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Ubiquitin is a versatile scaffold protein for the generation of molecules with *de novo* binding and advantageous drug-like properties

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ABSTRACT

In the search for effective therapeutic strategies, protein-based biologicals are under intense development. While monoclonal antibodies represent the majority of these drugs, other innovative approaches are exploring the use of scaffold proteins for the creation of binding molecules with tailor-made properties. Ubiquitin is especially suited for this strategy due to several key characteristics. Ubiquitin is a natural serum protein, 100% conserved across the mammalian class and possesses high thermal, structural and proteolytic stability. Because of its small size and lack of posttranslational modifications, it can be easily produced in *Escherichia coli*. In this work we provide evidence that ubiquitin is safe as tested experimentally *in vivo*. In contrast to previously published results, we show that, in our hands, ubiquitin does not act as a functional ligand of the chemokine receptor CXCR4. Cellular assays based on different signaling pathways of the receptor were conducted with the natural agonist SDF-1 as a benchmark. In none of the assays could a response to ubiquitin treatment be elicited. Furthermore, intravenous application to mice at high concentrations did not induce any detectable effect on cytokine levels or hematological parameters.

1. Introduction

1.1. Scaffolds

Targeted intervention at protein structures on cells or in serum is the principle of therapeutic approaches using specifically developed or designed binders like monoclonal antibodies, soluble receptor molecules and the new class of scaffold proteins. The latter are based on natural or artificial protein molecules which are engineered by randomizing surface exposed amino acid positions to generate new binding properties to selected targets. In this field several concepts are pursued. They range from small protein domains such as the protein A domain (Affibody, [1]), PDZ domains [2] and ankyrin repeat proteins (Darpins, [3]), through small full-length proteins, such as the commonly used thioredoxin scaffold [4,5] to higher-molecular-weight beta-barrels and Ig-like structures such as lipocalins (Anticalins, [6]), green fluorescent protein (GFP, [7]) and the T-cell receptor complex [8]. One particularly well-suited scaffold for therapeutic and other applications is ubiquitin, a protein naturally occurring intracellular as well as in serum [9].

1.2. Ubiquitin

Ubiquitin possesses unique features with respect to protein characteristics, production and safety aspects. It has favorable biochemical properties like a stable structure over a wide pH range, thermal shifts or proteolytic degradation [10–12]. As a human serum protein ubiquitin exhibits low immunogenic potential when applied in humans. Its sequence is fully conserved in mammals, enabling fast development tracks due to dispensability of species-specific surrogate molecules during preclinical development. Ubiquitin can be

Abbreviations: CXCR4, CXC motif chemokine receptor 4; SDF-1, stromal cell-derived factor 1; PBS, phosphate buffered saline; ^{125}I-SIB, N-succinimidyl 3-(^{125}iodo)-benzoate; ID, injected dose; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration

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easily produced as soluble protein in high yields in the cytoplasm of *Escherichia coli* [12]. Dimeric head-to-tail fusions of two derivatized ubiquitin molecules are the basis for so called Affilin® compounds currently under development [9].

Ubiquitin is present within the cell at concentrations in the low μM range as free monomer as well as in conjugated form [13]. The different states are mainly balanced by conjugation and deubiquitinylation [14]. Intracellular ubiquitin has a half-life of several hours. Its turnover is counter steered in the cell mainly by modulation of intracellular ubiquitin pools and *de novo* synthesis [15,16]. Cellular ubiquitin is involved in several functions in the cytoplasm and the nucleus respectively [17]. In the cytoplasm it is an important player in processes like autophagy, proteasome mediated proteolysis, endocytosis of activated transmembrane proteins, cargo sorting [18], innate immunity [19] and endoplasmic reticulum associated degradation (ERAD, [20]). In the nucleus ubiquitin is a key component for mRNA-transport, transcriptional control, DNA damage tolerance and DNA repair [21]. The interactions by which ubiquitin assists in the above mentioned processes are manifold and include covalent as well as non-covalent binding of ubiquitin to numerous cellular proteins. Non-covalent interactions between ubiquitin and target proteins are of low affinity with K_D values mostly in the high μM range [22,23]. With respect to these interactions, several partially overlapping epitopes on the surface of ubiquitin have been identified [24]. Covalent conjugation of ubiquitin to several proteins and to other ubiquitin entities is of importance in proteolytic and non-proteolytic processes of the cell. The initial step in this event is the activation of ubiquitin and its transfer to the indicated protein. In this process which is driven by a complex enzyme machinery (E1, E2 and E3 enzymes) the two C-terminal glycine residues at positions 75 and 76 of ubiquitin play a key role [25]. Modification of ubiquitin at these positions via amino acid exchange completely abolishes the transfer of ubiquitin to proteins and the formation of polyubiquitin chains [26]. In addition to conjugation to other proteins ubiquitin molecules are activated and covalently attached to distinct lysine residues of other ubiquitin entities. That way, polyubiquitinylated proteins are formed. Currently, at least eight different types of ubiquitin chains are known, with each individual linkage affecting distinct cellular processes [27–31].

Ubiquitin is detected in serum of healthy humans in a concentration of less than 100 ng/ml (<10 nM) [32]. An obvious source might be the passive release from cells undergoing physiological turnover [33], but there are also reports about ubiquitin release from intact cells [34–36]. On the other hand uptake of ubiquitin into cells of the hematopoietic system has been shown [35,37,38]. The exact function of extracellular ubiquitin is still under debate. A role in modulation of immune responses [39,40] and conditions of inflammation is discussed [41,42], for a comprehensive review see Majetschak [43]. Experimental findings suggest an influence on the ratios of the cellular components of the blood [35] and recently on myocardial remodeling [44–46]. In this context CXC motif chemokine receptor 4 (CXCR4) has been suggested as a putative receptor for ubiquitin with an affinity in the medium nM range [47–50]. Signaling and function are described as being similar but not equal to the hitherto known and well characterized CXCR4 ligand stromal cell-derived factor 1 (SDF-1) [51–53].

1.3. CXCR4

CXCR4 is a typical class A G-protein coupled receptor broadly expressed in the organism. It can be found especially in hematopoietic cells but also many other cell types for example of the central nervous system and the gastrointestinal tract. The biological functions of CXCR4 are crucial during development and hematopoiesis [54–56]. It plays pleiotropic yet not fully understood roles in the immune system and during tissue repair processes [57–60]. Particular interest for CXCR4 as a drug target is based on its role in HIV infection and metastatic diseases [61–66]. The receptor is bound by its natural ligand SDF-1 (or chemokine (C–X–C motif) ligand 12) and a non-cognate ligand MIF, migration inhibitory factor [67]. Receptor activation by SDF-1 leads to anti-inflammatory and organ protective effects in various disease models [68–71].

Because of these similarities in the overall effects to those of ubiquitin [72–75], CXCR4 was discussed as a possible receptor for ubiquitin in the literature [43,76,77]. In this paper we challenge this hypothesis and present significant results elucidating the signaling competence of ubiquitin via different branches of the complex CXCR4 downstream interaction network compared to the natural ligand SDF-1. Neither in cells naturally expressing CXCR4 nor in cells transfected with the CXCR4 gene ubiquitin dependent CXCR4 activity could be demonstrated. Furthermore comprehensive data concerning the *in vivo* application of ubiquitin are discussed.

2. Material and methods

2.1. Production and purification of ubiquitin proteins

Human ubiquitin for biodistribution and toxicity studies was produced with an F45W substitution [12] in *E. coli*. The cDNA of ubiquitin was subcloned into pSCIL008 expression vector and expressed in *E. coli* JM83 cells (DSMZ). After cell harvest and disruption via ultrasonication, cell lysate was heat denatured for 5 min at 75 °C in a water bath. Precipitated protein was removed and the supernatant was diluted in 50 mM sodium acetate pH 5.0 and loaded onto a SP-Sepharose FF column. Elution was performed by a sodium chloride gradient in 50 mM sodium acetate pH 5.0. Fractions of interest were pooled and purified via a Q Sepharose FF. The flow through was applied onto a SP Sepharose HP column and protein of interest was eluted by sodium chloride gradient in 50 mM sodium acetate pH 5.0.

A pharmacokinetic study of ubiquitin was realized using commercially available protein from R&D Systems (U-100H).

The genetic construct of di-ubiquitin was obtained via head to tail fusion of the DNA fragments of two F45W ubiquitin monomers. Di-ubiquitin was expressed from the expression vector pET20b (Novagen) in *E. coli* Nova Blue (DE3) cells. After cell disruption, solid $(NH_4)_2SO_4$ was added to 40% saturation. Precipitated protein was removed and the supernatant was applied to a Phenyl Sepharose HP column. Elution was performed by $(NH_4)_2SO_4$ gradient in 50 mM Tris/HCl, 1 mM EDTA, pH 7.5. Fractions containing the target protein were pooled and applied to a SP Sepharose HP column after cross-flow filtration in 50 mM acetic acid/NaOH, 1 mM EDTA, pH 5.5. Fractions of di-ubiquitin eluted in a sodium chloride gradient were concentrated and applied to a Superdex 75 prep grade column equilibrated in phosphate-buffered saline (PBS) for removal of monomeric ubiquitin fragments. Fractions containing the homogeneous target protein were concentrated, applied to a Q Sepharose FF anion exchange column and collected in the flow through.

Purified preparations of ubiquitin and di-ubiquitin were filtered through sterile 0.2 μm polyethersulfone disc filters (Millipore) and stored at −80 °C.

2.2. Protein analytics

Analytical size exclusion chromatography (SE-HPLC) was carried out on a Superdex 75 Tricorn 10/300 column (GE Healthcare) coupled to an Ultimate 3000 SD chromatographic system (Dionex GmbH, Idstein, Germany). PBS containing 0.05% sodium azide as

preservative was used as eluent at a flow rate of 0.5 mL/min. Molar masses were estimated by comparison with the elution profile of a BioRad gel filtration standard mix (BioRad). Reversed-phase chromatography analysis was carried out using a PLRP-S column (300 Å, 5 μm, 250 mm × 4.6 mm; Agilent) with an eluent system of 0–80% 2-propanol in 0.1% trifluoroacetic acid, a flow rate of 0.8 mL/min, and a column temperature of 65 °C. Endotoxin content was measured using the Endosafe®-PTS™ system (Charles River). The amount of residual *E. coli* host cell protein in purified protein preparations was analyzed using a commercially available ELISA kit (Cygnus Technologies).

2.3. Radio labeling and analytics

Ubiquitin was radio iodinated directly via Iodogen method [78]. In brief, 100 μg of protein and 180–200 μCi of $Na^{125}I$-solution (6.67–7.4 MBq, Perkin Elmer) were added to a reaction vial coated with 50 μg of Iodogen. The mixture was incubated for 20 min at room temperature followed by purification via gel filtration (Sephadex G25, PD10) and elution with PBS. An alternative labeling was performed for ubiquitin and di-ubiquitin by an indirect iodination method using N-succinimidyl 3-(^{125}iodo)-benzoate (^{125}I-SIB) as iodo-precursor [79]. For this reaction, ^{125}I-SIB and the protein of interest were added to a reaction vial in a molar ratio of between 1/20 and 1/30, followed by pH adjustment to pH 8.5 using borate buffer. The mixture was stirred at 4 °C for 20 min and purified accordingly.

Radio chemical purity was analyzed by instant thin layer chromatography (ITLC, Agilent Technologies). Purity of labeled proteins was determined by analytical size exclusion chromatography with radio detection. Serum stability was tested by incubation of the proteins in 90% mouse serum at 37 °C over 16 h followed by ITLC and SE-HPLC analyses with radio detection.

2.4. Regulatory and husbandry

All *in vivo* studies were performed at qualified contract research organizations under attendance of Scil Proteins scientists.

Biodistribution and pharmacokinetic studies were performed at Chelatec SAS (France) and carried out in strict accordance with the recommendations of the European Guide for the Care and Use of Laboratory Animals (2010/63/EU). The protocol was approved by the Committee on Ethics of Animal Experiments (CEEA PdL n°6, France).

Toxicity studies on ubiquitin and di-ubiquitin were performed in strict accordance to the recommendations of the German Guide for Use of Animals for Research Purposes (German Protection of Animals Act, 18.05.2006, EU Directive 86/609). The toxicity study on ubiquitin was conducted by Bayer Healthcare AG (Wuppertal, Germany) and approved by the State Office for Nature, Environment and Consumer Protection of Nordrhein-Westfalen in Recklinghausen, Germany (Permit Number: AZ 9.93.2.10.43.07). The toxicity study on di-ubiquitin was performed at the Laboratory of Pharmacology and Toxicology GmbH & Co. KG (LPT, Hamburg, Germany) and approved by the Public Authority for Health and Consumer Protection in Hamburg (Permit Number: V 1307-591-00.33). According to the German Basic Law (article 20a, 01.08.2002) animal protection is an explicit constitutional aim. The ethical tenability of animal studies in fundamental research is assessed and approved by the authorities (referred to above) with their own ethical advisors or committees. An additional ethical committee is not stipulated.

Generally all efforts were made to minimize suffering of the animals. Animals were allowed to acclimatize for at least one week. During acclimatization phase animals were inspected on a daily basis for physical welfare. Free access to food (certified commercial diet) and water was provided ad libitum. Animals were housed under standard conditions of 20 ± 2 °C temperature, a humidity of 55 ± 5% and a light/dark cycle of 12 h rhythm by a maximum of four (mice) and group wise per cage or individually (rats). At the day of dosing, animals were weighed and examined for physical abnormalities. Toxicity studies were performed as blinded studies whilst PK and biodistribution studies were non-blinded. Generally, in all *in vivo* studies animal allocation was performed by body weight stratification and randomization by block design. Only healthy animals were included in the studies. After the end of the studies, all animals were anesthetized under diethyl ether anesthesia or by an intraperitoneal injection of ketamine hydrochloride and xylazine hydrochloride in PBS followed by rapid sacrifice by exsanguination.

2.5. Biodistribution studies of ubiquitin and di-ubiquitin

For biodistribution experiments of ^{125}I-ubiquitin, 3 male Wistar rats (7 weeks of age, mean body weight 133 g; Janvier Laboratory, France) were treated by an intravenous slow bolus injection of radio iodinated ubiquitin into the tail vein. The protein formulated in PBS was administered at a dose of 355 nmol/kg, a volume of 3 ml/kg and at a specific activity of 0.13 mCi/kg.

Biodistribution of ^{125}I-SIB-labeled di-ubiquitin was performed using a total of 12 female CD1 mice (7 weeks of age, mean body weight 25 g; Charles River, France). Animals were divided into 4 groups (n = 3), equivalent to the different termination time points. Radiolabeled di-ubiquitin in PBS was injected into the tail vein at a dose of 11.4 nmol/kg, a volume of 5 ml/kg and at a specific activity of 0.4 mCi/kg. Of three groups blood samples were collected at different time points (5 min, 1 h and 3 h) post-injection from the saphenous vein of non-anaesthetized mice. Bleedings were performed in a staggered regime with one bleeding time point for each mouse and consistent bleeding time points of the animals within a group. Blood was collected in Microvette tubes with clotting activator (Sarstedt, Nürmbrecht, Germany) for serum preparation. For analysis of excretion balance and recovery of di-ubiquitin, 3 mice were individually housed in metabolic cages over 16 h.

At time of sacrifice (t = 15 min for ^{125}I-ubiquitin treated group; t = 0.5 h, 2 h, 8 h and 16 h for ^{125}I-SIB-di-ubiquitin-treated groups), blood was collected in Microvette tubes with clotting activator. Subsequently, the animals were anesthetized by an intraperitoneal injection of ketamine hydrochloride and xylazine hydrochloride in PBS followed by rapid sacrifice by exsanguination via intracardiac puncture. Organs of interest were excised, rinsed of residual blood, weighed and counted in a gamma counter (Wallace Wizard 2470, Perkin Elmer). Whole blood and serum radioactivity as well as urine and feces radioactivity were measured accordingly. Radioactivity was calculated as percent of injected dose (% ID) per g tissue and % ID/total tissue.

2.6. Pharmacokinetic studies of ubiquitin

For a PK study of ubiquitin, a total of 16 female CD1 mice (7 weeks of age, mean body weight 25 g; Charles River, France) were used. Animals were divided into four groups (n = 4). ^{125}I-SIB-labeled ubiquitin was injected into the tail vein at a dose of 23.5 nmol/kg, a volume of 4 ml/kg and at a specific activity of 2 mCi/kg. Blood samples were collected from the submandibular vein of non-anaesthetized mice at seven bleeding time points post-injection (1 min, 5 min, 10 min 15 min, 30 min, 45 min and 60 min). Bleeding was performed group wise in a staggered regime with a maximum of two bleeding time points for each animal, approximately 30 min between the bleedings and consistent bleeding time points of the animals within a group. Blood was

collected in Microvette tubes with clotting activator. Blood and corresponding serum fractions were analyzed in a gamma counter. Radioactivity was calculated as % ID/g tissue and quantified as equivalent of protein provided as ng/total blood or serum and ng/g blood or serum.

2.7. Toxicity studies

For a subacute toxicity study of ubiquitin and a pilot toxicity study of di-ubiquitin, Wistar rats were obtained from Harlan Winkelmann GmbH (Germany) and Charles River (Germany), respectively.

For a subacute toxicity study of ubiquitin, a total of 20 Wistar rats (10 male and 10 female, 6 to 8 weeks of age, body weight range: 172–191 g/male rats, 142–160 g/female rats) were divided into two groups (n = 5 female and 5 male). Animals were assigned to their groups by randomization according to their body weight using a software application. Rats exhibiting extreme body weights were excluded from randomization. In the ubiquitin treatment group, animals were intravenously injected into the tail vein with a daily dose of 3 mg PBS-formulated ubiquitin per kg body weight at a dosing volume of 5 ml/kg over 14 days. In the control group, animals were treated with PBS vehicle accordingly. Inspections on morbidity and mortality were performed twice daily. Clinical signs and body weights were recorded once daily prior to each treatment. Individual food and water intake were determined weekly. On day 15, 24 h after the last treatment, all animals were sacrificed by exsanguination under diethyl ether anesthesia. Animals were inspected and their organs and tissues were subjected to thorough gross pathological examination. Organs were weighed and fixed for histopathological examinations. Heart, liver, kidneys, spleen, thymus and popliteal lymph node were embedded in Paraplast (Sigma) followed by subsequent sectioning. All slices were stained with hematoxylin and eosin (H&E). Cryo cuts obtained from the liver were stained with Oil Red O (ORO). Clinical laboratory investigations on blood were performed on the day of sacrifice. Determination of hemoglobin, blood leukocytes, erythrocytes, reticulocytes, thrombocytes as well as white blood cell differentiation and calculations of hematocrit, MCV, MCH, MCHC were performed using ADVIA 120 Hematology System (Siemens). Blood coagulation was determined using a Hepatoquick® test kit (Roche) and a ball coagulometer KC10 (Amelung). For determination of blood alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, gamma -glutamyltransferase, creatine kinase, lactate dehydrogenase, glutamate dehydrogenase, albumin total protein, creatinine, urea, glucose, inorganic phosphate, triglyceride and total bilirubin commercially available test kits were used. Electrolytes (sodium, potassium, calcium) were determined by flame photometric assays using a flame photometer EFUX 5057 (Eppendorf-Netheler-Hinz GmbH). Chloride was estimated by coulometric analysis on a Chloridmeter 50 (Kreienbaum Wissenschaftliche Meßsysteme e.K.). The study was performed in a GLP-certified laboratory at Bayer Healthcare AG (Germany) according to established procedures and protocols. Statistical evaluations on body and organ weight as well as food and water intake were done using the Dunnett-test in connection with a variance analysis. Evaluation of biochemical parameters was performed primarily by analyses of variance followed by a Dunnett-test. For all tests SAS® routines were applied. For evaluation of statistical different effects the dose groups were compared to the treatment groups of the same gender.

For a pilot toxicity study of di-ubiquitin, a total of 32 Wistar rats (16 male and 16 female, body weight range: 145–186 g/male rats, 132–156 g/female rats) were divided into 4 groups (n = 4 male and 4 female). Animals were assigned to their groups by randomization according to their body weight using a software application. Animals of groups 2–4 were intravenously injected into the tail vein with a single slow bolus injection of PBS-formulated di-ubiquitin at a dose of 3 mg, 17 mg or 100 mg per kg body weight in a volume of 8 ml/kg. In the control group 1 animals were treated with PBS vehicle accordingly. Animals were observed for mortality and clinical signs throughout the day after test item administration. At predose and 0.5 h, 6 h and 24 h post administration blood samples were taken from the retrobulbar venous plexus under isoflurane anaesthesia and transferred to hematological examinations (see above but excluding coagulation). Cytokine levels (IL-2, IL-12 and IFN-gamma) were determined in blood serum via Bead Array on a Cytomics FC 500 (Beckman Coulter GmbH). 24 h post administration animals were sacrificed under ether anesthesia by exsanguination. Animals were inspected, weighed and their organs and tissues were subjected to thorough gross pathological examination. The study was performed at the Laboratory of Pharmacology and Toxicology GmbH & Co. KG (LPT, Germany) based on the OECD principles of good laboratory practice. Statistical analyses of potential significant effects between the treatment groups and the control group of the same gender were performed using the multiple t-test based on Dunnett and a limit of $p < 0.01$ and $p < 0.05$.

2.8. Cells and reagents

HEK293 (DSMZ) and COS-7 (DSMZ) cells were maintained in DMEM (Lonza) supplemented with 10% FBS (Gibco). THP-1 (DSMZ where not specified and ATCC as indicated) and Jurkat (DSMZ) cells were cultivated in RPMI1640 (Lonza) supplemented with 10% FBS (Gibco).

2.9. Cloning

The CXCR4 gene was purchased from Thermo Scientific and PCR amplified with specific primers: pCDNA3-CX forward 5′-GCA TGA ATT CGC CAC CAT GGA GGG GAT CAG TAT ATA C-3′ and pCDNA3-CX reverse 5′-GCA TCT CGA GTT AGC TGG AGT GAA AAC TTG AAG ACT C-3′. PCR fragments were digested with EcoRI and XhoI (NEB) and ligated into pCDNA3 (Life Technologies) digested with the same enzymes using the Rapid Ligation kit (Roche). Ligation reactions were purified with the MinElute Reaction Cleanup kit (Qiagen) and transformed into *E. coli* XL1 blue cells (Agilent). After antibiotic selection a positive clone was sequence verified and propagated for plasmid preparation with the QIAfilter Plasmid Midi kit (Qiagen).

2.10. FACS analysis

CXCR4 binding was analyzed using CXCR4 expressing suspension cell lines THP-1 and Jurkat. 2×10^5 cells were suspended in 100 µl cold PBS containing indicated N-terminally labeled FITC-Ubiquitin (R&D Systems) concentrations. Specific binding of FITC-ubiquitin to CXCR4 was measured in the presence of a 30-fold molar excess of unlabeled ubiquitin (Sigma). Samples were incubated 30 min at 4 °C and washed twice with 500 µl cold PBS. Fluorescence intensities of 1×10^4 cells were determined using flow cytometry (FACSCalibur, BD Bioscience) at 490 nm wavelength.

Furthermore, about 8×10^5 HEK293 cells in DMEM/HamsF12, 10% FBS were cultivated in 12 well plates and were allowed to grow to 80% confluence at 37 °C. Cells were transiently transfected with 1.28 µg pVITRO2-CXCR4, 0.32 µg chimeric G-protein G alphaΔ6qi4myr cDNA and 4.8 µl Metafectene per well in 1 ml cultivation medium overnight. As control non-transfected cells were maintained. 200 µl Trypsin/EDTA per well were used to detach HEK293 cells. 800 µl DMEM medium were pipetted to the cell

suspension and the samples were transferred into tubes and centrifuged for 2 min at 500×*g*. Pellets were resuspended in 200 µl cold PBS containing 1 µM FITC-ubiquitin or as positive control 1 µM Alexa647-labelled SDF-1 (labelling of SDF-1 according to NanoTemper L001 Monolith™ Protein Labeling Kit RED-NHS instructions). Incubation was carried out for 30 min on ice and for FACS analyzes (CyFLow ML, Partec) cells were washed twice with cold PBS. Fluorescence intensities of 5×10^4 cells were analyzed.

2.11. Calcium-flux assay

Intracellular calcium mobilization was monitored using the FLIPR 5 Calcium Assay Kit (Molecular Devices). 1.2×10^5 THP-1 or Jurkat cells were suspended in 200 µl RPMI-1640, 2% FBS and seeded into a 96 well plate (black/clear bottom). An equal volume of Kit component A (calcium sensitive dye and masking dye) was added to the cells and the samples were incubated for 1 h at 37 °C and 5% CO_2. Relative fluorescence signals were measured simultaneously to automated ubiquitin or SDF-1 (PeproTech) administration to the cells using a FlexStation3 microplate reader (Molecular Devices). Dose response curves were collected for ubiquitin concentrations ranging from 10^{-10} to 10^{-6} M (ubiquitin Sigma) and from 5×10^{-6} to 2×10^{-5} (ubiquitin Sigma) and for 5×10^{-5} M (ubiquitin R&D Systems). For evaluation, signals recorded after complete ligand addition were normalized to RFU = 1 and $t = 0$. All curves were baseline corrected. Data were obtained as triplicates in between two and four independent experiments and progress curves are shown and calculated EC_{50} values are given as mean ± SEM.

2.12. cAMP-ELISA

cAMP levels were quantified using the cAMP complete enzyme immunoassay kit, acetylated format (EnzoLifescience). The cAMP competitive ELISA was performed according to the manufacturer's protocol. In brief, 5×10^5 THP-1 cells were incubated 15 min at 37 °C with or without ubiquitin (Sigma), SDF-1 or mixtures of both proteins in 100 µl PBS. Next the cells were centrifuged at 500×*g* for 5 min. cAMP was extracted by lysing the cell pellets in 200 µl 0.1 N HCl and incubating the samples for additional 20 min at room temperature. Cellular debris was removed by centrifugation at 500×*g* for 5 min. 100 µl of the supernatant were used for the quantification of cAMP levels by ELISA. All incubation steps were performed at room temperature. Signals were read out using a THERMO Multiscan GO microplate reader at a wavelength of 405 nm. Assays were repeated at least three times in triplicates and data are shown as mean ± SEM.

2.13. cAMP reporter assay

HEK293 cells were transfected with pCDNA3-CXCR4 and selected with 400 µg/ml G-418 (Merck). Stable cell pools were then transfected transiently with pGL4.29 plasmid (Promega) containing a CRE element using Fugene HD transfection reagent (Promega) according to the manufacturer's specifications. After 24 h cells were seeded in a 96 well microtiter plate (TPP) and grown at 37 °C, 5% CO_2. 48 h after transfection 20 µM forskolin (Merck) and respective compounds in indicated concentrations were added to the cells and incubated for 1 h at 37 °C, 5% CO_2. 100 µM AMD3100 (Merck), a CXCR4 inhibitor, was applied 30 min prior to compound addition where stated. Subsequently, cells were equilibrated at room temperature for 10 min and an equal volume of One-Glo reagent (Promega) was added. After 10 min incubation at room temperature the luminescent signal was read out in an Infinite M200 plate reader (Tecan).

2.14. Inositol phosphate accumulation assay

To investigate CXCR4 activation by SDF-1 and ubiquitin (Sigma, F45W Scil Proteins), inositol phosphate accumulation assays with transiently transfected COS-7 and HEK293 cells were performed according to [80] with minor changes. Briefly 9×10^4 HEK293 cells in 500 µl DMEM/HamsF12, 10% FBS per well were cultivated in poly-lysine coated 48 well plates. For COS-7 6×10^4 cells per well were seeded in 500 µl DMEM, 10% FBS into 48 well plates. After incubation overnight cells were transiently transfected with 0.32 µg pVITRO2-CXCR4, 0.08 µg chimeric G-protein GalphaΔ6qi4myr cDNA [81] and 1.2 µl Metafectene (Biontex) per well in 250 µl of the respective cultivation medium according to the manufacturer's protocol. Cells were incubated for 6 h or overnight at 37 °C and subsequently labelled with 0.3 µCi ^{3}H-myo-inositol for at least 16 h and washed with 250 µl DMEM containing 10 mM LiCl. Various concentrations of ubiquitin (Sigma or F45W Scil Proteins) or SDF-1 [82] dissolved in 150 µl DMEM/10 mM LiCl were added to the cells and incubated for 2 h at 37 °C. After medium aspiration, cells were lysed in 100 µl 0.1 N NaOH for 5 min at room temperature. 50 µl of 0.13 M formic acid were added to the wells and the samples were subsequently diluted 1:6 with IP-dilution buffer (5.0 mM Na-borate, 0.5 mM Na-EDTA). Cell debris was removed with a truncated pipet tip. IP3 amounts were determined by anion exchange chromatography followed by a beta counter quantification as described previously [83]. Experiments with COS-7 cells were performed in duplicates and with HEK293 cells in triplicates. For both cell lines at least three independent data sets were generated. Data were normalized to receptor activation in% and calculated EC_{50} values are given as mean ± SEM.

2.15. Cell migration assay

CXCR4 expressing THP-1 and Jurkat cells were used to analyze induction of chemotaxis triggered by ubiquitin (Sigma) or SDF-1. *In vitro* cell migration was assessed using 96 HTS transwell plate chambers (Sigma; pore size: 5 µm for Jurkat and 8 µm for THP-1 cells) as described earlier [47]. Briefly, in the lower chamber 250 µl PBS with or without varying SDF-1 or ubiquitin concentrations (10^{-10}–10^{-6} M) were loaded, whereas the upper chamber contained 2×10^5 cells in 100 µl PBS. After 3 h of incubation at 37 °C, 200 µl of the lower chamber were diluted 1:2 with 5 µg/ml propidiumiodide and incubated for 10 min at room temperature. Propidiumiodide staining enabled identification of living cells that had actively migrated to the lower chamber and were counted using flow cytometry analysis (FACSCalibur BD Bioscience). The chemotactic index was calculated by dividing the number of migrated cells towards a specific ligand concentration by the number of migrated cells against PBS (control). Data were collected as triplicates in at least three independent experiments and shown as mean ± SEM.

2.16. Ubiquitination assay

To check for the activity of all ubiquitin proteins (derived from Sigma, R&D Systems or cloned and purified by Scil Proteins GmbH) used within this study an *in vitro* ubiquitination assay was performed. CHIP Ubiquitin Ligase Kit-Glow-Fold Substrate (R&D Systems) was applied and all experimental steps were conducted according to the manufacturer's protocol. Briefly, a mix of water, E3 ligase reaction buffer, Mg^{2+}-ATP solution, HSP40/HSP70 and Glow-Fold™ substrate was heated for 7 min at 43 °C and then cooled for 10 min on ice. Afterwards, E1 and E2 enzyme and CHIP were added. Beside the kit supplied ubiquitin (positive control) indicated ubiquitin proteins (each 300 µM) were analyzed.

As negative control ATP and ubiquitin were omitted. Reactions were incubated for 1 h at 37 °C and terminated by the addition of 5×SDS–PAGE sample buffer containing DTT. Reactions were heated 5 min at 90 °C and afterwards loaded and run at 100 V, 2 h on a 10% TGX stain-free gel (Bio-Rad). The gel was activated and imaged in the Gel Doc EZ Imager station (Bio-Rad). Proteins were transferred to a PVDF membrane with the TransBlot turbo system (Bio-Rad). Detection of ubiquitinated Glow-Fold™ substrate was via western blot analysis using α-Glow-Fold™ primary antibody and HRP-labeled secondary antibody (R&D Systems, HAF017) according to the kit protocol.

3. Results

3.1. Protein preparation

Ubiquitin and di-ubiquitin were produced by heterologous expression in *E. coli* and purified by a series of chromatographic steps. The obtained preparations were homogeneous, as judged by Coomassie-stained SDS-PAGE and SE-HPLC and more than 98% pure by RP-HPLC. For di-ubiquitin no response in an endotoxin assay could be observed up to a sensitivity of 0.007 EU/mg protein. Host cell protein content was determined to be 32 ppm. Endotoxin in the ubiquitin preparation was determined at 0.33 EU/mg.

For the investigation of biodistribution and pharmacokinetics, ubiquitin proteins were directly iodinated on tyrosine side chains or indirectly iodinated on lysine residues. The labeling efficiency of the proteins was between 60% and 80%. Labeled proteins were more than 95% pure as determined by SE-HPLC with more than 99% protein associated radio label.

3.2. Blood/serum analysis and pharmacokinetics

In vitro stability analysis of ^{125}I-SIB-labeled proteins incubated for 16 h at 37 °C in 90% mouse serum revealed no dehalogenation as determined by ITLC. In addition, SE-HPLC analyses of serum incubated proteins provided a single peak eluting at a retention time comparable to the proteins stored in PBS (Fig. S1A and B).

Determination of radioactivity of *ex vivo* blood samples obtained from CD1 mice treated with ^{125}I-SIB-ubiquitin (Fig. 1A) and ^{125}I-SIB-di-ubiquitin (Fig. 1B) showed that both proteins are rapidly cleared from the circulation. For ^{125}I-SIB-ubiquitin a half-life of 23.6 min at a basal serum level of 24.0 ng/ml was determined.

Comparison of radioactivity measured in whole blood and the corresponding serum samples revealed accumulation of both proteins in the serum (Fig. 2A and B), i.e. there is no measurable cellular uptake of ubiquitin from the serum.

3.3. Biodistribution

Biodistribution studies in healthy animals were conducted to provide information on potential ubiquitin accumulation in main organs as well as organs and tissues of expected relevance due to their known CXCR4-expression. Initial tissue distribution of ubiquitin determined 15 min after intravenous administration into Wistar rats was performed using directly labeled protein (Table 1). To reduce the risk of *in vivo* dehalogenation and the potential for related tissue background radio activity mediated by enzymatically released and metabolized ^{125}Iodide, especially when evaluating biodistribution over several hours

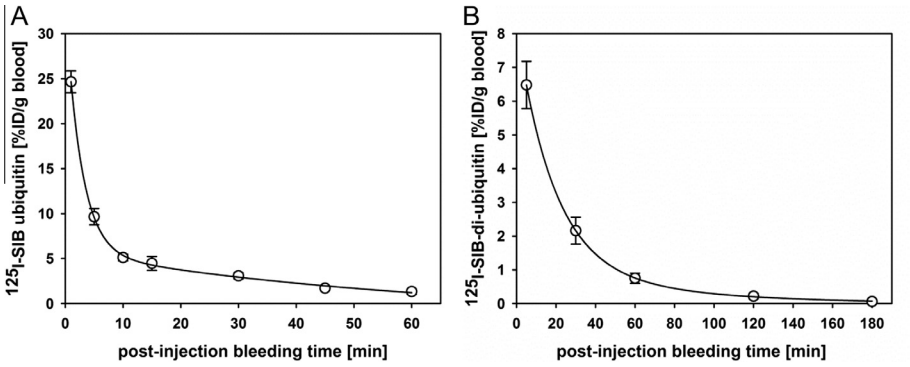

Fig. 1. Blood clearance of ubiquitin. (A) ^{125}I-SIB-labeled ubiquitin (R&D Systems) or (B) F45W di-ubiquitin (Scil Proteins) was injected into the tail vein of 7 weeks old, female CD1 mice. Blood samples of indicated bleeding time points were collected from three non-anaesthetized mice. After analysis of blood and serum in a gamma counter, radioactivity was calculated as % ID/g tissue. Error bars represent SD.

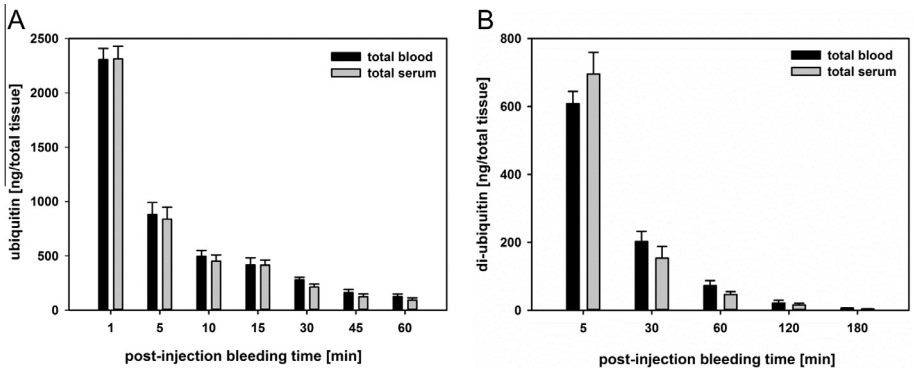

Fig. 2. Blood and serum accumulation of ubiquitin. (A) ^{125}I-SIB-labeled ubiquitin (R&D Systems) or (B) F45W di-ubiquitin (Scil Proteins) was injected into the tail vein of 7 weeks old, female CD1 mice. Blood samples of indicated bleeding time points were collected from three non-anaesthetized mice. After analysis of whole blood and serum in a gamma counter, radioactivity was calculated and quantified as equivalent of protein provided as ng/total blood or serum. Error bars represent SD.

Table 1
Distribution of ^{125}I-ubiquitin in Wistar rats after 0.25 h in % ID/g tissue.

^{125}I-ubiquitin	% ID/g tissue
Blood	1.02 ± 0.07
Heart	0.48 ± 0.02
Lungs	1.06 ± 0.13
Spleen	0.29 ± 0.02
Kidneys	33.07 ± 3.57
Liver	0.35 ± 0.04
GI tract	0.27 ± 0.01
Brain	0.04 ± 0.01

Table 2
Distribution of ^{125}I-SIB-di-ubiquitin in CD1 mice at different time points in % ID/g tissue.

^{125}I-SIB-di-ubiquitin	0.5 h	2 h	8 h
Blood	2.16 ± 0.40	0.22 ± 0.08	0.01 ± 0.01
Heart	0.78 ± 0.18	0.06 ± 0.02	0.01 ± 0.01
Lungs	2.73 ± 0.70	0.24 ± 0.10	0.04 ± 0.02
Spleen	1.09 ± 0.37	0.16 ± 0.06	0.04 ± 0.02
Kidneys	57.73 ± 7.20	4.74 ± 1.64	0.07 ± 0.02
Liver	2.12 ± 0.49	0.27 ± 0.51	0.03 ± 0.02
GI tract	2.02 ± 0.61	0.69 ± 0.32	0.02 ± 0.01
Brain	0.07 ± 0.01	0.006 ± 0.003	0.003 ± 0.004

post-injection, indirectly labelled di-ubiquitin was administered in CD1 mice (Table 2).

Tissue distribution of ^{125}I-ubiquitin and ^{125}I-SIB-di-ubiquitin showed that, except for the kidneys, no relevant accumulation of either protein is observed in main organs at any time after intravenous injection. ^{125}I-SIB-di-ubiquitin is cleared from the circulation and from organs revealing organ/blood ratios <1. For ^{125}I-ubiquitin and ^{125}I-SIB-di-ubiquitin 15 min or 30 min post-injection, total radioactivity of main organs, again kidneys excluded, accounted for approximately 15% and 8% of the injected dose, respectively. At the same time, 46% and 16% of injected doses of ubiquitin and di-ubiquitin were found in the kidneys. Excretion analysis revealed renal elimination as main path for di-ubiquitin clearance with 96.0% of radioactivity found in urine and 0.7% of radioactivity detected in feces 16 h post-injection. The carcass, constituting approximately 90% of the total animal weight and representing the sum of the remaining tissues, e.g. skin, fat and bones, accounted for 32% and 37% of the injected dose of the proteins at 15 min or 30 min post-injection. Independent biodistribution studies in mice pointed to an accumulation of less than 0.5% ID/g in these tissues at 2 h post-injection. Radioactivity in spleen was determined at 0.29% ID/g at 15 min post-injection of ^{125}I-ubiquitin and 1.09% ID/g at 30 min post-injection of ^{125}I-SIB-di-ubiquitin decreasing over time.

3.4. Toxicity studies

Toxicity studies in rats were performed to provide information on potential adverse effects after intravenous administration of ubiquitin and di-ubiquitin. A subacute toxicity study, based on daily intravenous dosing of 3 mg/kg ubiquitin over 2 weeks in Wistar rats, revealed no evidence for a treatment-related statistically significant effect on body weights, food and water consumption. Hematological examinations did not point to statistically significant effects on blood cell counts (Fig. 3A) as well as hemoglobin and blood coagulation. The same holds true for clinical chemistry examinations. Macroscopic post-mortem examinations did not point to pathological findings at the examined organs and tissues. Gross pathological and treatment-related changes were not recorded. Histopathological examinations of specified organs revealed some spontaneous findings which were evenly distributed between treatment and control groups and thus were not treatment-related. Lack of specific organ toxicity could be also verified for the kidney as organ of highest exposure and excretion.

A single dose toxicity study of di-ubiquitin administered in a dose range between 3 mg/kg and 100 mg/kg in rats revealed no

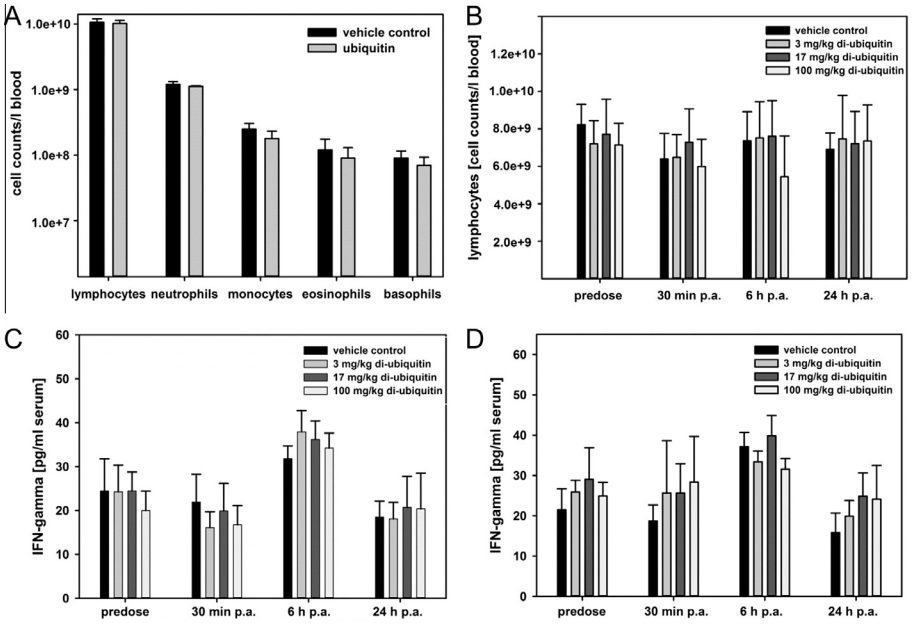

Fig. 3. Blood cell numbers and IFN-gamma levels after ubiquitin treatment. (A) In a subacute toxicity study blood samples of rats (n = 10/group, 5 male and 5 female) treated with F45W ubiquitin (Scil Proteins, grey bars) or the vehicle control (white bars) were analyzed and numbers of different blood cell types were determined. Bars represent cell counts from blood samples of male rats, error bars represent SD. (B) In a pilot acute toxicity study blood samples of rats (n = 8, 4 male and 4 female) treated with various doses of F45W di-ubiquitin (Scil Proteins, grey bars) or the vehicle control (white bars) were analyzed and numbers of lymphocytes were determined at indicated time points. Error bars represent SD. (C and D) IFN-gamma levels of male (C, n = 4) and female (D, n = 4) rats of B were determined with a bead array on a Cytomics FC 500 at indicated time points. Error bars represent SD.

influence on any examined parameter up to 24 h post-injection. No adverse effects on physiological parameters were found. Cage side observations of skin/fur, eyes, mucous membranes, respiratory and circulatory systems, somatomotor activity, and behavior patterns did not point to treatment-related effects. The treatment was well tolerated and there were no signs of local irritations at the injection site. The faeces of all rats showed a normal consistency during the entire experimental period. No premature deaths occurred during the course of the study. Body weights determined at predose and at day 1 did not point to statistically significant alterations between the treatment groups and the control group. Hematological examinations of the treatment groups on erythrocytes, leukocytes, platelets, differential blood count including lymphocytes (Fig. 3B) and hemoglobin, each determined predose and 0.5 h, 6 h and 24 h post administration, did not reveal any dose-dependent or time-dependent treatment-related changes. However, as can be expected for multiple comparisons, individual values at individual dosing groups or time points were found to deviate significantly from the control group. All of these values remained within the normal range for the individual parameters and where thus assessed as treatment-unrelated. Cytokine determination in serum samples at 0.5 h, 6 h and 24 h post-injection did not point to a treatment-related increase of pro -inflammatory cytokines. At any time point, IL-2, IL-12 (Fig. S2) and IFN-gamma (Fig. 3C and D) serum levels remained within the range of the control group serum levels of less than 4 pg/ml, 55 pg/ml and 40 pg/ml, respectively. Statistical comparison of treatment groups versus vehicle-treated control group at the indicated time point did not provide evidence for a statistically significant alteration of the assessed cytokines. Macroscopic post-mortem examinations did not point to any treatment -related pathological changes of the inspected superficial and subcutaneous tissues. Due attention was paid to organs of the thoracic viscera i.e., thymus, lymph nodes and heart and to organs of the abdominal viscera i.e., urinary bladder, gastrointestinal tract, stomach and caecum without hints for treatment-related effects. Examination of lungs, liver, kidneys, gonads, adrenal glands, uterus, intra-abdominal lymph nodes and accessory reproductive organs did not provide evidence for treatment-related effects on their appearance or size. No pathological findings were detected at any examined organ.

3.5. Ubiquitin binding to CXCR4 expressed on eucaryotic cells

Our *in vivo* experiments did not suggest any evidence for specific ubiquitin accumulation on the tested hematopoetic cells. However, previously it has been reported that extracellular ubiquitin is able to bind to and activate the CXCR4 receptor present on lymphocytic cell lines like THP-1 [48]. To corroborate our data from the *in vivo* studies and relate them to the existing literature we sought to investigate the binding of ubiquitin to CXCR4 in two relevant cell lines, THP-1 (Fig. 4A) and Jurkat (Fig. 4B). Cells were incubated with 1 or 10 μM FITC-labeled ubiquitin and binding was assessed through FACS measurements. To account for non-specific binding of FITC-ubiquitin to the cellular surface, unlabeled ubiquitin was added in a 30-fold molar excess to compete for specific binding. The addition of 10 μM labelled ubiquitin to both CXCR4 positive cell lines resulted in a small shift of the cell populations towards higher fluorescence intensities, whereas none or a very weak shift was observed after incubation with 1 μM FITC-ubiquitin in THP1 and Jurkat respectively. Binding at the higher FITC-ubiquitin concentration was most likely non-specific, as it could not be blocked with unlabeled ubiquitin and no decrease in the FACS signal was detected under these conditions. In addition, experiments with HEK293 overexpressing the CXCR4 were performed. Alexa Fluor 647 labelled SDF-1 showed increased binding to HEK293 cells after CXCR4 cDNA transfection (Fig. S3B), whereas no FITC-ubiquitin binding to the receptor could be detected (Fig. S3A). As a control, non-transfected HEK293 cells were used. These results are in contradiction to reports claiming that ubiquitin binds specifically to THP-1 or to CXCR4-transfected HEK293 cells at the examined conditions [48].

3.6. Influence of ubiquitin on CXCR4-mediated calcium-flux

To investigate the signaling of CXCR4 in response to the binding of its two reported ligands, SDF-1 [84,85] and ubiquitin [47], we measured intracellular calcium fluxes as part of the G-protein mediated signal cascade. THP-1 and Jurkat cells were labeled with the Calcium 5 fluorophore and treated with a concentration series of the two compounds in a FlexStation 3 (Molecular devices). This instrument was especially designed to precisely measure the very fast release of calcium ions indicated by an increase in fluorescent signal in real time. SDF-1 was effective in both cell lines (Fig. 4C and D) with an EC_{50} of 9 ± 1.4 nM, which is in good agreement with the existing literature [86]. In contrast, ubiquitin failed to induce any change in fluorescence signal intensity and accordingly in the cytosolic calcium ion levels. The results were further confirmed by additional measurements at very high ubiquitin concentrations. Ubiquitin from two different suppliers was applied at up to 20 or 50 μM on both cell types without eliciting any calcium signal (Fig. S4). This supports the conclusion that ubiquitin has no agonistic activity on the calcium flux response in THP-1 and Jurkat cells.

3.7. Impact of ubiquitin on intracellular cAMP levels

To further differentiate effects on the CXCR4 signaling cascade we made use of a cAMP ELISA kit (Enzo life sciences). Intracellular cAMP levels were quantified after incubation of the respective cells with the CXCR4 ligand SDF-1 or ubiquitin at two different concentrations, 1 μM and 100 nM. Since CXCR4 signals through G alpha i proteins, ligand binding and activation of the receptor should result in decreasing cAMP levels, which would correspond to an increase in signal intensity in this specific ELISA setup. Again, SDF-1 was able to decrease intracellular cAMP levels, whereas ubiquitin had no effect on intracellular cAMP levels (Fig. 5A). Because this is in contrast to already reported results, we verified several sources of experimental variability: (i) a THP-1 cell line from a different supplier (ATCC) revealed essentially the same result (Fig. S5A). (ii) To account for differences in incubation times we performed a time course experiment with increased incubation times of up to 60 min. For all tested conditions, there was no effect of ubiquitin on intracellular cAMP levels (Fig. S5B). (iii) Since we noticed slightly increased cAMP levels in some ubiquitin treated samples compared to controls, we looked at possible antagonistic effects of ubiquitin in the presence of SDF-1. Therefore, 1 μM SDF-1 and ubiquitin in various concentrations ranging from 1 to 10 μM were added simultaneously to THP-1 cells and incubated for 15 min followed by cAMP ELISA. In all tested concentrations ubiquitin had no antagonistic effect on SDF-1 treated THP-1 cells (Fig. S5C).

To further support the acquired data concerning cAMP levels we constructed a stable cell pool of 293T cells expressing the CXCR4 full length protein. Afterwards, we transiently transfected the cell pool with a pGL4.29 reporter plasmid (Promega), designed to monitor increases in cAMP levels through a cAMP response element (CRE) that drives transcription of a luciferase reporter gene. We stimulated cells with forskolin to non-specifically increase the cAMP levels through activation of adenylate cyclase and added in parallel different compounds to measure a change in luminescence. As seen for the cAMP ELISA, SDF-1 was able to inhibit the

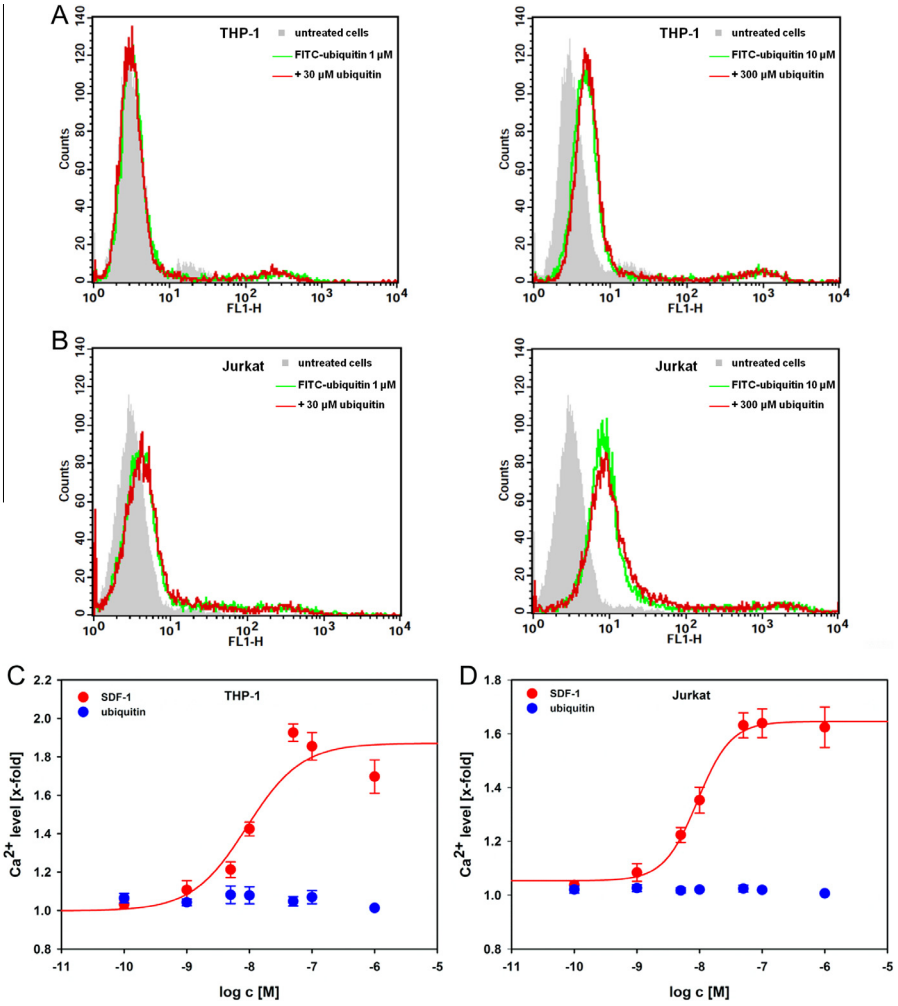

Fig. 4. Binding analysis and calcium signaling of ubiquitin on THP-1 and Jurkat cells. (A) Binding of 1 µM (left panel) or 10 µM (right panel) FITC-ubiquitin (R&D Systems) with (red) or without (green) competition with 30-fold molar excess of unlabeled ubiquitin (Sigma) to THP-1 cells was assessed by flow cytometry. Untreated cells are shown in grey. (B) Binding of 1 µM (left panel) or 10 µM (right panel) FITC-ubiquitin (R&D Systems) with (red) or without (green) competition with 30-fold molar excess of unlabeled ubiquitin (Sigma) to Jurkat cells was assessed by flow cytometry. Untreated cells are shown in grey. (C and D) THP-1 (C) or Jurkat (D) cells were labelled with the Calcium 5 fluorophore and incubated with different concentrations of SDF-1 or ubiquitin (Sigma). Increase in Calcium 5 signal intensity indicating calcium signaling was monitored in the FlexStation 3 instrument. Error bars represent SEM of four (THP-1) and three (Jurkat) independent experiments.

increase in cAMP levels caused by forskolin, whereas ubiquitin (F45W Scil Proteins) had no such effect (Fig. 5B). Similar as in the cAMP ELISA in some cases there was rather a tendency for slightly increased cAMP levels. This phenomenon was not concentration dependent and an agonistic activity of ubiquitin has been excluded with the ELISA. Additionally, we incubated the cells with the well-known CXCR4 inhibitor AMD100 [87] prior to SDF-1 and forskolin. This treatment resulted in a loss of activity of SDF-1 on cAMP levels due to inhibition of the CXCR4 receptor, showing the specificity of our assay (Fig. S5D). Furthermore, we compared different ubiquitin variants (F4A and V70A in Fig. S5D) or sources (F45W ubiquitin and di-ubiquitin Scil Proteins, ubiquitin Sigma, ubiquitin R&D Systems in Fig. S5E) but found no variation. Hence, ubiquitin has no impact on cAMP levels in the cell lines tested.

3.8. Influence of ubiquitin on IP3 levels

Next, we additionally investigated a different branch of the GPCR signaling cascade. We co-transfected HEK293 and COS-7 cells transiently with CXCR4 and chimeric G-protein G alphaΔ6qi4myr cDNA and labeled cells with ^{3}H-myo-inositol. G alphaΔ6qi4myr is a chimeric G-protein that switches CXCR4 signaling from the G alpha i pathway to the G alpha q pathway leading to release of inositol-3-phosphates (IP3) [88]. After successful labeling cells were treated with SDF-1 or ubiquitin (Sigma) for 2 h in the indicated concentrations and accumulation of radioactively labeled IP3 was determined. In both cell lines SDF-1 led to an increase in IP3 levels with an EC_{50} of 4 ± 1.4 nM for HEK293 (Fig. 5C) and 1.6 ± 1.5 nM for COS-7 cells (Fig. 5D) respectively. As in the previous assays ubiquitin had also no influence on IP3 levels through a postulated activation of the CXCR4 receptor. Similar results were obtained in an experiment with ubiquitin F45W from Scil Proteins (Fig. S6).

3.9. Effect of ubiquitin on cell migration

To verify our binding and signal transduction data with a more physiologically relevant read-out we chose a cell migration assay. THP-1 cells from two different suppliers or Jurkat cells were seeded in the upper chamber of 96 HTS transwell plates (Corning) with appropriate membrane pore sizes for the respective cell lines. The lower chamber was loaded with different concentrations of SDF-1 or ubiquitin (Sigma) and cell migration to the lower chamber after 3 h was determined by FACS analysis. SDF-1 showed an increase in the chemotactic index with specific kinetics for both

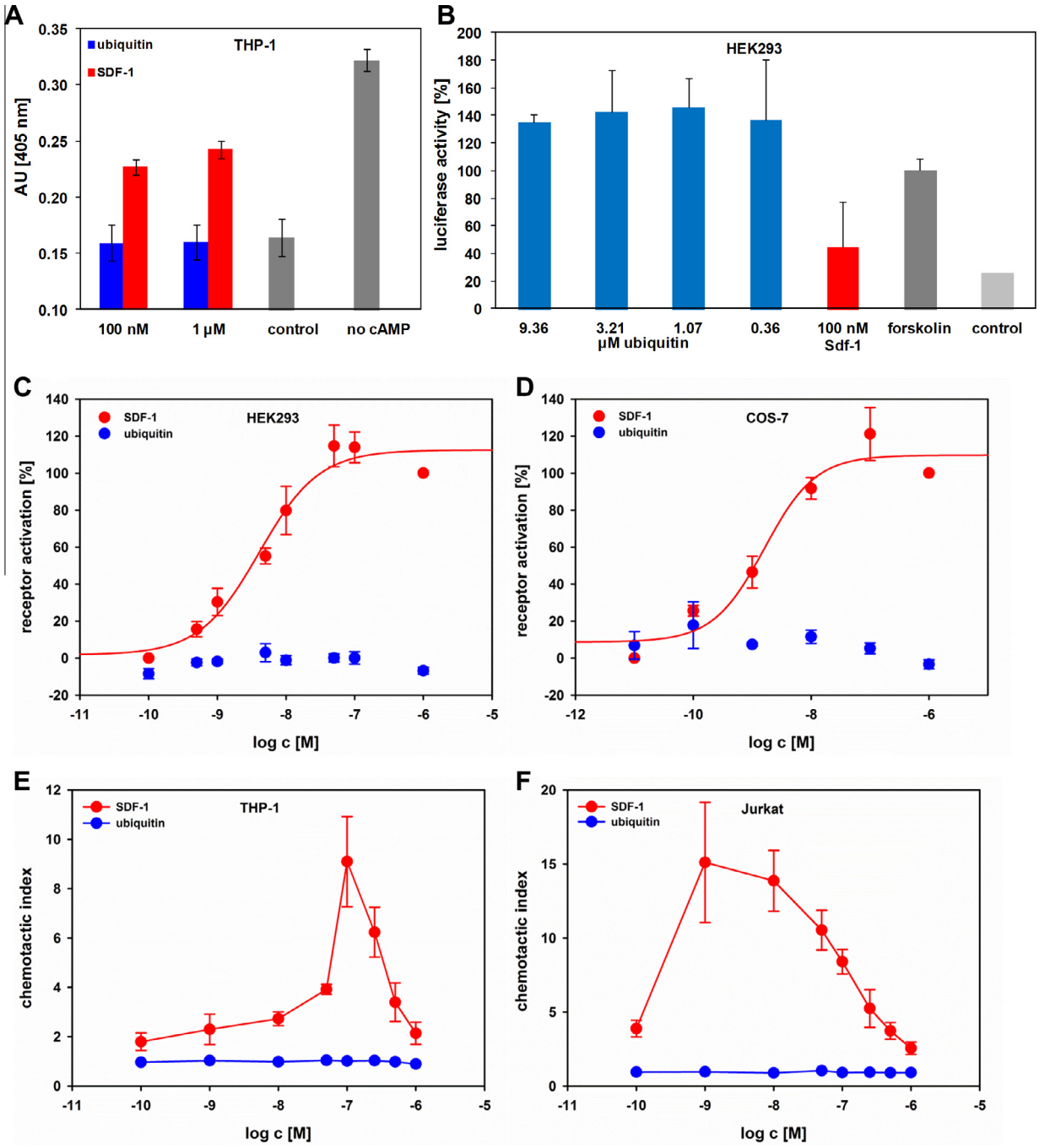

Fig. 5. Ubiquitin impact on the CXCR4 signaling cascade. (A) THP-1 cells were incubated with SDF-1 or ubiquitin (Sigma) in the indicated concentrations. cAMP content was analyzed after cell lysis with the cAMP complete enzyme immunoassay kit, acetylated format for higher sensitivity. Error bars represent SEM of three independent experiments. (B) HEK293 cells expressing CXCR4 were transfected with a pGL4.29 reporter plasmid and stimulated after 48 h with forskolin without (dark grey bar) or in combination with SDF-1 (red bar) or ubiquitin (F45W Scil Proteins, blue bars) in indicated concentrations. Unstimulated cells (light grey bar) served as negative control. Data were normalized on luciferase activity for forskolin treatment set to 100%. Error bars represent SD from three experiments. (C and D) HEK293 (C) or COS-7 (D) cells were transiently transfected with CXCR4 and the chimeric G-protein G alphaΔ6qi4myr. Afterwards cells were labelled with 0.3 µCi ^{3}H-myo-inositol for more than 16 h followed by incubation with SDF-1 or ubiquitin (Sigma) in the indicated concentrations. Accumulated IP3 was collected by anion exchange chromatography from cell extracts followed by a beta counter quantification. Error bars represent SEM from three (HEK) or four (COS-7) independent experiments. (E and F) THP-1 (E) or Jurkat (F) cells were seeded in the upper compartment of 96 HTS transwell plate chambers. The lower compartment was filled with PBS containing indicated concentrations of SDF-1 or ubiquitin (Sigma). After 3 h incubation cells in the lower chamber were collected and analyzed by flow cytometry. Chemotactic index was calculated as the ratio of the number of migrated cells at the indicated ligand concentration to the number of cells migrated to buffer. Error bars represent SEM from three independent experiments.

cell lines, whereas neither cell line showed any migration towards ubiquitin (Fig. 5E and F) in the tested, broad concentration range.

From all these *in vitro* experiments we conclude that ubiquitin induces no CXCR4 signaling in the tested lymphocytes or other CXCR4 overexpressing cell lines and had no effect on their migratory behavior. This result fits well to the *in vivo* data presented, as even very high concentrations of *in vivo* applied ubiquitin did not induce any significant biological activity, especially not any CXCR4-related reactions, in treated mice.

3.10. Bioactivity of ubiquitin variants

Ubiquitin is a naturally stable, fast folding protein. To finally exclude any doubt about the biological activity and equivalence of the ubiquitin species from different sources used in our work we performed ubiquitination studies (Fig. S7). A commercially available substrate and coupling kit was used for ligation reactions with monomeric ubiquitin from the R&D Systems' kit components, ubiquitin separately purchased from R&D Systems, ubiquitin purchased from Sigma and our in house produced F45W ubiquitin. Linear dimeric ubiquitin was run in parallel, again from R&D Systems and in house production. All monomeric ubiquitin preparations were very efficiently coupled to the substrate. Marginal deviations in the intensity of the detected bands can be correlated with the slightly fluctuating substrate amounts contained in the individual reactions and loaded on the gel. The phenomenon is due to technical features of the experiment and it has been repeated for confirmation with the same result. Ubiquitination with the dimeric forms was weaker as with the monomeric ones but again very similar among the two tested species.

4. Discussion

Ubiquitin is a highly abundant, omnipresent molecule in mammalian organisms. It exerts innumerable effects mainly inside the cell but is also present in the extracellular compartment. Its versatility is based on the many possible non-covalent and covalent interaction options. Ubiquitin occurs as monomer or multimer of diverse composition concerning numbers or type(s) of linkage of ubiquitin entities. The typical ubiquitin interaction has K_D values in the high nanomolar to low micromolar range. Specificity is given by compartmentalization or characteristics of the respective ubiquitin species.

The choice of (di)ubiquitin as scaffold for the generation of binding molecules with new properties for therapeutic application irrespective of the natural ubiquitin targets requires that the above described functions are unaffected by such compounds. A different or complementary ubiquitin based therapeutic approach was published by the Sidhu group, selecting ubiquitin variants after randomization of up to 19 amino acid positions as possible modulating agents for intrinsic ubiquitin targets [89]. The presented work scrutinizes the mandatory inertness or at least innoxiousness of ubiquitin as parenteral drug. In this context the recently published finding that ubiquitin is a hitherto unknown ligand of the chemokine receptor CXCR4 [48–50,76,77] is relevant and therefore investigated in depth.

Irrespective of our *in vitro* findings related to a postulated specific ubiquitin CXCR4 interaction, the general impact of high ubiquitin doses applied to the organism has been tested thoroughly in *in vivo* studies. Biodistribution experiments were performed with radio labeled proteins in the Wistar rat and CD1 mouse model. Distribution of radio labeled ubiquitin intravenously injected into Wistar rats did not provide any evidence for a specific ubiquitin accumulation in main organs, except the kidneys. Comparable data were obtained after intravenous administration of radio labeled di-ubiquitin into mice. After initial distribution into highly perfused organs like liver, spleen, lungs and gastrointestinal tract the proteins are quickly cleared from the circulation and from organs revealing organ/blood ratios <1. Ubiquitin and di-ubiquitin are small proteins sized below the glomerular filtration threshold. The normal pathway of elimination of proteins of this size is expected to be filtration in the glomerulus and subsequent reabsorption in the proximal tubules by means of endocytosis followed by lysosomal degradation. The high radioactivity detected in the kidneys at indicated time points and the accumulation within urine at 16 h post-injection point to the renal elimination as main route for clearance of ubiquitin and di-ubiquitin. Due to the quick renal clearance of ubiquitin a short half-life of 23.6 min was determined in mice. A similar fast blood clearance was observed for di-ubiquitin with approximately 7% of injected radioactivity measured in blood as early as 5 min post-injection. These data are well comparable to already published information on ubiquitin half-life [42,75].

While numerous physiological functions for intracellular ubiquitin species are reported, a physiological function of extracellular ubiquitin as contained in serum is so far not described. Recently, an interaction of extracellular ubiquitin with CXCR4 (in the range of K_D 100 nM) has been reported [47–50]. The CXCR4 receptor is expressed in almost all blood cells [47,90–93]. The basal ubiquitin serum level in mice was determined at 24.0 ng/ml corresponding to an ubiquitin concentration of 2.8 nM, which is similar to the human serum ubiquitin level with a mean concentration of 32.7 ng/ml [32]. This ubiquitin serum concentration is more than one order of magnitude below the K_D for the postulated ubiquitin CXCR4 interaction. However, administration of ubiquitin may lead to an increase to values around or above the K_D of a putative ubiquitin-CXCR4 interaction [37,51,77]. Five min after application of 0.2 mg/kg ubiquitin and 0.1 mg/kg di-ubiquitin serum levels of 50 and 20 nM are obtained, respectively. Concentrations of 500 nM were reached post-injection of 3 mg/kg di-ubiquitin. Although protein concentrations sufficient for protein internalization or physiologically relevant signals are available within the first minutes, our data on *ex vivo* analysis of blood and related serum samples do not support an uptake of ubiquitin into total blood cells. Moreover, radioactivity measured in spleen, the largest lymphoid organ with storage function for lymphocytes and monocytes [94], did not provide evidence for accumulation of ubiquitin. Therefore, the published data on ubiquitin-CXCR4 interaction obtained in *in vitro* experiments are not confirmed by a detectable ubiquitin accumulation in CXCR4 expressing cells or tissues under healthy conditions. One point that should be taken into account in this context is the rapid clearance and thus limited availability of a sufficient high ubiquitin concentration within the bloodstream.

Ubiquitin did not show toxic effects in reports from healthy and disease models in different animal species [41,42,74,75]. Application of ubiquitin was generally performed at a single low dose of between 1 and 3 mg/kg in these studies. Ubiquitin of all mammalian species investigated so far shares 100% sequence identity, which means freedom of choice regarding a relevant animal model. We selected the Wistar rat model for further toxicological evaluation of ubiquitin and di-ubiquitin. Rat CXCR4 shows 90% sequence identity and 96% sequence homology compared to human qualifying this species as relevant for these studies. Intravenous administration of up to 100 mg/kg of di-ubiquitin in an acute setting and repeated dosing of 3 mg/kg of ubiquitin in a subacute setting did not elicit any evidence for toxic effects. Moreover, no alterations of numbers of CXCR4 expressing blood cells were detected. Thus, for instance lymphocyte release from the spleen rapidly increasing the blood cell pool or cell depletion e.g. by diapedesis induced by inflammatory reactions do not occur. In addition, the endogenous level of several pro-inflammatory cytokines known to interfere with the regulation of CXCR4 [95–97] were not influenced by ubiquitin treatment.

To complement the assessment of ubiquitin or di-ubiquitin application *in vivo*, we were interested in the hypothesis of their direct interaction with CXCR4 in more detail. Indeed, we could detect some binding of FITC-labeled ubiquitin to CXCR4 expressing cell lines like THP-1 or Jurkat. This interaction was weak though and occurred only at high concentrations of up to 10 μM, much higher than expected for a binder with 100 nM affinity [47].

Moreover, the binding fraction could not be displaced by a 30-fold molar excess of unlabeled ubiquitin. We therefore consider this phenomenon as non-specific and unrelated to the actual presence of CXCR4. This was further confirmed in the comparison of CXCR4 transfected to non-transfected HEK293 cells. Binding of labeled ubiquitin to cells, namely platelets has also shown by Tan and colleagues [98]. Unfortunately the specificity of that effect has not been proven by competition with unlabeled ubiquitin. On the other hand uptake of a ubiquitin mutant fused to a BH3 peptide has been found and hypothesized to be mediated via the CXCR4 axis [38]. As this molecule deviates markedly from wild type ubiquitin not only in the primary sequence (K48R/K63R) but also by attached linker, BH3 and tag domains the mechanism of its uptake is not clearly elucidated. The presumably specific uptake could for example only be inhibited by very high amounts of ubiquitin as competitor. Recent papers discussing the structural basis of CXCR4 modulation do not imply the concept of ubiquitin being a ligand of the receptor [99,100].

To pursue the possibility for a notwithstanding signaling at very low receptor occupancies [101,102] we decided to inspect different aspects from the complex signaling network of CXCR4. Although the chemokine receptors are predominantly coupled to G alpha i, a known and frequently used indicator of receptor activity is an increase of free intracellular calcium. This response to ligand binding is probably elicited via the G beta-gamma subunits. As these calcium fluxes have a very fast kinetic an appropriate automated set up for exact real time measurements is mandatory. Moreover, manual handling often leads to artefacts like stretch activated calcium influx or instability through uneven mixing. We therefore used the Flex station 3 instrument for our analyses. With this reliable method a concentration dependent rise of calcium levels after CXCR4 stimulation with SDF-1 was observed in THP-1 and also in Jurkat cells. In clear contrast, ubiquitin, although tested several times and at high concentrations, did not show any response in the calcium flux assay. This contradicts the results of Saini et al. [49] but might be due to the different data acquisition.

As an independent criterion we analyzed inhibition of cAMP accumulation. We used a commercially available ELISA and THP-1 cells. SDF-1 led to a drop in cAMP levels, whereas ubiquitin had no such effect. In order to identify a source for the deviation from the data of Saini et al. [49] several controls were performed. THP-1 from a second supplier behaved exactly in the same way. In addition, we generated stable cell pools expressing the CXCR4 receptor in HEK293. These pools were transiently transfected with a reporter gene to monitor cAMP. SDF-1 showed specific inhibition, which could be blocked by AMD3100, while ubiquitin did not impair cAMP synthesis. No effects of addition of anti CXCR4 antibodies to epitopes in the postulated ubiquitin binding site or AMD3100 could be seen. The characteristic inhibitory signaling cascade was not initiated by any ubiquitin concentration or residence time. Results from several ubiquitin variants were in line with these conclusions. In some experiments we rather noticed a tendency for a slight increase of the cAMP levels after ubiquitin incubation. The phenomenon was not concentration dependent. Significance of or the mechanism responsible for this is speculative. A possible CXCR4 specific antagonistic effect was experimentally excluded.

The functional measurement of inhibitory G-proteins is somewhat challenging because the optimal timing of the activation–inhibition process is not fully understood. The order and time intervals of the treatment with the different compounds like forskolin and the ligand and/or inhibitor under investigation are not obvious and have to be individually established. For this reason a third independent route of second messengers was pursued. As a classical component in the GPCR downstream cascade, accumulation of inositol-3-phosphate was monitored in cells transfected with a chimeric G-protein switching G alpha i to G alpha q signaling. To broaden the approach, these analyses were performed in HEK293 and in COS-7 cells. Again, SDF-1 treatment led to the concentration dependent and saturable rise in intracellular IP3 while application of ubiquitin failed to evoke a detectable IP3 signal.

For a final attempt a more universal terminal signal of G alpha i activation was chosen, that is chemotaxis. Chemotaxis is a central mechanism for CXCR4 function involved in physiological and pathophysiological processes like stem cell homing, wound healing and metastasis. It is induced via different branches of the CXCR4 intracellular cascade. Therefore, independent of potential diversity in cell types or assay specificities, cell migration should be a general consequence of CXCR4 receptor activation. THP-1 and Jurkat cells were used in transwell experiments. SDF-1 showed the typical bell-shaped curves in the activation assay while ubiquitin did not initiate cell migration at all. Chemotactic activity of ubiquitin was observed on B16-F10, 4T1 and RM-9 cells as described in the literature [51]. A direct interaction of ubiquitin on CXCR4 has not been shown in this work.

In addition to the variation of aspects, assays and cell types we involved in our studies, different ubiquitin preparations from commercial sources and in house production were included. Relevant experiments were conducted with the Sigma ubiquitin used for the recently published CXCR4 studies, some of them also with ubiquitin from one or more other sources. Finally all species were enclosed in a ubiquitination assay showing equal bioactivity of the tested monomeric and dimeric forms respectively. This should exclude the notion, ubiquitin from variable sources might behave differently in a biological context.

Thus, in spite of applying a diverse set of assays, the hypothesis of ubiquitin being a ligand for CXCR4 could not be proven. There was not a faint tendency for effectiveness in any experiment. The function of known chemokines and their receptors usually implies a specific and high affinity interaction while ubiquitin is known for weak interactions at usually high concentrations *in vivo,* at least in the context of intracellular functions. In our hands, there is no evidence for ubiquitin-induced signaling through a sensitive chemokine receptor such as CXCR4. We have taken much effort to exclude variations in assay conditions and to include a representative set of cell lines and consistently found no activation. The effects of high doses or levels of ubiquitin and those of CXCR4 activation in the organism might be similar, but probably not directly interconnected. It could well be that CXCR4 is part of a more complex network of mechanisms where ubiquitin is also involved. For example transactivation events, receptor internalization and membrane plasticity are influenced by ubiquitin in general. Very current reports show compelling evidence for the ubiquitin dependent signaling and trafficking of CXCR4 without the assumption of a ligand–receptor complex formation [103–105]. This fits well into the known functions and regulation of chemokine receptors and ubiquitin alike. Surprising findings like the disruption of the SDF-1/CXCR4 axis by IL-24 [106] or the antagonism of a serum albumin fragment [107] show that our idea of possible underlying interconnections is far from being complete. The pathways, which lead to the observed and published effects and the physiological consequences in the interplay of ubiquitin and CXCR4 have to be further elucidated.

5. Conclusion

With the presented data the safety of ubiquitin as a scaffold for the development of therapeutic binding molecules has been underpinned in detail and in general. A specific binding of ubiquitin to CXCR4 or activation of the receptor could be experimentally

excluded. Adverse or unfavorable reactions after *in vivo* administration were not seen even at high doses.

Author contributions

Conceived and designed the experiments: F.J., F.S., S.L., C.R., U.H., H.L. and E.B.D. Performed the experiments: F.J., F.S. and L.B. Contributed reagents/materials/analysis tools: L.B. and A.B.S. Analyzed the data: F.J., F.S., S.L., C.R., L.B., H.L., A.B.S. and E.B.D. Wrote the paper: F.S. and E.B.D. Made substantive contributions to the manuscript: F.J. and S.L. Reviewed the final manuscript: F.J., F.S., S.L., C.R., L.B., A.B.S., U.H., H.L. and E.B.D. Edited the manuscript: E.B.D. Made substantive contributions to the final draft: F.J. S.L. C.R. U.H. and H.L.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.07.002.

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