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Specific *in vitro* binding of a new ^{99m}Tc-radiolabeled derivative of the C-terminal decapeptide of prothymosin alpha on human neutrophils

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ABSTRACT

Prothymosin alpha (ProTα) is a conserved mammalian polypeptide with intracellular functions associated with cell proliferation and apoptosis and an extracellular role associated with immunopotentiation. The N-terminal fragment [1–28], which is identical with the immunostimulating peptide thymosin α1 (Tα1), was earlier considered as the immunoactive region of the polypeptide; however, recent data suggest that ProTα may exert a discrete immunomodulating action through its central or C-terminal region, *via* targeting Toll-like receptor- 4 (TLR4). In this work, a derivative of the C-terminal fragment ProTα[100-109] (ProTα-D**1**) that can be radiolabeled with ^{99m}Tc was developed. The biological activity of the non-radioactive $^{185/187}$rhenium-complex of this derivative ([$^{185/187}$Re]ProTα-D**1**, structurally similar with [^{99m}Tc]ProTα-D**1**) was verified through suitable *in vitro* bioassays on human neutrophils. Subsequent cell-binding studies revealed specific, time-dependent and saturable binding of [^{99m}Tc]ProTα-D**1** on neutrophils, which was inhibited by intact ProTα and ProTα[100-109], as well as by a "prototype" TLR4-ligand (lipopolysaccharide from *Escherichia coli*). Overall, our results support the existence of ProTα-binding sites on human neutrophils, recognizing [^{99m}Tc]ProTα-D**1**, which might involve TLR4. [^{99m}Tc]ProTα-D**1** may be a useful tool for conducting further *in vitro* and *in vivo* studies, aiming to elucidate the extracellular mode of action of ProTα and, eventually, develop ProTα-based immunotherapeutics.

1. Introduction

Prothymosin alpha (ProTα) is a highly acidic polypeptide (MW ~12.5 kDa) with unique structural features, first isolated from rat thymus (Haritos et al., 1984). As revealed later on, ProTα is highly conserved in all mammalian species and expressed in all mammalian tissues, where it serves a dual role (Ioannou et al., 2012; Mosoian, 2011): intracellularly it is involved in cell proliferation (Eschenfeldt and Berger, 1986), chromatin remodeling (Gomez-Marquez and Rodriguez, 1998), regulation of apoptosis (Jiang et al., 2003) and expression of oxidative stress-protecting genes (Karapetian et al., 2005) as well as in triggering a response of the tumor suppressor p53 (Kobayashi et al., 2006); extracellularly it acts as a stimulator of immune responses, both *in vitro* and *in vivo* (Baxevanis et al., 1990; Pan et al., 1986; Pineiro et al., 2000), and promotes angiogenesis (Koutrafouri et al., 2001). More recently, ProTα was also reported to act as a necrosis-inhibitory factor with potent neuroprotective activity (Ueda et al., 2012).

Abbreviations: Aca, aminocaproic acid (6 amino-hexanoic acid); AcS, acetylated serine; dmGly, dimethylglycine; EDT, ethanedithiol; FC, flow cytometry; fMLP, N-formyl-methionine-leucine-phenylalanine; HATU, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; HBSS, Hank's balanced salt solution; IC_{50}, half maximal inhibitory concentration; LAL, Limulus amebocyte lysate; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NLS, nuclear localization signal; oxyma, ethyl 2-cyano-2-(hydroxyimino)acetate; PBMC, peripheral blood mononuclear cells; SPECT, single-photon emission computed tomography; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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An immunoactive peptide identical with the N-terminal fragment [1-28] of ProTα, known as thymosin alpha1 (Tα1), was isolated in late 1970's (Low et al., 1979) from thymus fraction 5 – a defined extract of the bovine thymus gland (Goldstein et al., 1966). Tα1 exhibits *in vitro* and *in vivo* immunoenhancing activity (Naylor et al., 1983; Romani et al., 2004) and stimulates angiogenesis (Koutrafouri et al., 2001). Tα1 formulations have been evaluated in clinical trials as immunopotentiating therapeutics for hepatitis B and C (Billich, 2002; Chien et al., 2006), cancer (Bodey, 2001), cytomegalovirus infection following renal transplantation (Ji et al., 2007), and AIDS (Billich, 2002).

Due to the structural identity between the N-terminal 28-amino acids of ProTα and Tα1, and the immunopotentiating *in vitro* and *in vivo* functions of both peptides, ProTα was initially considered as the natural precursor of Tα1 (Haritos et al., 1984), while Tα1 was considered as the main immunoactive fragment of ProTα. However, much evidence has been accumulated during the last years according to which ProTα may exert its immunomodulating role through different fragments of the polypeptide (Cordero et al., 1996), including the central, negatively charged region 50-89 (Mosoian et al., 2010) or the C-terminal fragment spanning residues 100-109 (Skopeliti et al., 2006). More specifically, ProTα[50-89] has been associated with anti-HIV-1 activity (Mosoian et al., 2007), and ProTα[100-109] has been reported to improve the functionality of immunogenic peptide-pulsed dendritic cells (DCs) *in vitro* (Ioannou et al., 2013), enhance the decreased cytotoxicity of tumor-associated lymphocytes against autologous tumor cells *in vitro* and inhibit tumor growth *in vivo* (Voutsas et al., 2013), and activate neutrophils of healthy individuals and breast cancer patients *in vitro* (Samara et al., 2013).

Limited information is available on the mechanisms underlying the extracellular mode of action of ProTα. Experimental data have indicated that ProTα exerts its activity *via* ligating Toll-like receptor- 4 (TLR4) on murine macrophages through its central region (Mosoian et al., 2010), and on human DCs through its C-terminal decapeptide (Ioannou et al., 2013). On the other hand, Tα1 was reported to act mainly through TLR9 present on human plasmacytoid DCs (Romani et al., 2004).

The main aim of the present work was to further elucidate the extracellular mode of action of ProTα by employing a suitable radiolabeled probe. Our team was the first to develop a derivative of the N-terminus of ProTα/Tα1 labeled with ^{99m}technetium ([^{99m}Tc]ProTα-D**2**) and study the structure of its corresponding complex with non-radioactive $^{185/187}$rhenium ([Re]ProTα-D**2**), (Benaki et al., 2012; Klimentzou et al., 2007). In the present work, we designed and synthesized a new [^{99m}Tc]ProTα-derivative ([^{99m}Tc]ProTα-D**1**), which contains the immunoactive C-terminal fragment [100-109] of the polypeptide (ProTα-D**1**; Table 1). By employing this new radiolabeled probe we subsequently performed cell binding studies, in the search of putative binding sites for ProTα/ProTα[100-109] on human neutrophils.

2. Materials and methods

2.1. Materials

The Fmoc-protected amino acids, the oxyma reagent and the Rink amide resin (0.6 mmol/g) were purchased from Novabiochem/Merck (Darmstadt, Germany). All HPLC solvents (analytical grade) and the other chemicals and biochemicals used were obtained from Merck (Darmstadt, Germany) or Sigma–Aldrich (MO, USA), unless otherwise mentioned. ProTα was a product of Thymoorgan (Vienenburg, Germany). Na[$^{99m}TcO_4$] was obtained in physiological saline as a commercial ^{99}Mo/^{99m}Tc generator eluate (Mallinckrodt, Petten, Netherlands).

2.2. Peptide synthesis

The ProTα fragments ProTα[100-109], ProTα[100-105], ProTα[1-14], ProTα[1-28] (Tα1), the ProTα derivatives ProTα-D**1** and ProTα-D**2**, as well as the negative controls NCP**1a** and NCP**1b** (Table 1) were synthesized manually following the Fmoc/tBu solid-phase peptide synthesis strategy (Amblard et al., 2006). More specifically, ProTα[1-14], ProTα[1-28] (Tα1), and ProTα-D**2** were synthesized following a protocol previously described by our team for ProTα-D**2** (Klimentzou et al., 2007). ProTα[100-109], ProTα[100-105], ProTα-D**1**, NCP**1a** and NCP**1b** were synthesized following a slightly modified protocol. Briefly, couplings were performed using a 4-fold molar excess of each Fmoc-protected amino acid, oxyma (Subiros-Funosas et al., 2009) and *N*,*N*′-diisopropylcarbodiimide (DIC) in *N*,*N*′-dimethylformamide (DMF), and incubating for 2 h at room temperature. Coupling of dmGly-OH was performed using a 3-fold molar excess of dmGly-OH and HATU in DMF and then by adding 9-fold molar excess of diisopropylamine (DIEA). Coupling efficiency was monitored with the Kaiser ninhydrin test and double coupling/capping was performed when necessary. Fmoc-protecting groups were removed by treating the resin with a piperidine solution in DMF (20% v/v) in three successive steps of 5 min, 5 min and 10 min. Cleavage of the peptides from the resin

Table 1
Amino acid sequences of the synthetic peptides used in the *in vitro* assays.

Synthetic peptides	Amino acid sequence
ProTα-fragments	
1. ProTα[100-109]	T^{100}KKQKTDEDD^{109}
2. ProTα[100-105] (NLS-containing fragment)	T^{100}KKQKT^{105}
3. ProTα[1-14] (identical with Tα1[1-14])	AcS^{1}DAAVDTSSEITTK^{14}
4. ProTα[1-28] (identical with Tα1)	AcS^{1}DAAVDTSSEITTKDLKEKKEVVEEAEN^{28}
5. ProTα[51-89][a]	E^{51}VDEEEEEGGEEEEEEEEGDGEEEDGDEDEEAESATGKR^{89}
6. ProTα[50-89]N50W[a]	W^{50}EVDEEEEEGGEEEEEEEEGDGEEEDGDEDEEAESATGKR^{89}
ProTα-derivatives that can form complexes with radioactive ^{99m}Tc/non-radioactive $^{185/187}$Re	
1. ProTα-D1 (d*m*GSC-Aca-ProTα[100-109])	dmGSC-Aca-T^{100}KKQKTDEDD^{109}
2. ProTα-D2 (d*m*GSC-K{Nε-ProTα[1-14]} -Aca)	dmGSC-K-Aca | AcS^{1}DAAVDTSSEITTK^{14}
Negative-control peptides	
1. NCP1a (scrambled ProTα[100-109] peptide)	KETDKDKTDQ
2. NCP1b (NCP1a-derivative that can form complexes with ^{99m}Tc/$^{187/185}$Re)	dmGSC-Aca-KETDKDKTDQ

dmG: dimethylglycine; Aca: aminocaproic acid (6-amino-hexanoic acid); AcS: acetylated serine; NLS: nuclear localization signal.
[a] Numbers correspond to prothymosin alpha isoform 1 [Homo sapiens] (NCBI Reference Sequence: NP_001092755.1).

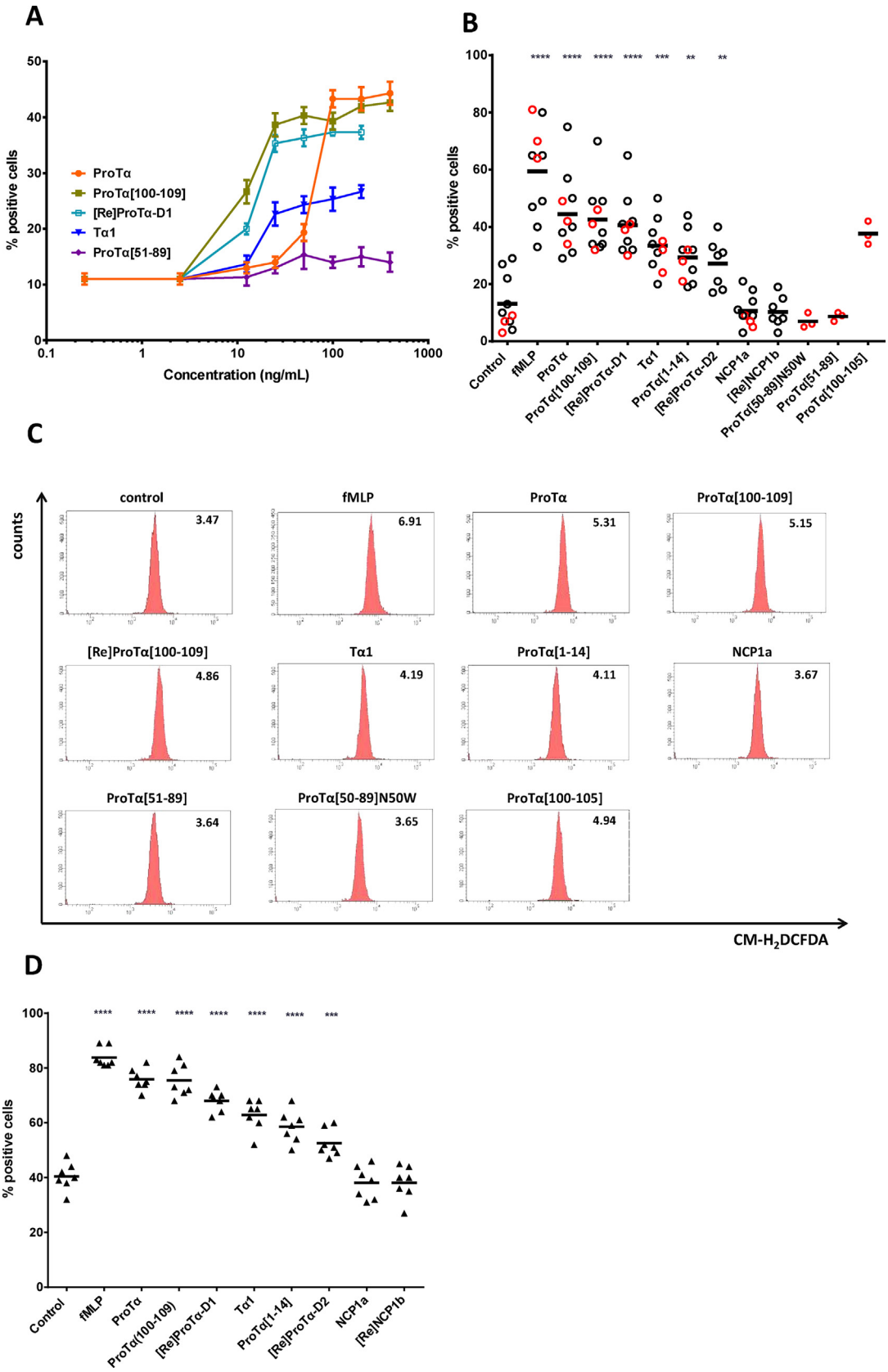

Fig. 1. A. Dose-dependent stimulation of human neutrophils by [Re]ProTα-D**1**, synthetic ProTα-fragments and intact ProTα. Neutrophils isolated from healthy donors ($n = 3$) were incubated with increasing concentrations of [Re]ProTα-D**1** (0.25–400 ng/mL), ProTα[100-109] (0.25–400 ng/mL), Tα1 (0.25–400 ng/mL), ProTα[51-89] (0.25–800 ng/mL) and intact ProTα (0.25–800 ng/mL) and tested for intracellular production of superoxide anion with the NBT reduction assay. B. [Re]ProTα-D**1** stimulates superoxide anion production in human neutrophils, as assessed by the NBT reduction assay. Neutrophils were incubated in HBSS-g (control), with fMLP (5 μM), intact ProTα (200 ng/mL), Tα1 (50 ng/mL), ProTα[51-89] and ProTα[50-89]N50W (100 ng/mL), other synthetic ProTα-fragments/[Re]complexes (25 ng/mL) and the negative controls NCP**1a** (25 ng/mL) and [Re]NCP**1b** (25 ng/mL). Cells with intracellular blue formazan deposits were counted as positive. Each symbol (◯) corresponds to the value obtained from a single donor;

was performed using a mixture of TFA/EDT/TIS/H_2O (95/2.5/2.5/2, v/v) and incubating for 2 h.

The crude peptides were purified with semi-preparative RP-HPLC on a Waters HPLC system (pump 600 E, detector UV-484) using a 10 Nucleosil 7 C18 column (250 × 12.7 mm ID; Macherey Nagel). Purity of the crude and the final products was tested with analytical RP-HPLC, on a Waters HPLC system (pump 616 E, detector 996 PDA) using a LiChrospher RP C18 column (250 × 4.6 mm ID; 5 μm particle size, Merck). Pure synthetic peptides were characterized with ESI-MS as previously described (Benaki et al., 2012; Klimentzou et al., 2007).

The fragments ProTα[51-89] and ProTα[50-89]N50W (Table 1) were synthesized on a multiple peptide synthesizer (Syro II, MultiSyn Tech, Witten, Germany) following a protocol previously described by Wilson et al. (Wilson et al., 2006) slightly modified, *i.e.*, by using a double coupling procedure. Crude synthetic peptides were first purified on a strong anion exchange chromatography column, also as previously described by Wilson et al. Desalting and final purification was successfully achieved using a C18 column (150 × 10 mm; Nucleosil 100, Macherey-Nagel, Düren, Germany) with the following solvent system: (A) 0.055% (v/v) trifluoroacetic acid in water and (B) 0.05% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile in water. Elution was performed using a linear gradient from 0% to 80% solvent B within 40 min. Detection was carried out at 214 nm. Pure peptides were characterized with MALDI-TOF MS analysis.

2.3. Preparation of [$^{185/187}$Re]complexes

$^{185/187}$Rhenium-complexes of the ProTα derivatives, ProTα-D**1** and ProTα-D**2**, as well as the negative-control peptide NCP**1b** (abbreviated as [Re]ProTα-D**1**, [Re]ProTα-D**2** and [Re]NCP**1b**, respectively) were prepared, purified and characterized as previously described by our team (Klimentzou et al., 2007).

2.4. Isolation of peripheral blood neutrophils

Venus blood (15–20 mL) was withdrawn from 43 healthy, drug-free volunteers into tubes containing heparin as anticoagulant. The isolation procedure consisted in layering slowly the blood over a Ficoll-Histopaque (Lonza, Cologne, Germany) density gradient in a Falcon-tube, centrifugating the tube at 500 × *g* for 25 min, recovering the erythrocyte/neutrophil pellet and applying 0.8% ammonium chloride for 10 min, which induced lysis of any contaminating erythrocytes. Isolated neutrophils were then washed three times with Hank's balanced salt solution (HBSS; Lonza) without Ca^{2+}/Mg^{2+}, to avoid cell priming, counted on a haemocytometer and resuspended to the desired concentration. In all cases, more than 95% of the isolated cells were neutrophils, as proved by optical microscopy, and more than 98% of them were alive, as shown by trypan blue exclusion.

The experiments were undertaken with written informed consent from each volunteer; the methodologies used in the study have conformed to the standards set by the declaration of Helsinki and are approved by the local ethics committee.

2.5. Endotoxin assay

ProTα and all synthetic ProTα fragments/derivatives/[Re]complexes were tested for endotoxin, with the LAL chromogenic Endotoxin Quantitation kit (Pierce Biotechnology, IL, USA), prior to their use in the immunostimulation bioassays; endotoxin levels were in all cases below the detection limit of the kit, *i.e.*, <0.1 Endotoxin Units/mL.

2.6. Nitroblue tetrazolium (NBT) reduction assay

The capacity of neutrophils to intracellularly produce superoxide anion in the presence of various ProTα-fragments/derivatives was tested using a protocol previously described (Samara et al., 2013). Optimal concentrations were selected with dose-dependent stimulation studies (Fig. 1A). In brief, neutrophils resuspended in HBSS containing 0.1% gelatin (HBSS-g, 2×10^6 cells/mL) were added to Eppendorfs containing 0.05% NBT solution in HBSS-g. Cells were incubated in HBSS-g (control group) or in HBSS-g containing the chemotactic peptide N-formyl-methionine-leucine-phenylalanine (fMLP) (final concentration 5 μM), as positive control, ProTα (200 ng/mL) and the ProTα-fragments/derivatives shown in Fig. 1B (final concentration 25 ng/mL, except for Tα1, which was used at 50 ng/mL, and for ProTα[51-89] and ProTα[50-89]N50W, which were used at 100 ng/mL). The samples were incubated for 10 min at 37 °C (humidified atmosphere, 5% CO_2) and then for 10 min at room temperature, fixed with 4% paraformaldehyde (10 min, 4 °C), centrifuged (190 x *g*, 10 min) and resuspended in HBSS-g. At least 100 cells per sample were counted under an optical microscope. Cells with intracellular blue formazan deposits were considered as positive. Samples were counted in a blinded fashion.

2.7. ROS determination by flow cytometry

ROS production by neutrophils in the presence of the ProTα-fragments/derivatives shown in Fig. 1C was quantified using a flow-cytometry-based assay. For this, the ROS sensitive fluorescent dye CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester; Life Technologies, CA, USA) was used. Briefly, neutrophils suspended in HBSS (2.5×10^6/mL) were pre-loaded with CM-H_2DCFDA (5.5 μg/mL) for 20 min, at 37 °C in the dark, under gentle orbital agitation. Cells were centrifuged (190 x *g*, 5 min) to remove excess of the dye, and resuspended in HBSS (2.5×10^6/mL). Ninety microliters of cell suspension were added to Eppendorfs, mixed with 10 μL HBSS (control group) or HBSS containing fMLP, as positive control, ProTα and the ProTα-fragments/derivatives shown in Fig. 1C (used at the concentrations aforementioned), and incubated for 10 min at 37 °C in the dark. At the end of the incubation period, Eppendorfs were placed on ice and 300 μL FACS buffer (DPBS – 0.5% BSA) were added in each tube. Samples were immediately measured using FACSCanto II and analyzed with FACSDiva software (Becton-Dickinson Biosciences, Erembodegen, Belgium).

horizontal lines indicate mean values ($n = 10$; for [Re]ProTα-D**2** and [Re]NCP**1b**,$n = 7$; for ProTα[51-89], ProTα[50-89]N50W and ProTα[100-105] $n = 3$). ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.05$ by one-way ANOVA *vs.* control group. C. [Re]ProTα-D **1** enhances ROS production by human neutrophils. Cells were incubated in HBSS-g (control group), with fMLP (positive control), [Re]ProTα-D**1**, synthetic ProTα-fragments, intact ProTα, and NCP**1a**, at the same concentrations as in 1B. Mean fluorescence intensity (MFI) values are shown on each histogram and correspond to 10^4 gated events. Representative results from one donor out of three analyzed are shown. D. [Re]ProTα-D**1** increases neutrophil phagocytosis, as assessed by the zymosan phagocytosis assay. Human neutrophils were incubated in HBSS-g (control group), or were stimulated with fMLP (positive control), [Re]ProTα-D**1**, other synthetic ProTα-fragments/[Re]complexes, intact ProTα, NCP**1a** and [Re]NCP**1b** at the same concentrations as in **1**B. Cells that engulfed at least one zymosan particle were counted as positive. Each symbol (▲) corresponds to the values obtained with neutrophils of a single donor; horizontal lines indicate mean values ($n = 7$). One-way ANOVA was used to compare mean values of each group with that of the control (****, $p < 0.0001$; ***, $p < 0.001$).

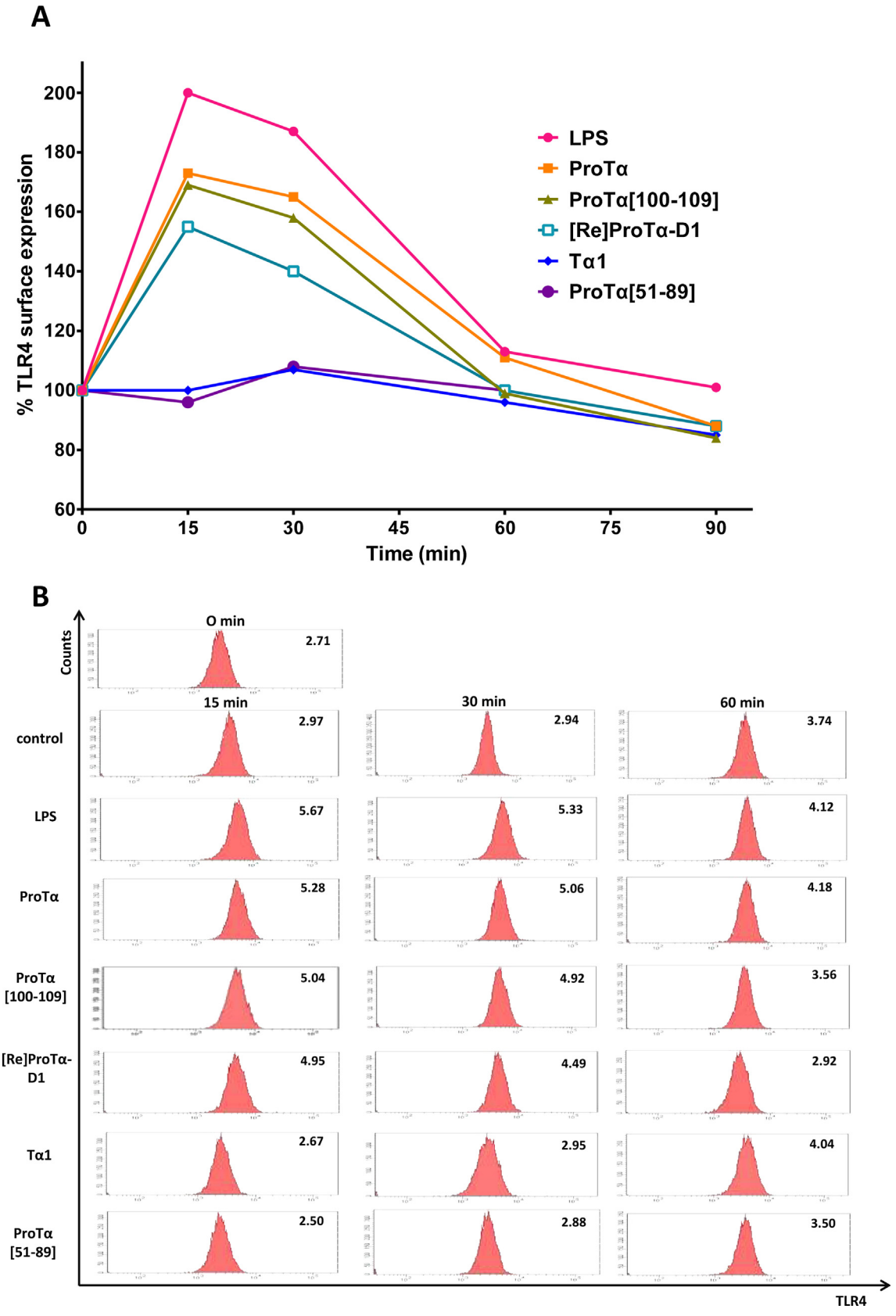

Fig. 2. Surface expression of TLR4 on human neutrophils stimulated with [Re]ProTα-D**1**, synthetic ProTα-fragments, intact ProTα and LPS. Cells were incubated with LPS, [Re] ProTα-D**1**, each ProTα-fragment or intact ProTα for 15–90 min, stained and analyzed by flow cytometry. A. Percentage of TLR4 expression was determined as (MFI experimental/MFI at 0 min) × 100. The data shown are mean values from three donors; SDs are omitted for clarity and were in all cases <15%. B. Results shown are from a representative donor out of three analyzed. Mean fluorescence intensity (MFI) values are shown on the histograms and correspond to 10^4 gated events.

2.8. Zymosan particle incorporation assay

The phagocytic capacity of neutrophils in the presence of the compounds shown in Fig. 1D was evaluated using a protocol previously described (Samara et al., 2013). In brief, neutrophils resuspended in HBSS-g (5×10^6 cells/mL) were added to Eppendorfs containing a suspension of 3% zymosan particles. Cells were incubated in HBSS-g (control group) or in HBSS-g containing fMLP, as positive control, ProTα and the ProTα-fragments/derivatives shown in Fig. 1D (at concentrations as aforementioned). The samples were incubated for 90 min at 37 °C (humidified atmosphere, 5% CO_2), then fixed with 4% paraformaldehyde (10 min, 4 °C), centrifuged (190 x *g*, 10 min) and resuspended in HBSS. Cells (at least 100 per sample) were observed under an optical microscope and samples were counted blindly. Cells having engulfed at least one zymosan particle were counted as positive.

2.9. TLR4 surface expression kinetics

For TLR4 surface expression experiments, freshly isolated neutrophils (2.5×10^6/mL) distributed in Eppendorfs were incubated with lipopolysaccharide (LPS, 1 μg/mL), ProTα (200 ng/mL), ProTα [100-109] (25 ng/mL), [Re]ProTα-D**1** (25 ng/mL), Tα1 (50 ng/mL) and ProTα[51-89] (100 ng/mL) for 15, 30, 60 or 90 min at 37 °C. At the end of each incubation period, the Eppendorfs were centrifuged and cells were resuspended in 50 μL FACS buffer. Subsequently, neutrophils were stained with human TLR4/PE mAbs (BioLegend, San Diego, CA, USA) (15 min, room temperature, in the dark) and immediately analyzed on FACSCanto II (Fig. 2A and B).

2.10. Radiolabeling with ^{99m}Tc

ProTα-D**1** was dissolved in water at a final concentration of 1 mg/mL. Aliquots of 50 μL were dispensed to glass vials and lyophilized. For radiolabeling, in a vial containing 50 μg of ProTα-D**1**, 700 μL of a Na[$^{99m}TcO_4$] solution (7.5–10 mCi) were added, followed by the addition of 80 μL physiological saline containing 40 mg calcium glucoheptonate and 4 μg stannous chloride. The mixture was allowed to react for 30 min at 37 °C; then samples of the reaction mixture were subjected to analytical radio-RP-HLPC (Benaki et al., 2012) to assess radiochemical purity and stability up to 24 h post-labeling.

2.11. Cell-binding studies

Human neutrophils, freshly isolated from peripheral blood of 33 healthy volunteers as described above, were used in the cell binding studies.

In general, cell-binding experiments were performed in RPMI 1640 medium, supplemented with L-glutamine (Biochrom AG.), 25 mM HEPES buffer (Biochrom AG.), 1 μg/mL aprotinin and antibiotics (Biochrom AG.). In tubes used for determining "total cell-bound radioactivity", neutrophils and the radiolabeled derivative [^{99m}Tc]ProTα-D**1** were added and incubated in a waterbath adjusted to 37 °C. The final volume of the incubation mixture was 1 mL. The tubes were mixed gently by inversion every 10 min during incubation. After incubation, the cells were pelleted by centrifugation (1500 x *g*; 5 min; 4 °C) and washed twice with cold saline to separate cell-bound from free radioactivity. Cells were then dissolved in 2N NaOH at room temperature, and the radioactivity of the cell lysates, assigned as "total cell-bound radioactivity", was measured in a gamma counter (Packard Minaxi). In tubes used to determine "non-specific radioactivity", neutrophils and [^{99m}Tc]ProTα-D**1** were incubated in the presence of a 100-fold excess of the non-radioactive peptide ProTα[100-109]. Total radioactivity and non-specific radioactivity tubes were run in parallel. Specific cell-bound radioactivity was calculated by subtracting non-specific from the total cell-bound radioactivity.

Optimal number of cells was selected through cell-titration experiments conducted in advance, in which increasing numbers of neutrophils (3×10^5 – 10×10^5/tube) were incubated with [^{99m}Tc]ProTα-D**1** (~2.0×10^6 cpm/tube) for 60 min (Fig. 3A). In

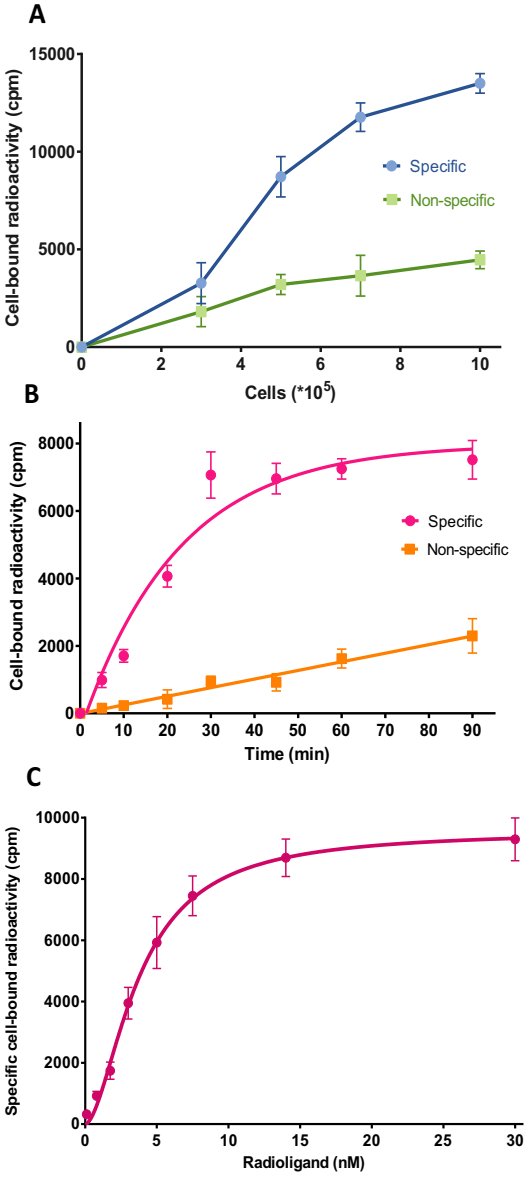

Fig. 3. A. Cell-binding studies: cell-titration experiments. Specific *vs.* non-specific cell-bound radioactivity after incubation (60 min, 37 °C) of the radiolabeled derivative [^{99m}Tc]ProTα-D**1** (~2×10^6 cpm/tube) with increasing numbers (3×10^5 – 10×10^5 cells/tube) of human neutrophils. Specific radioactivity was calculated by subtracting non-specific from total cell-bound radioactivity; non-specific radioactivity corresponds to cells incubated with [^{99m}Tc]ProTα-D**1** in the presence of 100-fold excess of the non-radioactive peptide ProTα[100-109], while total radioactivity corresponds to cells incubated only with [^{99m}Tc]ProTα-D**1**. Total and non-specific radioactivity was measured in tubes run in parallel. B. Cell-binding studies: time-kinetic experiments. Specific *vs.* non-specific cell-bound radioactivity after incubation (37 °C) of human neutrophils (7×10^5 cells/tube) with [^{99m}Tc] ProTα-D**1** for various time periods (5–90 min). Specific and non-specific radioactivity were calculated as above mentioned. C. Cell-binding studies: saturation experiments. Specific cell-bound radioactivity after incubation (60 min, 37 °C) of human neutrophils (7×10^5 cells/tube) with increasing concentrations (0.01–30 nM) of [^{99m}Tc]ProTα-D**1**. All experiments (3A, 3B, 3C) were performed in triplicate; data represent mean values ± SD of three independent experiments.

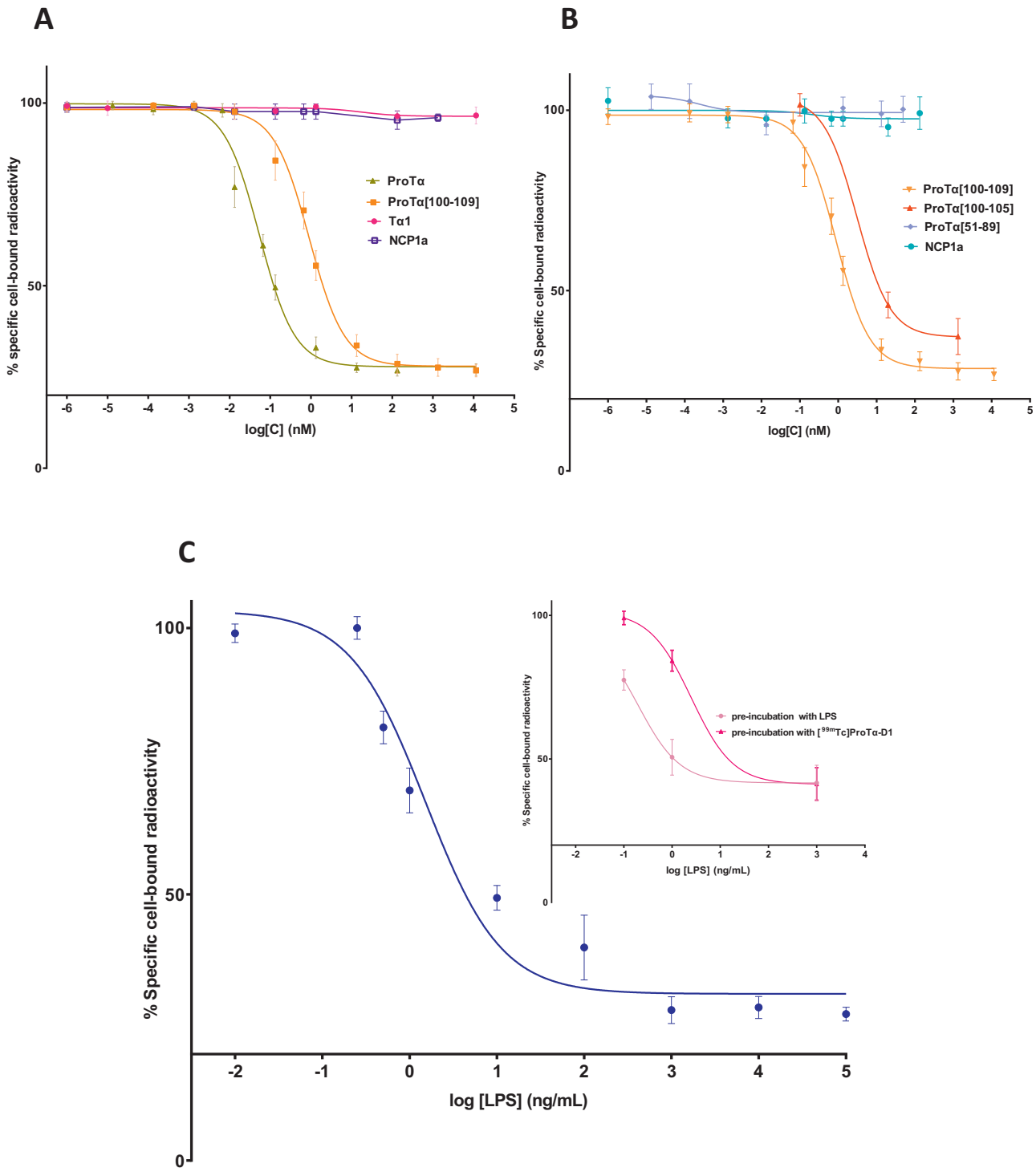

Fig. 4. Cell-binding studies: cold competition experiments. Specific cell-bound radioactivity after incubation (60 min, 37 °C) of human neutrophils (7×10^5 cells/tube) with [^{99m}Tc]ProTα-D**1** (14 nM), in the presence of increasing concentrations of cold (non-radiolabeled) putative competitors. A. Intact ProTα (1 fM–100 nM), ProTα[100-109] (1 fM–10 μM), Tα1 (1 fM–10 μM), and NCP**1a** (1 fM–10 μM) were used as putative cold competitors. Experiments were performed in triplicate; data represent mean values ± SD of three experiments. B. ProTα[100-105] (1 fM–10 μM) and ProTα[51-89] (1 fM–10 μM) were used as putative cold competitors, while ProTα[100-109] (1 fM–10 μM) and NCP**1a** (1 fM–10 μM) were used as positive and negative controls, respectively. Results obtained with ProTα[50-89]N50W were identical with those of ProTα[51-89] and thus not shown. Experiments were performed in triplicate; data represent the mean values ± SD of two experiments. C. Non-radiolabeled LPS was used as putative cold competitor. Experiments were performed in triplicate; data represent the mean values ± SD of three independent experiments. C, inset. Results from an independent experiment are shown, in which human neutrophils (7×10^5 cells/tube) had been pre-incubated (15 min, 37 °C) with [^{99m}Tc]ProTα-D**1** (14 nM) or LPS (100 pg/mL–1 μg/mL). Data represent mean values ± SD of six replicates.

time-kinetic experiments (Fig. 3B), neutrophils (7×10^5 cells/tube) were incubated with [^{99m}Tc]ProTα-D**1** ($\sim 1.4 \times 10^6$ cpm/tube) for different time periods (5–90 min). In saturation experiments (Fig. 3C), neutrophils (7×10^5 cells/tube) were incubated with increasing concentrations of [^{99m}Tc]ProTα-D**1** (0.01–30 nM/tube) for 60 min.

In cold (non-radiolabeled) competition experiments (Fig. 4A–C), neutrophils (7×10^5 cells/mL) and the radiolabeled derivative

[^{99m}Tc]ProTα-D**1** (14 nM) were incubated for 60 min (except as otherwise indicated) in the presence of increasing concentrations of putative non-radiolabeled competitors, *i.e.*, intact ProTα, ProTα [100-109], ProTα[100-105], Tα1, ProTα[51-89], ProTα[50-89] N50W, the negative control peptide NCP**1a**, as well as in the presence of LPS.

The equilibrium dissociation constant (*K*d) was estimated by analyzing the results of the cell-binding saturation experiments with a non-linear regression, fitting the Hill equation. Statistical analysis was performed in order to determine if the experimental data were consistent with the one-site or the two-site binding model. The IC_{50} values were calculated by plotting the data of the inhibition experiments.

All sets of experiments were performed in triplicate and each set of experiment was repeated three times, in three different days, except as otherwise indicated.

2.12. Statistical analysis

All data were analyzed using Graphpad Prism 6 software (Graphpad software, San Diego, CA, USA).

One-way analysis of variance (ANOVA) was used to compare the mean values of each group evaluated in the *in vitro* bioassays (Fig. 1B and D) with the control one; p values <0.05 were considered statistically significant.

3. Results

3.1. Peptide synthesis and preparation of [$^{185/187}$Re]complexes

ProTα[1-14], ProTα[1-28] (Tα1) and ProTα-D**2** (Table 1) were synthesized as previously reported by our group (Klimentzou et al., 2007), following a protocol according to which Fmoc-deprotection was carefully monitored by UV absorption spectroscopy at 301 nm (Chao et al., 1993) and, when necessary, suitably elongated until a baseline optical absorbance was recorded. ProTα[100-109] and ProTα-D**1** (Table 1), both containing three aspartic acid residues at neighboring sites in their C-termini, were synthesized as described in Section 2, *i.e.*, following a short (20-min) Fmoc-deprotection step. Initial attempts to synthesize ProTα[100-109] and ProTα-D**1** following the former protocol (Klimentzou et al., 2007) led to highly impure crude products and, consequently, low yields, possibly due to aspartimide formation (Yang et al., 1994). ProTα [100-105] and the negative control peptides NCP**1a** and NCP**1b** (Table 1) were also synthesized as described in Section 2. Crude synthetic peptides were purified by semi-preparative RP-HPLC. As revealed with analytical RP-HPLC, the final purity of all peptides exceeded 98% and the overall yield of the synthesis ranged between ~10% (ProTα[1-28] (Tα1) and ~35% (ProTα-D**2**). ESI-MS analysis confirmed the theoretically calculated molecular weight values (data not shown).

The highly acidic ~40-mer fragments ProTα[51-89] and ProTα [50-89]N50W (Table 1) were synthesized on a multiple peptide synthesizer and purified with a combination of anion exchange chromatography and RP-HPLC, following a protocol previously described by Wilson et al. (Wilson et al., 2006) with slight modifications. The overall yield of the synthesis was ~14% and ~11% for ProTα[51-89] and ProTα[50-89]N50W, respectively. MALDI-TOF analysis confirmed the theoretically calculated molecular weight values (data not shown).

The synthetic derivatives ProTα-D**1** and ProTα-D**2**, as well as the negative-control peptide NCP**1b** can form complexes, through their tripeptidyl dmGSC-moiety (Smith et al., 2003), with metals, such as Re and Tc. Preparation of the non-radioactive [$^{185/187}$Re] complexes of ProTα-D**1**, ProTα-D**2** and NCP**1b** ([Re]ProTα-D**1**, [Re] ProTα-D**2** and [Re]NCP**1b**, respectively) was performed according to a well-established method, as previously described by our team for ProTα-D**2** (Benaki et al., 2012; Klimentzou et al., 2007). The crude products were purified by semi-preparative RP-HPLC. As revealed with analytical RP-HPLC, the purity of the final complexes was ≥95%. Formation of two interconverting diastereoisomers (*syn*- or *anti*-) was previously shown by our group for [Re]ProTα-D**2** with NMR studies and a slow analytical RP-HPLC gradient (Benaki et al., 2012). Accordingly, two overlapping product peaks were observed in the analytical RP-HPLC chromatograms of each of the [Re]complexes, corresponding probably to the *syn*- and *anti*-diastereoisomers. The [Re]complexes were also characterized with ESI-MS and the data were consistent with the expected molecular weight values – considering the presence of two Re isotopes (data not shown).

3.2. Activity of [Re]ProTα-D1 *in* in vitro *immunostimulation bioassays, in comparison with intact ProTα and other synthetic ProTα-fragments/derivatives/[Re]complexes*

The immunostimulating activity of [Re]ProTα-D**1** was assessed in comparison with intact ProTα, the synthetic fragments ProTα [100-109], ProTα[100-105], ProTα[1-14],Tα1, ProTα[51-89] and ProTα[50-89]N50 W, the [Re]complex of a derivative encompassing the N-terminal fragment [1-14] of ProTα/Tα1 previously developed by our group ([Re]ProTα-D**2**), the positive-control peptide fMLP (Wittmann et al., 2002), the negative-control peptide NCP**1a**, and the negative-control [Re]complex, [Re]NCP**1b**. Two *in vitro* bioassays measuring superoxide anion production *i.e.*, the nitroblue tetrazolium (NBT) reduction assay (Kremer et al., 1987) and reactive oxygen species (ROS) by flow cytometry (FC) using CM-H_2DCFDA (Saito et al., 2013) were performed. Moreover, the zymosan particle incorporation assay was used to evaluate phagocytosis (Carvalho et al., 2000) in the presence of some of the compounds. Human neutrophils obtained from overall ten healthy individuals were used as the specific cell-system in the above bioassays, performed following the protocols recently described (Samara et al., 2013).

Titration experiments were initially conducted, to estimate minimum optimal concentrations of the substances (Fig. 1A). The concentrations determined for ProTα and ProTα[100-109] were consistent with those previously reported by Samara et al. (Samara et al., 2013) (200 ng/mL and 25 ng/mL, respectively). Minimum optimal concentrations for [Re]ProTα-D**1** and Tα1 were 25 ng/mL and 50 ng/mL, respectively, whereas ProTα[51-89] and ProTα[50-89]N50W did not show any activity even at high concentrations (800 ng/mL) and thus, were used at 100 ng/mL. fMLP was used at 5 μM (Samara et al., 2013).

According to the experimental results of the NBT reduction assay (Fig. 1B), fMLP induced the highest intracellular superoxide anion production in comparison with the control group (59.4% *vs.* 13.0%, respectively; $p < 0.0001$), followed by ProTα and ProTα[100-109] (44.5% and 42.6%, respectively; $p < 0.0001$). ProTα[1-14] and Tα1 increased superoxide anion production to 29.3% ($p < 0.05$) and 33.4% ($p < 0.001$), respectively. [Re]ProTα-D**1** induced increase to 41.0% ($p < 0.0001$), *i.e.*, higher than ProTα[1-14] and Tα1, while [Re] ProTα-D**2** also increased superoxide anion production, but to a lower extent (27.1%, $p < 0.05$). ProTα[51-89] and ProTα[50-89] N50W did not stimulate neutrophils to produce superoxide anions and, as expected, both negative controls (NCP**1a** and [Re]NCP**1b**) were inactive, as well.

The results of the NBT reduction assay were further confirmed by FC analysis of ROS. In indicative results, shown in Fig. 1C, fMLP induced the production of the highest levels of ROS (mean fluorescence intensity [MFI] 6.91) in comparison with the non-stimulated cells (control group), followed by ProTα (5.31), ProTα [100-109] (5.15), ProTα[100-105] (4.94), [Re]ProTα-D**1** (4.86), Tα1

(4.19) and ProTα[1-14] (4.11). The two central peptides, ProTα[51-89] and ProTα[50-89]N50W, and the negative control peptide NCP**1a** did not stimulate ROS production by neutrophils, since in all cases the recorded MFI values were at the level of the control group.

According to the experimental results obtained from the zymosan particle incorporation assay (Fig. 1D), fMLP induced the highest increase in neutrophil phagocytosis, in comparison with the control group (83.9% *vs.* 40.4%, respectively; $p < 0.0001$). Intact ProTα and ProTα[100-109] also significantly increased phagocytosis (75.9% and 75.4%, respectively; in both cases $p < 0.0001$, in comparison with the control group), which is in agreement with recently reported data (Samara et al., 2013). ProTα[1-14] and Tα1 induced 58.6% ($p < 0.0001$) and 62.9% ($p < 0.0001$) increase in neutrophil phagocytosis, respectively. On the other hand, [Re]ProTα-D**1** induced 68% ($p < 0.0001$) increase, *i.e.*, higher than ProTα[1-14] and Tα1, while [Re]ProTα-D**2** also increased cell phagocytosis, but at a lower level (52.6%, $p < 0.001$). As expected, negative controls (NCP**1a** and [Re]NCP**1b**) did not induce any statistically significant increase in phagocytosis, in comparison with the control group.

3.3. Effect of [Re]ProTα-D1 on the kinetics of TLR4-surface expression on human neutrophils

Ioannou et al. (Ioannou et al., 2013) have previously reported that ProTα and ProTα[100-109] can affect the kinetics of TLR4-surface expression in human dendritic cells. In the present work, human neutrophils were incubated for 15, 30, 60 and 90 min with [Re]ProTα-D**1**, intact ProTα, ProTα[100-109], Tα1 or ProTα[51-89]. The latter has been previously shown to stimulate the production of type I IFNs in murine macrophages following ligation with TLR4 (Mosoian et al., 2010). LPS from *Escherichia coli*, the prototype TLR4 ligand (Akira and Takeda, 2004), was used as positive control. Changes in TLR4 expression are shown in Fig. 2A and B. Incubation of neutrophils with LPS led to an early (≤15 min) and significant (≥90%) increase in TLR4 expression and a subsequent decrease thereafter (from 30 to 90 min). [Re]ProTα-D**1**, ProTα[100-109] and intact ProTα induced a similar effect over the same time, though less potent than that of LPS ([Re]ProTα-D**1** < ProTα[100-109] < intact ProTα < LPS). ProTα[1-28] (Tα1) and ProTα[51-89] did not significantly upregulate TLR4 expression, under the conditions used.

3.4. Radiolabeling of ProTα-D1 with ^{99m}Tc

ProTα-D**1** was radiolabeled with ^{99m}Tc as described in Section 2. Radio-RP-HPLC analysis of [^{99m}Tc]ProTα-D**1** indicated radiolabeling yield ≥98% and specific activity ranging from 150 to 200 μCi/μg ($2.4 - 3.0 \times 10^{11}$ cpm/μmol). Formation of two interconverting diastereomers (*syn-* or *anti-*) was previously shown by our group for [^{99m}Tc]ProTα-D**2**, using a slow HPLC gradient (Benaki et al., 2012). In accordance with that, two overlapping product peaks were observed in the analytical radio-RP-HPLC chromatograph of [^{99m}Tc]ProTα-D**1,** corresponding probably to *syn-* and *anti-*diastereoisomers. Stability studies with analytical radio-RP-HPLC showed that [^{99m}Tc]ProTα-D**1** remained stable for at least 24 h post-labeling.

3.5. Cell-binding studies with [^{99m}Tc]ProTα-D1

Human neutrophils, freshly isolated from the peripheral blood of 33 healthy volunteers, were used as target immune cells.

Preliminary cell-titration experiments were performed, in which increasing numbers of neutrophils ($3 \times 10^5 - 10 \times 10^5$ per tube) were incubated with [^{99m}Tc]ProTα-D**1** (~2.3×10^6 cpm/tube, 20 nM) for 60 min at 37 °C. Specific cell-bound radioactivity could be well discriminated from non-specific when at least 5×10^5 cells were used (Fig. 3A). The final assay volume was also titrated (0.5–1.2 mL/tube) and optimal results were obtained for 1 mL assay volume. Thus, 7×10^5 neutrophils per milliliter per tube were used in the cell-binding experiments.

Time-kinetic experiments were conducted (37 °C) over a period of 90 min (0–90 min); as revealed (Fig. 3B), specific cell-bound radioactivity reached a maximum between 20 and 30 min of incubation, which remained practically stable for at least another 60 min. Specific binding accounted for at least 75% of the total cell-bound radioactivity measured.

In saturation experiments (Fig. 3C), specific cell-bound radioactivity was measured in the presence of increasing concentrations of the radiolabeled derivative [^{99m}Tc]ProTα-D**1** (0.01–30 nM). Data analysis with the non-linear regression fitting Hill equation resulted in an apparent equilibrium dissociation constant (*K*d) value of 3.76 ± 0.40 nM. Statistical analysis of the saturation curve data supports a single-site rather than a two-site binding model.

In order to acquire further evidence verifying that [^{99m}Tc]ProTα-D**1** was bound on human neutrophils (i) specifically, (ii) on binding sites recognizing parental ProTα, and (iii) through its ProTα[100-109] region, cold-competition experiments were conducted in the presence of increasing concentrations (1 fM–100 nM) of intact ProTα as well as increasing concentrations (1 fM–10 μM) of synthetic ProTα[100-109], synthetic Tα1 and the negative-control peptide NCP**1a** (scrambled ProTα[100-109], Table 1). As clearly shown, cell-binding of [^{99m}Tc]ProTα-D**1** was inhibited in the presence of ProTα and ProTα[100-109] in a concentration-dependent manner, but not in the presence of either the scrambled peptide or Tα1, even when high concentration of the peptides were used (Fig. 4A). The calculated IC_{50} values were 0.056 ± 0.011 nM and 0.91 ± 0.14 nM for intact ProTα and ProTα[100-109], respectively.

Cold-competition was also performed in the presence of increasing concentrations (1 fM–100 nM) of ProTα[100-105], which encompasses the positively charged NLS-fragment of ProTα (Manrow et al., 1991; Rubtsov et al., 1997) as well as of ProTα[51-89] and ProTα[50-89]N50W); ProTα[100-109] and NCP**1a** were used as positive and negative controls, respectively. As shown (Fig. 4B), ProTα[100-105] was able to displace cell-bound radioactivity, but the variants of ProTα-central region or NCP**1a** were not.

Taken altogether, the above experimental data (Fig. 4A and B) indicate the presence of binding sites on human neutrophils that recognize the radiolabeled derivative of ProTα [^{99m}Tc]ProTα-D**1**, its active part ProTα[100-109], the NLS-fragment ProTα[100-105] and intact ProTα. Noticeably, intact ProTα exhibited the highest affinity for these binding sites.

To gain insight into the nature of the putative binding sites on human neutrophils that ligate ProTα, cold-competition experiments were also conducted in the presence of increasing concentrations (1 pg/mL–10 μg/mL) of LPS from *E. coli*. As shown in Fig. 4C, LPS was able to prevent binding of [^{99m}Tc]ProTα-D**1** on human neutrophils (IC_{50}: 1.71 ± 0.69 ng/mL). In an independent experiment, pre-incubation of neutrophils for 15 min with LPS led to a shifted-to-the-left inhibition curve (Fig. 4C, inset), which might be attributed to reduced availability of putative binding sites for "competition", due to their early engagement by LPS. Overall, based on the above results we propose that specific binding of ProTα or the C-terminal fragments of the polypeptide on human neutrophils involves TLR4.

4. Discussion

ProTα and its N-terminal 28-mer fragment Tα1 have been reported to exhibit a well-established extracellular

immunostimulating function in various *in vitro* and *in vivo* systems (Ioannou et al., 2012; Mosoian, 2011; Naylor et al., 1983; Romani et al., 2004), presumably through specific receptors of the immune cells. In the last decade, ProTα was shown to exert its immunopotentiating activity in association with TLR4 (Ioannou et al., 2013; Mosoian et al., 2010), while Tα1 was reported to activate immune resistance pathways to fungal infection mainly downstream TLR9 (Romani et al., 2004); nevertheless, the exact mechanism of action of alpha thymosins has not yet been fully elucidated. The identification of the immunoactive fragment of ProTα also remains elusive. Although initially immunoreactivity was limited to Tα1, more recently the highly acidic central part 50-89 (Mosoian et al., 2010) and, the C-terminal decapeptide 100-109 (Ioannou et al., 2013) were proposed to comprise the immunoactive region of ProTα. It is worthwhile noting that the latter decapeptide is identical with the fragment cleaved from ProTα by caspases during apoptosis (Evstafieva et al., 2000, 2003); a putative association between excretion of the cleaved decapeptide from dying apoptotic cells with the perception of acting as a "danger-signal" sensed by neighboring innate immune cells was earlier suggested (Skopeliti et al., 2009; Ioannou et al., 2012).

Radiolabeled probes have been used to investigate putative specific binding of alpha thymosins on various subpopulations of immune cells. Thus, in middle 1990's, both ProTα and Tα1, as well as the peptide spanning their common sequence [16-23], were reported to compete with ^{125}I-radiolabeled human interferon-α2 for the same binding sites on mouse thymocytes (Zav'Yalov et al., 1995). On the other hand, in middle 1990's, too, ^{125}I-radiolabeled ProTα was used in cell-binding studies with human peripheral blood mononuclear cells (PBMC) (Cordero et al., 1994) and two homogeneous lymphocytic populations, *i.e.*, human lymphoblasts and YT cells (Cordero et al., 1995). These experiments revealed two cell-surface binding sites for ProTα, one of low- and one of high-affinity for the polypeptide. Lack of competition between ProTα and Tα1 supported the assumption that these binding sites were specific for ProTα (Cordero et al., 1996).

In the present study, a new ^{99m}Tc-radiolabeled derivative of the C-terminal decapeptide 100-109 of ProTα ([^{99m}Tc]ProTα-D**1**) was developed and evaluated indirectly for its *in vitro* immunostimulating activity on human neutrophils. More specifically, the non-radioactive [Re]complex, [Re]ProTα-D**1**, which is structurally similar with the radiolabeled derivative [^{99m}Tc]ProTα-D**1** according to well-established data on the structures of rhenium and technetium complexes (Deutsch et al., 1986), was tested *in vitro* and found to retain the immunostimulating activity of the non-radiolabeled decapeptide; this finding assures reliability of the results obtained when using its radiolabeled analog, [^{99m}Tc]ProTα-D**1**, in subsequent direct *in vitro* binding studies on the same type of immune cells.

Human neutrophils were selected for conducting both, biological evaluation and direct binding studies, since their functions were previously reported to be modulated by exogenously administered ProTα (Heidecke et al., 1997; Samara et al., 2013). Moreover, neutrophils express all TLRs, including TLR4 and TLR9, except for TLR3 (Hayashi et al., 2003). Finally, neutrophils can be easily isolated from human peripheral blood in adequate numbers.

[Re]ProTα-D**1** was immunologically evaluated in parallel with intact ProTα, the C-terminal decapeptide ProTα[100-109], the NLS-containing fragment ProTα[100-105], the two N-terminal fragments ProTα[1-14] and Tα1, and two variants of the central region of the polypeptide, namely ProTα[51-89] and ProTα[50-89]N50W. ProTα[50-89]N50W, which contains a tryptophan residue at its N-terminus, was first used by Mosoian et al. (Mosoian et al., 2007) and reported to be associated with the anti-HIV-1 activity of ProTα; the tryptophan residue is not present in native ProTα, which does not contain any aromatic residues in its sequence. Synthetic ProTα[51-89], on the other hand, spans the sequence of the natural central region of the polypeptide. To our knowledge, the concomitant evaluation of intact ProTα, ProTα[100-109], Tα1, and ProTα[51-89]/ProTα[50-89]N50W in the same *in vitro* immunostimulation bioassays is reported for the first time. As shown (Fig. 1) and in agreement with recent data (Samara et al., 2013), ProTα[100-109] (tested at 25 ng/mL, ~20 nM) was almost as effective as intact ProTα (tested at 200 ng/mL, ~17 nM) in stimulating neutrophil phagocytosis and ROS production; ProTα[100-105] (tested at 25 ng/mL, ~34 nM) though less effective than intact ProTα and ProTα[100-109], was still immunoactive, which is in agreement with previous data showing immunoenhancing synergistic effect of both ProTα[100-109] and ProTα[100-105] with IL-2 on PBMC cytotoxicity (Skopeliti et al., 2009). Tα1 (tested at 50 ng/mL, ~16 nM) was less effective than all aforementioned peptides, but still exerted a statistically significant stimulatory effect on human neutrophils. On the other hand, none of the central ProTα-fragments showed any activity, even at the highest concentrations tested (800 ng/mL, ~180 nM), which is in agreement with previous findings reporting marginal upregulation in the functionalities of PBMC following treatment with ProTα[31-87], a peptide isolated after tryptic cleavage of bovine ProTα (Skopeliti et al., 2006).

Based on reported data (Ioannou et al., 2013), [Re]ProTα-D**1** was also evaluated for its effect on the kinetics of TLR4-surface expression on neutrophils, in parallel with intact ProTα and fragments thereof, in comparison with the prototype TLR4-ligand, LPS. As shown (Fig. 2A and B), the increase in TLR4-surface expression induced by [Re]ProTα-D**1** followed a similar pattern with that induced by LPS, intact ProTα and ProTα[100-109], which further supports that the radioactive counterpart [^{99m}Tc]ProTα-D**1** is suitable for conducting cell-binding studies. As expected, Tα1 did not upregulate TLR4-surface expression, since its activity has not been associated with TLR4-triggering (Romani et al., 2004). Interestingly, the central fragment ProTα[51-89] did not affect TLR4-surface expression, though a previous report has associated this ProTα-region with TLR4 (Mosoian et al., 2010).

Direct cell-binding studies were subsequently conducted with [^{99m}Tc]ProTα-D**1**. Under the experimental conditions used herein, cell-surface bound radioactivity could not be discriminated from any putative cell-internalized radioactivity; however, exact differentiation was beyond the scopes of the present study. A specific, time-dependent and saturable binding of [^{99m}Tc]ProTα-D**1** on human neutrophils was obtained (Fig. 3A–C). Cell-binding was inhibited in the presence of increasing concentrations of intact ProTα, ProTα[100-109], ProTα[100-105], but not of Tα1, ProTα[51-89] or ProTα[50-89]N50W (Fig. 4A and B). A significantly lower IC_{50} was calculated for intact ProTα, in comparison with that of ProTα[100-109], which supports higher binding affinity of intact ProTα on neutrophils. On the other hand, the prototype ligand of TLR4, LPS, prevented binding of [^{99m}Tc]ProTα-D**1** on human neutrophils (Fig. 4C). Taken together, the above results support the existence of binding sites for ProTα on human neutrophils, which recognize [^{99m}Tc]ProTα-D**1** and ProTα[100-109] and might be associated with TLR4.

Further studies with the new ^{99m}Tc-labeled probe are expected to significantly enrich our basic knowledge on the mode of extracellular action of ProTα, both *in vitro* and *in vivo*, through animal biodistribution and imaging studies with a SPECT camera (Schwochau, 1994). Additionally, such data will facilitate future clinical exploitation of the polypeptide or smaller active fragments thereof, such as ProTα[100-109], which, due to their short length, are likely to present a desirable pharmacokinetic profile.

5. Conclusion

In the present work, we developed a ^{99m}Tc-labeled derivative of the C-terminal decapeptide of ProTα ([^{99m}Tc]ProTα-D**1**). The corresponding non-radioactive rhenium-complex, [Re]ProTα-D**1**, which is structurally similar with [^{99m}Tc]ProTα-D**1**, was also prepared. Parallel evaluation of [Re]ProTα-D**1** and intact ProTα in bioassays using neutrophils revealed that the former still retains biological activity. The radiolabeled probe [^{99m}Tc]ProTα-D**1** had adequate half-life (6 h), was stable for at least 24 h post-labeling and had high specific radioactivity, features which – along with the biological activity shown indirectly, *via* the evaluation of [Re]ProTα-D**1** – ensured suitability of [^{99m}Tc]ProTα-D**1** for *in vitro* cell-binding studies. The experimental results of these studies support the existence of binding sites for ProTα/ProTα[100-109] on human neutrophils which are putatively associated with TLR4.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

C.-E. K.: performed the experiments, analyzed data, carried out statistical analyses and wrote the manuscript. C.L.: helped in ^{99m}Tc-radiolabeling, cell-binding experiments and statistical analysis. C.T.: performed ^{99m}Tc-radiolabeling. C.Z.: designed the peptide synthesis experiments, except for ProTα[51-89] and ProTα[50-89]N50W. P.S.: helped in the *in vitro* bioassay experiments. O.E. T.: coordinated the *in vitro* biological evaluation and critically edited the manuscript. H.K.: synthesized the two different variants of the central segment of prothymosin alpha (ProTα[51-89], ProTα[50-89]N50W), designed the negative control peptides and edited the manuscript. W.V.: designed the two different variants of the central segment of prothymosin alpha (ProTα[51-89], ProTα[50-89]N50W) and critically edited the manuscript. M.P. and I.P: designed the radiolabeled technetium-complex and the corresponding non-radioactive rhenium complex; evaluated all data of ^{99m}Tc-radiolabeling. E.L.: designed and coordinated the whole study, drafted and reviewed the manuscript.

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