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Nanostructured recombinant cytokines: A highly stable alternative to short-lived prophylactics

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ABSTRACT

Cytokines have been widely used as adjuvants and therapeutic agents in treatments of human diseases. Despite their recognized potential as drugs, the medical use of cytokines has considerable drawbacks, mainly related to their low stability and short half-life. Such intrinsic limitations imply the administration of high doses, often prompting toxicity, undesirable side effects and greater production costs. Here, we describe a new category of mechanically stable nanostructured cytokines (TNFα and CCL4/MIP-1β) that resist harsh physicochemical conditions *in vitro* (pH and temperature), while maintaining functionality. These bio-functional materials are produced in recombinant cell factories through cost-effective and fully scalable processes. Notably, we demonstrate their prophylactic potential *in vivo* showing they protect zebrafish from a lethal infection by *Pseudomonas aeruginosa*.

1. Introduction

In the quest for safe, effective, practical prophylactics and therapeutics, recombinant proteins are provoking wide interest. Prophylactics such as subunit vaccines or therapeutics such as immunostimulants, are examples of successfully produced proteins. Both are functionally well-characterized products, which are superior to DNA vaccines or inactivated/attenuated vaccines in terms of biosafety. Additionally, they are poorly reactogenic and can be designed *a la carte* [1]. Heterologous protein expression systems allow for easy and highly reproducible protein production at lower cost and at large scale [2]. Such systems have been developed in bacteria (*E. coli*), yeast (*S. cerevisiae*), insect cells (*D. melanogaster* cells), protozoa (*Tetrahymena thermophila*), mammalian cell cultures or plants [3,4]. In bacterial expression systems, in many cases, the desired protein product needs to be extracted from inclusion bodies (IBs) and renaturalized to obtain the soluble form. Recently IBs, *per se*, have attracted interest as bio-materials due to their high stability, withstanding extreme temperatures and lyophilization [5]. IBs are protein clusters in the upper size of the nanoscale, ranging from 50 to 700 nm [6], highly enriched with the recombinant protein itself but complex in composition, including a spectrum of macromolecules from the producing cell. Nevertheless, IBs are not inert and when exposed to mammalian cells penetrate the membrane and they issue a sustained release of the protein they are comprised of, in a functional form, over time [7]. This fact has been previously demonstrated with a diversity of proteins forming IBs, including hormones, chaperones, enzymes, growth

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factors and cytoskeleton components [7–10]. Most of the biological and physicochemical properties of IBs are moldable, such as size, bio-adhesiveness, net charge, density and the release of functional protein inside cells upon exposure [5,11–13]. Importantly, the recombinant protein production of IBs is fully scalable and the biofabrication process is cost-effective.

Cytokines are proteins with a central role in immunity [14] and have been used as adjuvants, immunostimulants and therapeutic agents in the treatment of human diseases [15–17]. Their biomedical use is associated to their involvement in the pathogenesis of many diseases; the fact that they are unlikely to elicit an allergic response and that they are able to stimulate the immune system without the presence of a pathogen. Different cytokines have been tested as treatments for various diseases. Interferon alpha (IFNα) is one of the best characterized cytokines for human disease treatment [18] and can be naturally derived (leukocyte or lymphoblastoid derived) or recombinantly produced (*E. coli*-derived). Therapeutic uses of IFNα include hepatitis B and C (antiviral), condyloma (immunomodulation), Kaposi's sarcoma (anti-angiogenic) and hairy cell-leukemia (antitumor) [17,18]. Interleukin-2 (IL-2) is another example of a cytokine that has been obtained from *E. coli* and evaluated as a therapeutic in bladder carcinoma and melanoma [16,17]. Tumor necrosis alpha (TNFα) has been produced recombinantly in *E. coli* and different studies have revealed it exhibits antitumor properties and strong hemodynamic effects. In fact, TNFα has been used to treat cancer in phase III studies in melanoma patients [19]. Chemokine (C-C motif) ligand 4 (CCL4) induces monocyte cell recruitment at inflammatory sites [20] but clinical studies are still being conducted to confirm the pathophysiological role of CCL4 as an active factor in ischaemic stroke, as a neuroprotective agent or as an anti-HIV agent [21,22]. Additionally, other cytokines have been explored as a potential therapeutics, such as CCL5, IL-6, IL-7, IL-15, IFNβ [23–25] but only a few recombinant cytokines such as G-CSF, GM-CSF, erythropoietin or IL-2 have been licensed for human use [1,26]. Despite all their appealing therapeutic properties, cytokines have significant drawbacks for medical use, in particular their short half-life (minutes or hours), which implies the administration of high doses, prompting toxicity and systemic effects as cytokines are administrated intravenously. Further, high doses entail the expensive production of large quantities [27,28].

In this study, we have explored the ability of TNFα and CCL4 produced as nanostructured functional IBs, to act as immunostimulants and potential adjuvants. By using cytokines in the form of nanostructured IBs, we hope to overcome the fundamental difficulties of poor stability and short half-life, which hinder cytokine use. We show that nanostructured recombinant cytokines are easy to produce by cost-effective and highly reproducible procedures. Furthermore, we provide detailed evidence in the model organism zebrafish (*Danio rerio),* that they can be used *in vivo* as efficient immunostimulants or adjuvants, either injected or orally administrated, inducing excellent immune protection levels against an otherwise lethal bacterial challenge.

2. Materials and methods

2.1. Bacterial strains and plasmids

Trout CCL4 sequence (acc. number AY561709.1) was used to design specific cloning primers (Suppl. Table 1). The CCL4 full length was amplified from cDNA synthesized from trout head kidney total RNA purified using TriReagent (Sigma). The PCR product was excised from the gel, ligated into pET-30Xa/LIC vector (Novagen) and transformed into *E. coli* DH5α (Invitrogen). The CCL4-pET-30 Xa/LIC was then purified using the Nucleo-Spin Plasmid Quick-Pure (Macherey-Nagel), quantified using a Nanodrop ND-1000 (Thermo Scientific) and sequenced in order to check the sequence orientation. Finally, for recombinant protein expression the CCL4-pET-30Xa/LIC was subcloned into *E. coli* BL21(DE3) pLysS strain. The bacterial strain used for the production of TNFα IBs was *E. coli* M15[pREP4] (Qiagen). This strain was transformed with the TNFα-pQE30 vector (Qiagen) as described [29]. CCL4-pET-30Xa/LIC and iRFP-H6-pET22b were transformed into *E. coli* BL21(DE3) for the recombinant production of IB^{CCL4} and infrared fluorescent protein, $IB^{iRFP-H6}$ respectively. Note that $IB^{iRFP-H6}$ were produced as control nanoparticles with irrelevant biological activity regarding immunostimulation. All the recombinant proteins contained a His-tag and could be detected by western blot using an anti-His tag antibody (GenScript).

2.2. IBs production, purification and fluorescent labelling

The *E. coli* transformed with TNFα-pQE30, CCL4-pET-30 Xa/LIC and iRFP-H6-pET22b were cultured in LB medium supplemented with required antibiotics. Expression was induced when $OD_{550\ nm}$ reached 0.5 for 3 h at 1 mM of IPTG (Panreac). For IB purification the bacterial cultures were processed through a combination of enzymatic and mechanical disruption. First, lysozyme at 1 μg/ml (Roche) and PMSF at 0.4 mM (Roche) were added to bacterial suspensions and incubated for 2 h at 37 °C and 250 rpm. Then, the cells were frozen and thawed and Triton X-100 (Sigma) was added (0.2% (v/v)), the suspension was incubated 1 h under gentle agitation at RT. IBs were harvested by centrifugation and resuspended in PBS (10 times concentrated with respect to the original culture volume). Next, samples were incubated with DNAse at 0.6 μg/ml (Roche), 1 h at 37 °C under agitation. Several freeze/thaw cycles were carried out until no viable bacteria were detected. Samples were centrifuged at 15.000 × *g* for 15 min and pellets containing purified IBs stored at −80 °C until use. The IBs were quantified by western blot using an anti-His-tag antibody and the protein concentration was inferred from a standard curve made with recombinant protein.

To visualize $IB^{TNFα}$ and IB^{CCL4} by flow cytometry and confocal microscopy, Atto-488 NHS ester (Sigma) was conjugated at a molar ratio 1:2 (protein/dye) following manufacturer's instructions. Labeling efficiency was calculated using Nanodrop ND-1000.

2.3. $IB^{TNFα}$ and IB^{CCL4} nanoparticle characterization

$IB^{TNFα}$ and IB^{CCL4} were characterized by Field Emission Scanning Electron Microscopy (FESEM, Zeiss Merlin). The samples were resuspended in distilled water at a final concentration of 100 μg/ml and 20 μl were deposited on silicon chips and air dried O/N. FESEM images were processed using the software ImageJ software v1.5 (National Institute of Health, USA) length measures of at least 300 particles per sample were taken and size distribution graphs were generated using Past3 software v3.03 (University of Oslo). Particle stability was evaluated under the same conditions found in the trout gastrointestinal tract [30]. The $IB^{TNFα}$ at 0.5 mg/ml were incubated in PBS at pH 2.5 (adjusted with HCl) for 3 h and then in PBS at pH 8.0 (adjusted with NaOH) for 6 h at RT under orbital shaking. Particle thermostability was also evaluated at high temperature. The $IB^{TNFα}$ at 0.5 mg/ml were incubated at 100 °C for 30 s. Finally, we analyzed the stability of TNFα IBs after lyophilization and storage at room temperature. $IB^{TNFα}$ were lyophilized in a Telstar Lyoquest-80 lyophilizer for 8 h at −80 °C and stored at RT for 3 weeks. The $IB^{TNFα}$ were analyzed by FESEM and the diameter and length of 300 nanoparticles were measured. Statistical differences were analyzed using a *t*-test (GraphPad Prism software v5, USA).