that improved pharmacokinetic properties as well as enhanced cytokine activity might contribute to the stronger antitumor effect observed for RLI. In our study, scFv_IL-15 and RD_IL-15 achieved similar antitumor effects; an outcome probably influenced by the tumor targeting properties of scFv_IL-15. Enhanced antitumor activity by directing IL-15 to the tumor in form of an antibody-cytokine fusion protein has been described previously (18). Here, IL-15 was fused to a fibronectin EDB domain-specific antibody in a diabody format, resulting in a homodimeric molecule. Accumulation at the tumor site as well as target-mediated tumor growth inhibition was shown in an s.c. F9 tumor mouse model, ascribing a crucial role for CD8+ T cells in the therapeutic effect. Tumor directed targeting of IL-15/IL-15Rα as a complex or fusion protein has not been reported so far. Here, by means of scFv_RD_IL-15, we could show that the strategy of combining tumor targeting by a recombinant antibody and trans-presentation of IL-15 in the IL-15Rα domain context was able to further enhance the antitumor potential of this powerful cytokine. Thus, scFv_RD_IL-15 appears as a promising
reagent that sets itself apart from other recombinant molecules developed for the application of IL-15 in cancer immunotherapy.

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References


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<td>1,4 ± 0,3</td>
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Figure legends

**Fig. 1** Schematic illustration of IL-15 trans-presentation (A) and the assembly of the fusion proteins (B). scFv: FAP-specific antibody fragment; VḤ: variable region of the heavy chain; VL: variable region of the light chain, RD: IL15Rα(sushi domain + 12 amino acids of exon 3); L1: G4SG4SG2SA; L2: SG4SG4; L3: G3SG4SG3SG4SLQ; L4: SGSG4SLQ; black square: hexahistidyl-tag; FAP: fibroblast activation protein Characterization of the fusion proteins by (C) 10% SDS-PAGE under reducing conditions (Coomassie staining) and (D) Western blot. Recombinant protein (2µg/lane) was detected via hexahistidyl-tag. (E) Antibody-mediated binding properties analyzed by flow cytometry. Binding specificity was determined by incubating the fusion proteins (350nM) on FAP-positive (B16-FAP) and FAP-negative (B16) cells, respectively. (F) Binding affinity was compared by titrating the fusion proteins on B16-FAP cells. Bound constructs were detected via the hexahistidyl-tag. Gray filled: cells; dotted line: detection system; black line: antibody construct. Graphic shows mean ± SD, n=3.

**Fig. 2** Cytokine-dependent proliferation assays with the fusion proteins in solution was performed with CTLL-2 (IL-15Rαβγ) (A) and Mo7e (IL-15Rβγ) (B) cells. Cytokine activity of target-bound fusion proteins was demonstrated by incubation on B16-FAP (C) or B16 (D) cells, followed by washing and the addition of CTLL-2 cells. Indicated are concentrations of the constructs during the incubation step on target cells. After 3 (CTLL-2) or 4 (Mo7e) days, proliferation was measured by MTT assay. Graphics show mean ± SD, n=3.

**Fig. 3** Cytokine activity of fusion proteins induces PBMC proliferation. CFSE labelled PBMC were incubated either with fusion proteins in solution (A) or fusion proteins bound to recombinant FAP coated on ELISA plate (B). After 5 days, proliferation of PBMC was
measured by flow cytometry. (C) PBMC were incubated with fusion proteins in solution as indicated above and proliferation of CD4+ T cells (CD3-PerCP/CD4-PE), CD8+ T cells (CD3-PerCP/CD8-PE) and NK cells (CD3-PerCP/CD56-PE) determined by appropriate antibody staining, followed by flow cytometry analysis. Graphics show mean ± SD, n=3-4, **p<0.01, ***p<0.001.

**Fig. 4** Effect of scFv_IL-15 and scFv_RD_IL-15 in soluble and targeted form on T cell proliferation and cytotoxicity. PBMC were incubated with the fusion proteins either in solution (A and C) or targeted to B16-FAP cells (B and D) for 5 days. Then, proliferation (A and B) was determined for T cells identified by mAb (CD3-PerCP) staining by CFSE measurement in flow cytometry analysis. Alternatively, cytotoxicity of T cells (C and D) was assessed by incubating PBMC with B16-FAP cells in presence of 30pM scDbFAPCD3 and monensin for 6h. Degranulating T cells were identified by mAb (CD3-PerCP/CD107a-FITC) labelling via flow cytometry. Unstimulated or preactivated PBMC were used for the approach with fusion proteins in soluble and targeted form, respectively. Data of assays with targeted fusion proteins are presented as relative to the effect of prestimulated PBMC in absence of fusion protein. Graphics show mean ± SD, n=3-6. *p<0.05, **p<0.01, ***p<0.001.

**Fig. 5** (A) Pharmacokinetics: fusion proteins (1nmol) were injected i.v. into C57BL/6 mice (n=4-5) and serum concentration determined at different time points. (B) Therapeutic experiment: 8.5x10^5 B16-FAP cells were injected i.v. into C57BL/6 mice on day 0. Mice were treated with a single dose (0.2nmol) fusion protein (i.p.) on day 1, 2 and 10. Lungs were removed on day 20 and tumor foci counted. Graphics show mean ± SD, n=5-6. *p<0.05, **p<0.01, ***p<0.001.
Fig. 2

A

B

C

D

![Graphs showing OD values vs. fusion protein concentration for different proteins.](image-url)
Fig. 4

A

![Graph showing proliferation percentage for different fusion protein concentrations.]

B

![Graph showing relative proliferation for different fusion protein concentrations.]

C

![Graph showing CD107a expression for different fusion protein concentrations.]

D

![Graph showing relative CD107a expression for different fusion protein concentrations.]

Legend:
- scFv_IL-15
- scFv_RD
- RD_IL-15
- scFv_RD_IL-15

Each graph presents data for different concentrations of fusion proteins with statistical significance indicated by asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.
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An antibody fusion protein for cancer immunotherapy mimicking IL-15 trans-presentation at the tumor site

Vanessa Kermer, Volker Baum, Nora Hornig, et al.

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