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Abstract

Polymorphonuclear neutrophils (PMNs) are potent effectors of inflammation and their attempts to respond to cancer are suggested by their systemic, regional and intratumoral activation. We previously reported on the recruitment of CD11b+ leukocytes due to tumor site-specific enrichment of TNF activity after intravenous administration of a dimeric TNF immunokine with specificity for fibroblast activation protein (FAP). However, TNF-induced chemo-attraction and extravasation of PMNs from blood into the tumor is a multistep process essentially mediated by interleukin 8. With the aim to amplify the TNF-induced and IL-8-mediated chemotactic response, we generated immunocytokines by N-terminal fusion of a human anti-FAP scFv fragment with human IL-8 (IL-8(72)) and its N-terminally truncated form IL-8(3-72). Due to the dramatic difference in chemotaxis induction in vitro, we favored the mature chemokine fused to the anti-FAP scFv for further investigation in vivo. BALB/c nu/nu mice were simultaneously xenografted with FAP-positive or -negative tumors and extended chemo-attraction of PMNs was only detectable in FAP-expressing tissue after intravenous administration of the anti-FAP scFv-IL-8(72) construct. As TNF-activated PMNs are likewise producers and primary targets for IL-8, we investigated the therapeutic efficacy of co-administration of both effectors: Sequential application of scFv-IL-8(72) and dimeric IgG1-TNF fusion proteins significantly enhanced anti-tumor activity when compared either to a single effector treatment regimen or sequential application of non-targeted cytokines, indicating that the tumor-restricted sequential application of IL-8(72) and TNF is a promising approach for cancer therapy.
Sequential cancer immunotherapy: targeted activity of dimeric TNF and IL-8

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Polymorphonuclear neutrophils (PMNs) are potent effectors of inflammation and their attempts to respond to cancer are suggested by their systemic, regional and intratumoral activation. We previously reported on the recruitment of CD11b+ leukocytes due to tumor site-specific enrichment of TNF activity after intravenous administration of a dimeric TNF immunokine with specificity for fibroblast activation protein (FAP). However, TNF-induced chemotraction and extravasation of PMNs from blood into the tumor is a multistep process essentially mediated by interleukin 8. With the aim to amplify the TNF-induced and IL-8-mediated chemotactic response, we generated immunocytokines by N-terminal fusion of a human anti-FAP scFv fragment with human IL-8 (IL-81-72) and its N-terminally truncated form IL-83-72. Due to the dramatic difference of the anti-FAP scFv-IL-8 construct. As TNF-activated PMNs are likewise producers and primary targets for IL-8, we investigated the therapeutic efficacy of co-administration of both effectors: Sequential application of scFv-IL-83-72 and dimeric Igal-TNF fusion proteins significantly enhanced anti-tumor activity when compared to a single effector treatment regimen or sequential application of non-targeted cytokines, indicating that the tumor-restricted sequential application of IL-83-72 and TNF is a promising approach for cancer therapy.

Keywords: mice, anti-FAP scFv, IL-8, TNF, recombinant fusion protein, polymorphonuclear neutrophils

Introduction

The therapeutic use of cytokines as anticancer drugs is greatly limited in the clinic by their lack of tumor specificity causing unselective side effects outside the tumor environment. These non-specific activities result in a narrow therapeutic window with a low therapeutic index. Therefore, the most critical issue in order to improve therapeutic efficacy is the increase of local cytokine concentrations at the tumor site, together with the minimization of unwanted side effects. Fusing cytokines to tumor-associated antigen-specific antibodies offers an alternative strategy to achieve the goal of locally restricted cytokine enrichment. Fusion proteins can preserve their targeting properties (determined by the monoclonal antibody domain), remain mostly inactive in circulation and induce cytokine-mediated anti-tumor effects when reaching the target site (1). The prototype of a cytokine that possesses a wide range of potent biological anti-tumor activities is tumor necrosis factor (TNF). Since its systemic administration mediated the regression of sarcomas in mice (2), TNF has attracted attention as a potential anti-tumor reagent (3). However, the problem of dose-limiting toxicity has been particular apparent in human trials with a 40-fold lower maximal tolerated dose in humans than in mice (4, 5) and systemic administration resulted in life-threatening side effects (6). Nevertheless, its more localized delivery by isolated limb perfusion mediated very impressive tumor regressions (7) and spurred the design of a clinically applicable cytokine format. To this aim, our strategy relied on the alteration of the natural TNF homotrimeric symmetry by linking a TNF molecule to the C-terminus of each CH1 domain of a human IgG antibody. These fusion proteins preserved the characteristic IgG-dimeric structure with two TNF subunits (8). The dimeric TNF structure resulted in significantly reduced toxicity as seen both in immunocompetent and -deficient mouse strains and could, therefore, display significantly stronger anti-tumor activity when compared with trimeric TNF and its conjugates (9). Moreover, the IgG-derived dimeric design represented a structural principle for targeted immunotherapy of multiple tumor entities, as accumulation of TNF depends on the antigen specificity of the fused antibodies (10). Accumulation of dimeric TNF in the microenvironment of xenotransplanted tumors in nude mice activated multiple tumorcidal effector mechanisms, including alteration of the natural tumor to polymorphonuclear neutrophils (PMNs) balance due to recruitment and activation of CD11b+ leukocytes (8). However, TNF-induced chemotraction and extravasation of PMNs from blood into the tumor is a multistep process that is mainly IL-8-dependent (11, 12). The most abundant naturally occurring form of IL-8 is a 72-residue protein derived by processing a 99-residue precursor (13, 14). In addition, several alternatively processed amino-terminal variants have been detected; the IL-81-72 variant was described to display a two-fold higher neutrophil chemotaxis activity (15). Therefore, we fused a human anti-FAP scFv fragment to either the 72-residue human IL-8 or the human IL-83-72 variant in...
Preparation and characterization of scFv constructs. (A) Scheme of the constructs for anti-FAP scFv, anti-FAP scFv-IL-8<sub>72</sub> and anti-FAP scFv-IL-8<sub>3-72</sub> as they were inserted into the mammalian expression vector pEAK8. The anti-FAP scFv antibody vector consisted of the promoter-leader cassette coding for an 18-aa secretory leader sequence, the variable heavy chain region followed by a 10-aa linker with the light chain variable region and a DNA sequence encoding a c-myc and His6 tag at the C-terminus for detection and affinity purification. IL-8 variants are N-terminally linked to anti-FAP scFv by (Ser<sub>4</sub>Gly)<sub>3</sub>. (B) Anti-FAP scFv-IL-8 (lanes 1 to 3) and anti-FAP scFv-IL-8<sub>3-72</sub> (lanes 4 to 6) were purified by immobilized metal chelate chromatography. Culture supernatant (lanes 1 and 4), flow through (lanes 2 and 5) and purified constructs (lanes 3 and 6) were analyzed by SDS-PAGE under reducing conditions. As expected, bands corresponding to scFv IL-8 variants were detected at 39 kDa (lane 3 for anti-FAP scFv-IL-8<sub>72</sub> and lane 6 for anti-FAP scFv-IL-8<sub>3-72</sub>). (C) Western blot analysis of anti-FAP scFv (lanes 1 and 4), anti-FAP scFv-IL-8<sub>72</sub> (lanes 2 and 5) and anti-FAP scFv IL-8<sub>3-72</sub> (lanes 3 and 6) stained with anti-c-myc (lanes 1 to 3) and anti-hu IL-8 antibody (lanes 4 to 6).

Results

Generation, biochemical characterization and binding of IL-8-derived fusion proteins

To generate the single-chain IL-8-derived fusion proteins, the cDNA of hu IL-8<sub>72</sub> and hu IL-8<sub>3-72</sub> were fused to a 45-bp linker at the 3’ end of the previously described anti-FAP scFv (9) (Figure 1A). Constructs were cloned into the pEAK mammalian expression vector and confirmed by DNA sequencing. Transfection of suspension-adapted HEK293 EBNA cells (16) was performed in spinner flasks using an optimized calcium phosphate precipitation method (17). Fusion proteins were purified from the supernatant with a final yield of 40 mg/l culture. Constructs were analyzed by gel electrophoresis and
Figure 2

Binding properties of scFv constructs. (A) Flow cytometry analysis of constructs [anti-FAP scFv (thick line), anti-FAP scFv-IL-83-72 (dotted line) and anti-FAP scFv-IL-872 (thin line)] was performed on FAP-transfected HT1080 cells. An anti-CD30 scFv-IL-8 fusion protein served as negative control. Binding was detected by monoclonal anti-c-myc antibody. (B) Recognition of targeted antigen by scFv constructs was performed by incubation with rabbit anti-human IL-8 and visualized by PE-conjugated goat anti-rabbit serum. Uncoupled parental anti-CD30 scFv-IL-8 was used as negative control. (C) Binding of scFv variants to immobilized anti-FAP-anti-ID was visualized by rabbit anti-IL-8 serum using ELISA. Uncoupled anti-FAP scFv and anti-CD30 scFv-IL-8 were used as controls. Measurements were carried out in triplicate samples; standard deviations are indicated by the bars.

Figure 3

Chemotactic potential of anti-FAP scFv-IL-8 variants in vitro. We used a fluorescence-based end-point assay to measure PMN migration in vitro. Chemoattractants (rhu IL-8, anti-FAP scFv, anti-FAP scFv-IL-83-72 and anti-FAP scFv-IL-872) and controls were diluted in PBS-HSA 0.1%. PBS-HSA 0.1% alone was used to determine random migration. In order to define the total fluorescence of PMNs, calcem-AM labeled PMNs were placed directly at defined concentrations (80000, 8000, 800 and 80 cells) in three separate wells. 80000 PMNs were placed directly onto the top of the filter and the chamber was incubated for 60 minutes. The total number of PMNs that had migrated to the lower chamber was measured with a fluorescence plate reader. Measurements were carried out in triplicate samples. The standard deviation of three assays is shown.

Western blotting using an anti-IL-8 and anti-c-myc antibody for detection. The IL-8-derived single chain antibody constructs revealed a molecular size of 39 kDa under reducing conditions (Figure 1B). Western blotting of SDS gels and staining with anti-c-myc antibody identified the tagged constructs. As expected, staining with anti-IL-8 antibody failed to detect anti-FAP scFv (Figure 1C).

The immunoreactivity of the IL-8 fusion proteins was assessed by flow cytometry. Anti-FAP moieties were demonstrated on FAP- and mock-transfected HT1080 cells using either the anti-c-myc (Figure 2A) or anti-IL-8 antibody (Figure 2B), respectively. Affinity of anti-FAP scFv was higher when compared to both IL-8 immunocytokines, showing equal binding properties. The integrity of the IL-8-derived constructs was confirmed by sandwich ELISA with simultaneous recognition of the IL-8 moiety by an anti-human IL-8 antibody and binding of the antibody moiety to a murine anti-idiotype anti-FAP mAb (Figure 2C).
Figure 4

Kinetics of FAP expression of transfected HT1080-FAP+ fibrosarcoma cells in vitro and in vivo. (A) FAP transfected HT1080 cells were cultured without G418 selective pressure and the levels of antigen density were measured after 3 weeks by FACSscan analysis. (B to E) Immunohistochemical staining of HT1080-FAP+ xenograft fibrosarcoma cells showing positive staining over a period of four weeks. 5 x 10⁶ cells were injected s.c. in BALB/c nu/nu mice. Solid tumors were harvested after 14 (B, C) and 28 days (D, E), respectively. Tissue sections were stained with anti-FAP IgG (B, D) or with anti-G250 IgG (C, E) as a control. Bars correspond to 10 µm.

Chemotactic potential of anti-FAP scFv-IL-8 fusion proteins in vitro

PMNs from the venous blood of healthy human donors were labeled with calcein and PMN migration towards IL-8-derived constructs was quantified in vitro using a standardized fluorescence-based assay in a chemotaxis chamber in disposable 96-well microplates. With regard to different molarities of scFv-fused IL-8 derivates and recombinant human IL-8, chemotaxis assays were performed at IL-8-equivalent doses (Figure 3). The chemotactic activities of recombinant human (rhu) IL-8 and anti-FAP scFv-IL-8_72 were nearly identical. Both substances revealed concentration-dependent migration of PMNs. Surprisingly, migration of PMNs towards the scFv-coupled 3-72 isoform of IL-8 was not superior to the migration towards anti-FAP scFv which lacks the capability of specific chemotraction. In accordance with published data from other groups (18), we could not detect PMN migration towards a TNF gradient, neither when used as trimeric rhu TNF nor as dimeric antibody-fused TNF. Furthermore, we did not observe additive effects of TNF and IL-8 in stimulating PMN migration across the filter (data not shown).

Tumor model, PMN recruitment and therapeutic efficacy of anti-FAP scFv-IL-8_72

In cell culture, incessant FAP expression of transfected HT1080 cells was detectable over several weeks without G418 selective pressure (Figure 4A). The stability of FAP expression was the prerequisite for in vivo targeting of FAP in BALB/c nu/nu mice xenografted with FAP- or mock-transfected human HT1080 fibrosarcoma cells. FAP-positive and -negative tumors were simultaneously established on opposite flanks of mice and FAP expression could be demonstrated for the entire period of targeted treatment (Figure 4B). Due to its superior chemotactic
Figure 5

Targeted antigen-dependent recruitment of PMNs to FAP-expressing tumors. Fibrosarcomas were established in BALB/c nu/nu mice by s.c. injection of 5 x 10^6 FAP-transfected and mock-transfected HT1080 cells, respectively. FAP-positive (left column) and -negative (right column) tumors were simultaneously established at the opposite flanks of the animals. Tumors were harvested 24 hours after i.v. injection of a total dose of 300 µg scFv-IL872 (equivalent to 100 µg rhu IL-8). (A, B) FAP expression of xenografts was confirmed by anti-FAP mAb staining. PECAM-1-positive tumor vessels were visualized to define comparable areas of FAP-positive (C) or -negative (D) fibrosarcomas to exclude unspecific PMN accumulation. Staining with anti-CD11b mAb demonstrated the targeted antigen-specific delivery of scFv-IL872 by chemokine-triggered enrichment of PMNs (E, F). Bars correspond to 100 µm.

Potency in vitro, the full-length IL-872 scFv construct was tested for FAP-specific chemo-attraction of PMNs. Mice simultaneously bearing FAP-positive and -negative tumors were treated with effector construct equivalent doses of intravenously administered anti-FAP scFv-IL-872, rhu IL-8 or anti-FAP scFv, respectively. Enrichment of CD11b+ PMNs within FAP-expressing tumors was only seen in mice properly treated with anti-FAP scFv-IL-872. Furthermore, PMN recruitment was specific for the targeted antigen as no migration was seen towards FAP-negative tumors. Neither the application of uncoupled rhu IL-8, nor of the anti-FAP scFv alone could induce PMN recruitment towards fibrosarcoma cells, irrespective of their FAP expression status (Figure 5).

As PMNs are likewise producers and primary targets for IL-8, sequential targeted enrichment of scFv-IL-872 and IgG-TNF might raise the chance to initiate an activation loop in chemo-attracted PMNs, thereby improving the focused therapeutic activity. To test the efficacy of the targeted TNF and IL-872 combination regimen, we adopted our well-proven schedule for the treatment of BALB/c nu/nu mice with dimeric anti-FAP IgG-TNF (8). Both the TNF-equivalent dose of anti-FAP IgG-TNF and the treatment schedule were excellently tolerated by the animals without significant side effects and PMN recruitment at targeted tumor sites was detectable within 24 hours following administration (8, 9). Since TNF and IL-8 are key components in the promotion of generalized endothelial activation leading to systemic inflammatory response syndrome (SIRS) (19), we favored a sequential schedule with an eight-hour time interval between the administrations of each targeted cytokine to minimize unwanted peripheral activation of PMNs and endothelial cells. The delay of eight hours was feasible since the half-life of IgG antibodies is mainly determined by the CH2/CH3 constant regions and their exchange by TNF causes a rapid clearance rate of dimeric antibody-fused TNF immunocytokines (10). Treatment of mice started on day nine after tumor cell inoculation and was carried out over a period of two weeks with five consecutive treatment days (Figure 6A). Repetitive administration of anti-FAP scFv-IL-872 alone at its chemo-attractive dose already results in significant anti-tumor effects when compared to the application of unconjugated anti-
Figure 6

Effects of sequential immunotherapy on the growth pattern of tumor xenografts. (A) FAP positive tumors were established in BALB/c nu/nu mice by s.c. injection of 5 x 10^6 HT1080 FAP+ cells and mice randomly assigned to different treatment groups at day nine. For sequential immunotherapy, effector constructs were injected i.v. and followed eight hours later by i.v. injection of the second effector construct as indicated (100 µl per injection). This treatment regimen was repeated every three days as highlighted by the arrows (five times in total). Starting on day 21, the growth delay was statistically significant for the anti-FAP TNF & anti-FAP scFv-IL-8 group (*, \( P < 0.001 \)) when compared to all other treatment groups. Comparison of tumor sizes between the anti-FAP TNF and the anti-FAP TNF & rhu IL-8 group and the remaining groups was also significant at this time point (**, \( P < 0.001 \)), but tumor sizes did not differ significantly between the anti-FAP TNF and the anti-FAP TNF & rhu IL-8 group. Single agent treatment with anti-FAP scFv-IL-8 also resulted in significant (***, \( P < 0.001 \)) anti-tumor effects when compared to the rhu IL-8-containing regimens that did not differ from the PBS group (data not shown). Treatment results stayed significant over the observation period. Animals were taken off study when tumor volume exceeded 1 cm³. (B) Delay of tumor growth of mice receiving anti-FAP TNF & anti-FAP scFv IL-8 was linked to antigen expression. Simultaneous growth of two phenotypically different xenografts [FAP-positive (closed symbols) and FAP-negative (open symbols)] was established in nude mice and animals assigned to their respective treatment group when tumor diameter reached 5 mm. Again, animals were treated with anti-FAP TNF & anti-FAP scFv-IL-8 following the sequential application schedule indicated by the arrows. Mice had to be taken off study when the volume of FAP-negative xenografts exceeded 1 cm³. At this time point, differences in tumor size between FAP-positive and -negative xenografts were statistically significant (*, \( P < 0.001 \)).

FAP-antibody. However, its therapeutic activity was inferior when compared to the TNF-derived anti-FAP fusion protein. Attempts to enhance the therapeutic potency of anti-FAP IgG-TNF by adding sequential applications of rhu IL-8 did not provide the desired effect. The addition of rhu TNF to scFv-IL-8 did not be cause because of lethal TNF toxicity at this dose level. Only the combination of both cytokines as part of the anti-FAP fusion protein and administere by a sequential application regimen enhanced the therapeutic impact of the single construct and led to a significant delay in tumor growth. In addition to its improved therapeutic activity, anti-tumor effects were dependent on the presence of the targeted antigen and strongly restricted to FAP-expressing tumors (Figure 6B).

Discussion

Genetic fusion of cytokines and tumor-associated antigen-specific antibodies is a widely accepted strategy for targeted anti-cancer therapy (20, 21). Further development of monotherapeutic approaches has led to the combined application of cooperatively acting fusion proteins displaying superior anti-tumor effects when compared to single effector molecule treatment (22, 23). We hypothesized that successful immunotherapy of FAP-positive tumors with our dimeric anti-FAP IgG-TNF construct could be further enhanced by sequential application of anti-FAP scFv-fused IL-8. Based on data about chemically synthesized IL-8 analogues (15), we generated two anti-FAP scFv-IL-8 fusion proteins carrying different IL-8 isoforms. The structural integrity of N-terminally fused IL-8-derived constructs was demonstrated after transient mammalian expression and purification. While FAP binding was the same for both fusion proteins, the superior antigen binding capacity of the parental anti-FAP scFv suggests an alteration in the binding specificity by the N-terminal fusion of IL-8 variants or by the second linker (24, 25) or a combination of both. When fused to anti-FAP scFv, only the mature IL-8_2 chemokine displayed chemotactic potency comparable to native rhu IL-8, while the truncated variant totally failed to induce...
neutrophil attraction. Recently reported genetic engineering combining an anti-epidermal growth factor receptor F(ab) antibody with an N-terminally truncated form of IL-8 (aa 2-72) resulted in reduced receptor binding capacity and chemotactic activity when compared to rhu IL-8. In this construct, the C-terminus of the CH1 domain and the N-terminus of IL-8 were closely linked by three amino acids and produced in E. coli. These factors are thought to be responsible for impaired biological activity (26). However, comparison of the biological activity of our anti-FAP scFv IL-8R construct and rhu IL8 clearly revealed equal potency regarding induction of chemotaxis. The fact that we did not observe any chemotactic activity induced by TNF in vitro is in accordance with data from other groups demonstrating the IL-8 dependence of PMN migration: i.e. PMN migration across TNF-pre-treated endothelium needs active secretion of IL-8 (27, 28). Furthermore, transmigration of PMNs properly stimulated by TNF could even be completely inhibited by anti-IL-8 antibodies (18). In addition to chemotactic activity in vitro, PMN recruitment by scFv-fused IL-8R construct could be demonstrated in vivo using a nude mouse model. Species cross-reactivity is known for human IL-8 and infiltration of xenotransplanted tumors in nude mice by murine PMNs has been described upon transfection of human ovarian cancer cells with an expression vector coding for human IL-8. Furthermore, the study demonstrated a striking correlation between IL-8 production, neutrophil-monocyte infiltration and regression of xenotransplanted ovarian cancer (29). The antibody-based approach simplifies the strategy of therapeutic IL-8 release with regard to the clinical situation. Administration of anti-FAP scFv-IL-8R construct resulted in focused PMN recruitment localized to xenotransplanted tumors and was strictly dependent on the presence of the targeted FAP antigen. However, the role of PMNs in anti-tumoral immune responses still harbors a striking dichotomy and is currently a matter of intensive debate. Under inflammatory conditions, PMNs may promote tumor growth and progression, primarily in advanced disease (30). In contrast, PMNs can exert important tumor-inhibitory activities in the context of therapeutic interventions (31, 32). Even in the clinical situation of bladder cancer patients treated with bacillus Calmette-Guérin (BCG), PMNs are the first and most abundant immune cell subpopulation immediately following BCG instillation (33). They produce high levels of pro-inflammatory cytokines and chemokines, such as IL-8 (34). Furthermore, the extent of leukocyte accumulation correlates with a favorable outcome after BCG instillation (35) and local concentrations of IL-8 in the bladder were shown to predict clinical responses to BCG (36), due to its functional role as an effector cell recruiting chemokine (37).

In preclinical models, pivotal anti-tumor mechanisms involved in dimeric TNF-based immunotargeting approaches also rely on recruitment and side-specific activation of PMNs (8, 9). In the present approach, sequential administration of IL-8 and TNF-derived FAP-targeting fusion proteins could significantly enhance the therapeutic impact of antibody-bound dimeric TNF. This effect is based on the antigen-restricted enrichment of PMNs to FAP-positive tumors mediated by IL-8 and the consecutive specific activation of recruited effector cells mediated by TNF. In conclusion, the sequential treatment schedule was well tolerated without severe side effects and the cytokine-induced activation loop in PMNs displayed significant therapeutic activity.

**Abbreviations**

FAP, fibroblast activation protein; PMN, polymorphonuclear neutrophil; rhu, recombinant human

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**References**


Materials and methods

Cell lines and reagents

RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and glutamine (0.3 mg/ml, all obtained from Invitrogen Life Technologies) was used as standard medium. Suspension-adapted HEK 293 EBNA cells (ATCC) were cultivated in spinner flasks in serum-free Ex-Cell VPro medium (JRH, Lenexa, USA) at 37°C in a humidified 5% CO₂ atmosphere. HTI1080 FAP-transfected (HTI1080–FAP), mock-transfected (HTI1080) cells and anti-FAP IgG TNF were previously described (8). Rat anti-mouse CD11b (clone M1/70) and rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1; clone MEC13.3) were purchased from BD PharMingen (San Diego, CA). Rabbit anti-human IL-8 was from Dianova (Hamburg, Germany). Murine anti-idiotypic anti-FAP was from Boehringer Ingelheim. Human recombinant TNF and human recombinant IL-8 were purchased from Genzyme (Neu-Isenburg, Germany).

Construction of antibody-derived constructs

Reverse transcription PCR on mRNA isolated from peripheral blood mononuclear cells for amplification of different human IL-8 cDNA sequences was previously described (8). The cDNA sequences coding for the mature 72-residue (IL-872) or the N-terminal truncated 69-residue form (IL-83–72) were cloned into the eukaryotic pEAK8 vector (Edge Biosystems, Gaithersburg, MD, USA). The final vector coding for anti-FAP was cloned into the eukaryotic pEAK8 vector (Edge Biosystems, Gaithersburg, MD, USA). The final vector coding for anti-FAP was from Boehringer Ingelheim. Human recombinant TNF and human recombinant IL-8 were purchased from Genzyme (Neu-Isenburg, Germany).

Expression, purification and characterization of antibody-derived constructs

Suspension-adapted HEK293 EBNA cells were cultured in DMEM/F12 medium (Life technologies, BRL) supplemented with 29 mM sodium bicarbonate, 10 mM HEPES, 2.5 mg/l insulin, 2.5 mg/l human transferrin, 0.1 mM proline, 0.1 mM diethanolamine and 1% FCS, at a density of 3 x 10⁵ cells/ml. Transfection was performed in spinner flasks with an optimized calcium phosphate precipitation method (16, 17), using 2.5 μg DNA (99% construct DNA and 1% pEGFP C1, as a reporter for transfection efficiency) per 1 ml transfection medium. Approximately 80% transfection efficacy was observed. The supernatant was harvested 8–10 days after transfection, filtered through a 0.2 μm cellulose acetate membrane and purified via its C-terminal His₆ tag using affinity chromatography (metal ion affinity chromatography on Ni²⁺ loaded HiTrap Chelating HP columns; Amersham, Freiburg, Germany). Potential endotoxin contamination was excluded by Limulus amebocyte assay (QCL 1000; BioWhittaker). Recombinant proteins were analyzed by SDS-PAGE as previously described (9) and protein bands were visualized by staining with Coomassie brilliant blue (Sigma, Munich, Germany). Proteins were transferred to PVDF membranes (Millipore, Bedford, MA) for Western blot analysis and blotted with 10% non-fat dry milk before incubation with rabbit anti-human IL-8 (0.2 μg/ml) antibody. Membranes were either stained by goat anti-rabbit IgG conjugated to HRP (1:3000; BioRad, Munich, Germany) or mouse anti-c-myc 9E10 antibody (2 μg/ml), followed by biotinylated rabbit anti-mouse IgG conjugated to HRP (1:1000; BioRad) and visualized by chemiluminescence (ECL-Kit, Amersham).

Binding analysis

Flow cytometry was performed as previously described (9). In brief, 1 x 10^⁶ FAP- or mock-transfected cells were incubated (30 min, 4°C) with purified constructs at the specificity and concentration indicated. Cells were washed twice with PBS, incubated with anti-c-myc 9E10 or rabbit anti-IL-8 antibody and, finally, the complex visualized by adding PE-conjugated rabbit anti-mouse or goat anti-rabbit serum (dilution 1:100; DAKO, Hamburg, Germany). For each sample 10000 cells were counted and analyzed. Binding of IL-8 fusion proteins was also assessed by ELISA. 96-well flat-bottomed microtiter plates (Maxisorp Immuno microwell plates, Nunc, Roskilde, Denmark) were coated (overnight, 4°C) with murine anti-idiotypic anti-FAP antibody (1 μg/ml) in 30 μl coating buffer per well. Plates were blocked with 1.5% gelatin in PBS and then the indicated reagents dissolved in PBS were added in serial dilutions and incubated for 1 h at room temperature (RT). After incubation with rabbit anti-human IL-8 antibody (0.5 μg/ml, 1 h, RT), biotinylated goat anti-rabbit serum (1:2000; 1 h, RT) and horseradish peroxidase (1:50000, 15 min, RT; Boehringer Mannheim, Germany) were added. Plates were developed by the addition of o-phenylenediamine substrate (Sigma, Deisenhofen, Germany). The reaction was stopped with 3 M HCl and the plates were then read on a Fluorimeter (Wallac 1420 Victor 2, Turku, Finland) at 490 nm.

Fluorescence-based determination of PMN migration in vitro

PMNs were purified from the venous blood of healthy human donors as previously described (8) and labeled with 5 μg/ml calcein-AM (Molecular Probes, Eugene, OR). Cells were washed twice and resuspended in RPMI-1640 (without phenol red) and 10% FCS at a concentration of 3.2 x 10⁶ cells/ml. PMN migration was measured in triplicates using a non-disposable 96-well ChemoTx-system with a polycarbonate filter (3.2 mm in diameter, 8 μm pore size, 8 mm² exposed filter area per well; Neuroprobe, USA). Chemo-attractants were diluted in 0.1% PBS-HSA to a final volume of 29 μl per well. Pure PBS-HSA 0.1% served as a control to determine random migration. In order to define the total fluorescence of the PMNs added to the top side of the filter and to correlate fluorescence with the number of PMNs that migrated, cell suspensions at
concentrations of 80000, 8000, 800 and 80 cells per 25 µl, respectively per well, were placed directly in three wells in the bottom chamber. The filters were positioned on the loaded microplate and labeled PMNs were placed directly onto the top of the filters (25 µl/well). The plates were then incubated (at 37°C, 5% CO2) for 60 minutes. Cells which had not migrated from the top of the filter were removed and migrated PMNs residing within and under the filter were spun down into the bottom chamber (450 x g, 10 min). The filter was removed and plates were read on a fluorimeter (model 1420, Victor 2; Wallac, Turku, Finland) at 485 nm. Statistical analyses were performed using Pearson’s correlation coefficient (38).

Tumor model and treatment protocols

Adult male and female BALB/c nu/nu mice (6-8 wk; Charles River Laboratories) were used for efficacy assessment. Tumors were simultaneously engrafted by s.c. injection of 2 x 10⁶ FAP-or mock-transfected HT1080 cells, respectively, at the opposite flanks of the animals. FAP antigen expression on xenografts was confirmed over four weeks. In the first trial, tumors were established in four groups of mice (three animals per group) and harvested 24 hours after i.v. injection of either 300 µg anti-FAP scFv-IL-8, 200 µg anti-FAP scFv, 100 µg rhu IL-8, or PBS at equal volume. In a second trial, seven groups of mice (five animals per group) received five repeated i.v. injections of either 100 µg anti-FAP IgG TNF followed eight hours later by 300 µg anti-FAP scFv-IL-8, 100 µg anti-FAP IgG TNF followed eight hours later by 100 µg rhu IL-8, 100 µg anti-FAP IgG followed eight hours later by 100 µg rhu IL-8, 100 µg anti-FAP IgG or 100 µg rhu IL-8. Control mice received equal volumes of PBS alone. Treatment started when tumors reached 3 mm in diameter. Tumor growth and signs of treatment-related side effects were checked daily. The studies were approved by the local Animal Welfare Committee and performed in accordance with their guidelines.

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