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Biological activity of heterologous murine interleukin-10 and preliminary studies on the use of a dextrin nanogel as a delivery system

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ABSTRACT

Interleukin-10 (IL-10) is an anti-inflammatory cytokine, which active form is a non-covalent homodimer with two intramolecular disulphide bonds essential for its biological activity. A mutated form of murine IL-10 was successfully expressed in *E. coli*, recovered and purified from inclusion bodies. Its ability to reduce tumor necrosis factor α synthesis and down-regulate class II major histocompatibility complex molecules expression on endotoxin-stimulated bone marrow-derived macrophages was confirmed, and shown to be similar to that of a commercially available IL-10. Given the potential of IL-10 for application in various medical conditions, it is essential to develop systems that can effectively deliver the protein. In this work it is shown that a dextrin nanogel effectively incorporate IL-10, stabilize, and enable the slow release of biologically active IL-10 over time. Altogether, these results demonstrate the suitability of dextrin nanogel to be used as a system for the controlled release of IL-10.

1. Introduction

Interleukin-10 (IL-10) is produced by various cell types including T and B cells, monocytes, and macrophages (Moore et al., 2001; Pestka et al., 2004). This cytokine is highly pleiotropic in its biological activity that includes: inhibition of the synthesis of several cytokines, including IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) (de Waal Malefyt et al., 1991a,b); immunosuppressive effects on monocytes/macrophages (Bogdan et al., 1991; Fiorentino et al., 1991; Gazzinelli et al., 1992); as well as immunostimulatory activity on a broad range of cells types, including T cells (MacNeil et al., 1990), B cells (Defrance et al., 1992), and mast cells. Furthermore, IL-10 down-regulates constitutive and IFN-γ- or IL-4-induced class II major histocompatibility complex (MHC-II) molecules expression on monocytes, dendritic cells, and Langerhans cells (de Waal Malefyt et al., 1991a,b; Groux et al., 1998) as well as adhesion and co-stimulatory molecules on antigen-presenting cells (APCs) (Willems et al., 1994; Creery et al., 1996) and, suppresses the release of reactive oxygen intermediates (Bogdan et al., 1991; Fiorentino et al., 1991). IL-10 conditioned APC may also promote the differentiation of counter-inflammatory regulatory T cells (Steinbrink et al., 1997; Steinbrink et al., 2002). IL-10 has thus a strong anti-inflammatory activity and may act as a general suppressor factor of immune responses. Due to its immunoregulatory properties, this cytokine has been proposed to be used in several clinical applications (Asadullah et al., 2003).

Interleukin-10 pleiotropic activities are conveyed to cells by a high-affinity interaction with its cell surface receptor (IL-10R). The IL-10R is a member of the class 2 cytokine receptor family and is composed of at least two subunits, IL-10R1 and IL-10R2, which are members of the interferon receptor family (Moore et al., 2001; Pestka et al., 2004). The interaction of IL-10 with IL-10R seems to be highly complex (Asadullah et al., 2003) and the receptor neutralization blocks IL-10 activity (Moore et al., 2001).

IL-10 DNA sequence from different species is highly conserved and contains an open reading frame encoding for a secreted polypeptide of about 178 amino acids, with an N-terminal hydrophobic leader sequence of 18 amino acids, with well conserved domains (Moore et al., 2001). Biologically active IL-10 has been shown to exist in solution predominantly as a non-covalent symmetric homodimer (Walter and Nagabhushan, 1995; Zdanov

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et al., 1995). Human IL-10 is composed of two polypeptide chains of 160 amino acids each. The very similar murine IL-10 consists of 157 amino acids and presents a 73% overall sequence identity with the human IL-10. Biophysical characterization of the human and murine IL-10 (Windsor et al., 1993; Bondoc et al., 1997) showed the presence of two disulfide bonds that link the first to the third and the second to the fourth cysteine residues, bonds that are essential to the biological activity of the protein (Zdanov et al., 1995, 1996; Moore et al., 2001; Asadullah et al., 2003; Pestka et al., 2004). The murine IL-10 also revealed a fifth cysteine that exists as a free sulfhydryl (Windsor et al., 1993). Rat IL-10 has a 83% identity with murine IL-10, including the existence of a fifth unpaired cysteine, and it has been shown (Ball et al., 2001) that, due to the high reactivity of the fifth unpaired cysteine, rat IL-10 produced from the native sequence presents a severely reduced biological activity. The same work (Ball et al., 2001) showed that a mutated form of rat IL-10, where the fifth unpaired cysteine at position 149 was substituted with tyrosine, possesses the full biological activity of the native physiological rat IL-10 homodimer.

As already stated, cytokines, like IL-10, have attracted great attention due to their potential application in various medical fields, such as: vaccines (Berzofsky et al., 2001), allergies (Pullerits, 2002), infectious diseases (Hubel et al., 2002), acute inflammatory diseases (Asadullah et al., 2003), etc. Normally, proteins are expensive to produce on a large scale, are easily denatured loosing their bioactivity, and have a quite short half-life *in vivo*. So, it is essential to develop new delivery systems that allow efficient therapeutic effects at a minimum dosage. A promising method is the encapsulation using polymeric nanohydrogels or nanogels (also called polymeric or macromolecular micelles), which, by trapping the proteins in a hydrated polymer-network, minimizing denaturation, and enabling slow-release, while maintaining an effective concentration for the necessary period of time (Murthy et al., 2002, 2003; Leonard et al., 2004; Kim et al., 2009).

Dextrin is a very promising biomaterial, available in medical grade and accepted by the United States Food and Drug Administration (FDA) (Hreczuk-Hirst et al., 2001; Treetharnmathurot et al., 2009) for human application. In previous work, we have developed and characterized a nanogel obtained by self-assembling of hydrophobized dextrin (Gonçalves et al., 2007; Gonçalves and Gama, 2008). The nanogel obtained have high colloidal stability and spherical shape. Size distribution, obtained by dynamic light scattering, showed two distinct populations, with 25 and 150 nm, the former being the predominant one. *In vitro* studies, by Gonçalves et al., showed that dextrin nanogel is non-toxic and does not elicit a reactive response when in contact with macrophages (Gonçalves et al., 2009). Therefore, the dextrin nanogel presents itself as a promising carrier for IL-10.

This work describes: (1) the expression in *E. coli* and purification of a mutated murine IL-10 form (rIL-10). In this mutated form, the unpaired cysteine (Cys 149) was replaced with tyrosine, as in the human IL-10 homologue; (2) the evaluation of rIL-10 biological activity, using bone marrow derived macrophages (BMDM), and its comparison with that of a commercially available IL-10 (cIL-10); (3) the incorporation/release of IL-10 into/from the dextrin nanogel, and the stability and bioactivity of rIL-10 in the nanogel/rIL-10 complex.

2. Experimental

2.1. Materials

All reagents used were of laboratory grade and purchased from Sigma–Aldrich (USA), unless stated otherwise. Commercial IL-10 (cIL-10) was purchased from eBioscience (San Diego, CA, USA). Purified anti-human CD210 (IL-10 R) (denominated as AntIL-10R) was obtained from BioLegend (San Diego, CA, USA).

2.2. Recombinant IL-10 expression and purification

2.2.1. Construction of the expression plasmid

The DNA encoding for murine IL-10 was synthesized by GenScript Corporation (NJ, USA) with optimized codons for bacterial expression and delivered cloned, into the *Nde*I and *Xho*I restriction sites, on the expression vector pET28a(+) (Novagen, USA), yielding pET28aIL-10.

2.2.2. Site directed mutagenesis

To remove the hexahistidine tag (HisTag) coded by the pET28aIL-10 construction, the HisTag and the linker coding sequences from the N-terminus were deleted using a site directed mutagenesis kit. The same kit was used to obtain the Cys149Tyr mutant (Ball et al., 2001). For that purpose, the primers listed in Table 1 were used.

The mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA) according to the manufacturer's instructions. The deletion of the HisTag and the linker coding sequences was accompanied by the loss of *Nco*I and *Nde*I restriction endonuclease sites. The primers designed to obtain the C149Y mutant also induced a novel *Hind*III restriction endonuclease site. These changes allowed the identification of the positively deleted/mutated clones by restriction endonuclease analysis. Automated DNA sequencing analysis later confirmed the positive clones identified.

2.2.3. Recombinant IL-10 expression, refolding, and purification

The culture of *E. coli* BL21 star (Invitrogen, CA, USA), transformed with the expression construct, was grown in Luria-Bertani (LB) medium supplemented with 50 μg/ml kanamycin at 37 °C, 185 rpm. The expression of IL-10 C149Y (termed rIL-10 ahead in this paper) was induced by the addition of isopropyl-β-D-thiogalactopyranoside into the culture medium at mid-log phase ($O.D._{600\,nm} = 0.6$) to 0.5 mM final concentration. After 3 h incubation, cells were harvested, resuspended in 50 mM Tris, 50 mM NaCl (pH 7.4) and lysed by adding 100 μg/ml lysozime. After freeze and thawing, 100 μg/ml deoxyribonuclease I and 100 mM $MgCl_2$ were added and the extract incubated at 4 °C for 1 h. The inclusion bodies were then washed for 3 h with 50 mM Tris, 50 mM NaCl (pH 7.4), pelleted at 10,000 × *g* and then washed again for another 3 h with 50 mM Tris, 50 mM NaCl (pH 7.4), 0.1% triton X-100 (v/v) followed by centrifugation at 10,000 × *g*. The pelleted inclusion bodies were then dissolved in 6 M guanidine. HCl, pH 8.5 and dithiothreitol was added to a final concentration of 5 mM and, after a ultracentrifugation step (100,000 × *g*, 20 min, 4 °C) to remove any insoluble material, the protein was refolded by rapid dilution (20-fold) into

Table 1
Primers used for the deletion of HisTag (dHisTag) and to obtain the Cys149Tyr mutant (C149Y).

Name	Sequence
dHisTag-forward	5′-CTTTAAGAAGGAGATATACCATGTCTCGTGGCCAGTACTCTCGTGAAG-3′
dHisTag-reverse	5′-CTTCACGAGAGTACTGGCCACGAGACATGGTATATCTCCTTCTTAAAG-3′
C149Y-forward	5′-GAATTTGACATCTTCATCAACTATATTGAAGCTTACATGATGATCAAAATGAAAAGC-3′
C149Y-reverse	5′-GCTTTTCATTTTGATCATCATGTAAGCTTCAATATAGTTGATGAAGATGTCAAATTC-3′

50 mM Tris pH 8.0, 2 mM glutathione (reduced form), 0.2 mM glutathione (oxidized form), 50 mM NaCl, 5 mM EDTA. The refolded solution was kept in cold room until purification (usually between 3–4 days to a week).

The refolded rIL-10 solution was first concentrated by tangential flow ultrafiltration (Sartocon Slice – Sartorius, Germany) to approximately 150 ml, followed by a N_2 pressurized stirred cell concentrator (Amicon 8200 – Millipore, MA, USA) to 12–15 ml. After ultracentrifugation to clarify the solution (100,000 × *g*, 20 min, 4 °C), the protein was applied to a Superdex 200 26/60 column (Amersham, England) pre-equilibrated at room temperature with 25 mM phosphate buffer, pH 7.25 at 2.0 ml/min. The collected fractions with elution volume corresponding to rIL-10 dimer were pooled and applied to a Mono S HR 5/5 (Amersham, England) column equilibrated with 25 mM phosphate buffer, pH 7.25 at 0.75 ml/min. The elution was done by a linear NaCl gradient from 0 to 0.5 M.

The purity of the protein was assessed by SDS-PAGE using 12.5% gels in a BioRad Mini Protean III (CA, USA) electrophoresis apparatus according to the method of Laemmli, and stained with Coomassie brilliant Blue R-250.

2.3. Cytokine analysis

IL-10 and TNF-α were quantified by a enzyme-linked immunosorbent assay (ELISA) commercial kit, Mouse IL-10 ELISA Ready-SET-Go!, and Mouse TNF-a ELISA Ready-SET-Go! (eBioscience, San Diego, CA, USA), respectively, following the manufacturer's instructions.

2.4. Culture of murine bone marrow-derived macrophages (BMDM)

Macrophages were obtained from mouse bone marrow as follows: mice were sacrificed and femurs and tibias removed under aseptic conditions. Bones were flushed with Hanks' balanced salt solution. The resulting cell suspension was centrifuged at 500 × *g* and resuspended in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS), 60 μg/ml penicillin/streptavidin, 0.005 mM β-mercaptoethanol (complete RPMI [cRPMI]), and 10% L929 cell conditioned medium. To remove fibroblasts or differentiated macrophages, cells were cultured, on cell culture dishes (Sarstedt, Canada), overnight at 37 °C in a 5% CO_2 atmosphere. Then, nonadherent cells were collected with warm cRPMI, centrifuged at 500 × *g*, distributed in 96-well plates (Sarstedt, Canada) at a density of 1×10^5 cells/well, and incubated at 37 °C in a 5% CO_2 atmosphere. Four days after seeding, 10% of L929 cell conditioned medium was added, and the medium was renewed on the seventh day. After 10 days in culture, cells were completely differentiated into macrophages. This method allows for the differentiation of a homogenous primary culture of macrophages that retain the morphological, physiological and surface markers characteristics of these phagocytic cells (Tushinski et al., 1982; Mosmann, 1983; Zhang et al., 2008).

2.5. Bioassay of IL-10

IL-10 bioactivity was assayed by its ability to inhibit the production of TNF-α and the surface expression of major histocompatibility complex class II (MHC-II) molecules in lipopolysacharide (LPS) and IFN-γ activated macrophages (endotoxin-stimulated macrophages).

IL-10, recombinant (rIL-10) and commercial (cIL-10), in concentrations ranging from 0.1 ng/ml to 250.0 ng/ml, were added to the BMDM, and the cells incubated at 37 °C in a 5% CO_2 atmosphere for 1 h. Then, 0.1 ng/ml LPS and 1.0 ng/ml IFN-γ were added to the cells to promote macrophage activation, and incubated for 24 h. As a positive control of macrophage activation, cells stimulated with LPS and IFN-γ, without IL-10, were used; as a negative control, cells cultured in cRPMI alone were used. After incubation, culture supernatants were removed and stored at −80 °C until TNF-α quantification. The attached cells were collected, with 5 mM EDTA in PBS, for MHC-II expression analysis by flow cytometry (FACS analysis).

2.5.1. Anti-IL-10R assay

The inhibition of rIL-10 biological activity by anti-IL-10R monoclonal antibody was tested in BMDM cultures performed as in the previous assay.

rIL-10 (100.0 ng/ml) and anti-IL-10R monoclonal antibody (AntiIL-10R) (0, 12.5, 25.0, 50.0 μg/ml) were added to the cells, and incubated for 1 h at 37 °C in a 5% CO_2 atmosphere. Then, 0.1 ng/ml LPS and 1.0 ng/ml IFN-γ were added to the cells, to induce macrophage activation, that were incubated for further 24 h. After incubation, culture supernatants were removed and stored at −80 °C until TNF-α quantification. The attached cells were collected, with 5 mM EDTA in PBS, for FACS analysis of MHC-II expression as described below.

2.6. FACS analysis

To determine the surface expression of MHC-II molecules, cells were labeled for 20 min on ice with 1:300 MCHII-PE antibody (BD Pharmingen™, USA) in FACS buffer (PBS, 1% BSA). Then, cells were washed with FACS buffer and transferred to FACS-tubes containing 1:100 5 μg/ml propidium iodide in FACS buffer.

Labeled cell samples were analyzed in a FACScan (Becton Dickinson, San Jose, CA, USA) with the CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.7. Nanogel/rIL-10 complex formation and stability

2.7.1. Preparation of the nanogel/rIL-10

Dextrin-MVA-SC_{16} (MVA: vinyl methacrylate; SC_{16}: alkyl chain) was synthesized as previously described (Carvalho et al., 2007; Gonçalves et al., 2007), with the exception that the transesterification was made with vinyl methacrylate instead of vinyl acrylate.

The size distribution was determined with a Malvern Zetasizer MODEL NANO ZS (Malvern Instruments Limited, UK). A dispersion of the nanogel was analyzed at 25 °C in a polystyrene disposable cell, using a He–Ne laser-wavelength of 633 nm and a detector angle of 173°.

To form the self-assembled nanogel, lyophilized dextrin–VMA–SC_{16} was dissolved in cRPMI, at a concentration of 1.0 mg/ml. The dissolution was accomplished after approximately 16 h at room temperature with stirring. The nanogel formation was confirmed by dynamic light scattering. All suspensions were sterilized by filtration through a 0.45 μm membrane.

The complex nanogel/rIL-10 was formed by dissolving rIL-10 in cRPMI, without FBS, and then by mixing lyophilized dextrin–VMA–SC_{16} (1.0 mg/ml). The dissolution was accomplished after approximately 16 h at room temperature with stirring.

2.7.2. Evaluation of rIL-10 incorporation into the nanogel/rIL-10 complex

rIL-10 incorporation into the dextrin nanogel was verified quantifying the amount of rIL-10 free in solution by ELISA. Briefly, the complex nanogel/rIL-10 was formed, as described previously, using concentrations of rIL-10 ranging from 10 ng/ml to 10,000 ng/ml. Then, the complex was washed three times, using the Ultra-Filtration device Amicon®Ultra-15 100 kDa (Millipore, MA, USA),

to elute unbound protein. Finally, rIL-10 free in solution was quantified, by ELISA, to evaluate the amount of rIL-10 incorporated.

2.7.3. Circular dichroism (CD) measurements

The secondary structure of rIL-10, free or complexed with the nanogel, was investigated using CD. Spectra were obtained on a Olis DSM 20 circular dichroism spectropolarimeter continuously purged with nitrogen, equipped with a Quantum Northwest CD 150 temperature-controlled cuvette and controlled by the Globalworks software.

CD spectra of free rIL-10 (0.5 mg/ml or 0.25 mg/ml) and rIL-10 complexed with the nanogel (1 mg/ml), in PBS, were collected using a 0.2 mm path length cuvette, between 190 and 260 nm at 1 nm intervals. Three scans with an integration time of 4 s were averaged for each measurement. Spectra were acquired at 4 or 37 °C. The results are expressed in terms of mean residue ellipticity $[\theta]_{MRW}$ in deg cm^2 dmol^{-1}, according to the equation $[\theta]_{MRW} = \theta_{obs} \times 100\,MW/(lcN)$, where θ_{obs} is the observed ellipticity in mdeg, MW is the protein molecular weight in g/mol, l is the cuvette path length in cm, c is the protein concentration in g/l and N is the number of residues of the protein.

rIL-10 stability, free or complexed with the nanogel (0.25 mg/ml rIL-10, 1 mg/ml nanogel), at 37 °C and at 4 °C, was accessed by obtaining CD spectra of the samples, at determined intervals of time.

2.8. Bioactivity of the complex nanogel/rIL-10

2.8.1. Evaluation of rIL-10 release from the nanogel/rIL-10 complex

The release of rIL-10 from the complex nanogel/rIL-10 was assessed in a BMDM culture.

The complex nanogel/rIL-10 was formed with 2000 ng/ml rIL-10, as described above, and 20% FBS was added to the suspension. Then, the suspension (200 μl/well) was added to BMDM cells (1×10^5 cells/well) and incubated at 37 °C in a 5% CO_2 atmosphere. At determined times, supernatants were collected and stored at −80 °C until IL-10 quantification.

2.8.2. Bioactivity of rIL-10 released from the nanogel/rIL-10 complex

Suspensions of nanogel and nanogel/rIL-10 complex were prepared as described previously. Then, 20% FBS was added and the suspensions (200 μl/well) added to BMDM cells (1×10^5 cells/well). After 2 h of incubation at 37 °C in a 5% CO_2 atmosphere, 0.1 ng/ml LPS and 1.0 ng/ml IFN-γ were added to the cells to promote macrophage activation, and incubated for further 24 h. After incubation, culture supernatants were removed and stored at −80 °C until TNF-α quantification. The attached cells were collected, with 5 mM EDTA in PBS, for MHC-II expression analysis by flow cytometry. As controls, wells without LPS and IFN-γ stimulation were used.

3. Results and discussion

3.1. IL-10 expression and purification

Interleukin-10 is a very important immunoregulatory protein hence drawing great interest for its potential regarding biomedical applications (Berzofsky et al., 2001; Hubel et al., 2002; Pullerits, 2002; Asadullah et al., 2003).

The active form of IL-10 is known to be a non-covalent homodimer and, as already stated, human and murine IL-10 have two disulfide bonds that are essential to the biological activity of the protein (Windsor et al., 1993; Bondoc et al., 1997). However, murine IL-10, and alike the rat IL-10, has also a fifth unpaired cysteine (Cys149). In the rat, this additional cysteine has been shown to reduce significantly the IL-10 biological activity (Ball et al., 2001). In order to eliminate the unwanted and predictable reactivity of Cys149, this amino acid was replaced by directed mutagenesis with tyrosine. Amino acid sequence and structure of human, murine, rat and porcine IL-10 dimer, suggests that both amino acids surrounding cys149 are conserved (Zdanov et al., 1995) and therefore, the replacement of cysteine 149 for tyrosine, the corresponding amino acid in human and porcine IL-10, was least likely to disrupt the dimer active conformation (Ball et al., 2001).

After deletion of the hexahistidine tag and the C149Y mutagenesis, the recombinant vector was transformed into *E. coli* BL21 star cells. The mutated murine IL-10 protein (rIL-10) was expressed in bacteria, yielding about 50% of the total cell protein, bearing an apparent molecular weight close to the expected (18 kDa), as shown in the SDS-PAGE analysis (Fig. 1A, lane 1). However, the expression of soluble and functional eukaryotic proteins in heterologous systems is not always straightforward (Dyson et al., 2004) and usually overexpressed recombinant proteins accumulate either in the cytoplasm and/or periplasmic space in form of inclusion bodies, which are amorphous granules of misfolded protein with no biological activity (Sorensen and Mortensen, 2005). The formation of inclusion bodies led to the recovery of rIL-10 by a process of solubilization in 6 M guanidine, renaturation and re-oxidation of the dissulphides. Then, the retrieval of the soluble protein from the renaturation solution was made by gel filtration, using the stationary phase Superdex 200 (Fig. 1B), to collect the dimeric form of the recombinant protein. The fractions containing the rIL-10 dimer, collected between 205 and 225 ml (Fig. 1A, lane 2), were pooled and combined for further purification by ion-exchange chromatography, in a Mono S column (Fig. 1C). SDS-PAGE (Fig. 1A, lanes 3, 4, 5, and 6) analysis confirmed the apparent molecular weight of the proteins as well as their purity. The oligomerization state of the collected fractions (either from the Superdex 200 column as from

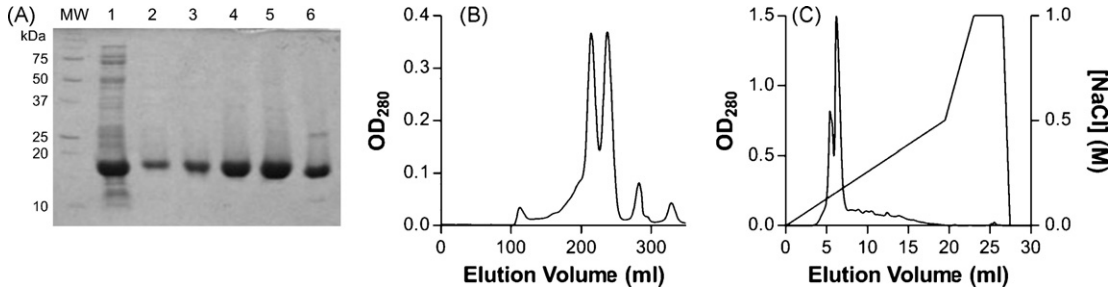


Fig. 1. (A) SDS-PAGE: MW, molecular weight marker; (1) insoluble fraction applied to Superdex 200; (2) fractions eluted from Superdex 200 and applied to Mono S; (3, 4) first peak eluted from Mono S; (5, 6) second peak eluted from Mono S; (B) chromatogram obtained using Superdex 200; (C) purification of the rIL-10 dimer in a Mono S column.

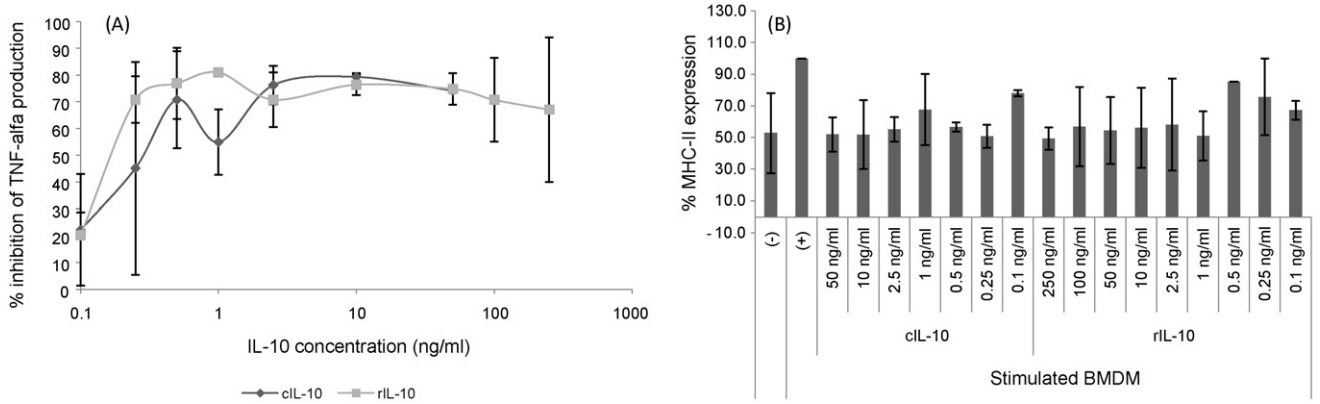


Fig. 2. Biological activity of rIL-10 and cIL-10. (A) Percentage of inhibition of TNF-α production, by 0.1 ng/ml LPS and 1.0 ng/ml INF-γ stimulated BMDM. (B) Percentage of MHC-II expression induced by cIL-10 and rIL-10 treatment of stimulated BMDM. Data points are the means ± SD (standard deviation) of duplicate independent assays with triplicate cell incubations each. (+) Positive control of macrophage activation; (−) negative control of macrophage activation.

Mono S column) was verified by analytical gel filtration and shown to be dimeric (data not shown).

So, rIL-10 was recovered as the active non-covalent homodimer and the yield of protein (about 1.0–1.5 mg/l culture, quantified by ELISA) was high enough to the subsequent assays, and stable between batches. Both fractions eluted from Mono S column were shown to be in dimeric state, and were then tested for biological activity.

3.2. Biological activities of rIL-10

In order to evaluate whether the rIL-10 recovered from the inclusion bodies was biologically active, BMDM cultures were used. Active macrophages exhibit a higher production of several cytokines, as well as oxygen reactive intermediates. IL-10 is known to deactivate macrophages, reducing the production of cytokines and oxygen reactive intermediates, and also to down-regulate MHC-II expression (Bogdan et al., 1991; de Waal Malefyt et al., 1991a,b; de Waal Malefyt et al., 1991a,b; Fiorentino et al., 1991; Groux et al., 1998; Moore et al., 2001; Pestka et al., 2004).

As it can be seen in Fig. 2A, in the range of 0.1–1.0 ng/ml, rIL-10 showed a dose-dependent ability to reduce the TNF-α production. At 1.0 ng/ml, TNF-α is reduced by about 80%; increasing the rIL-10 concentration does not seem to further reduce TNF-α production significantly. It is also noticeable that rIL-10 ability to reduce TNF-α production is similar to that of tested cIL-10, in the analyzed range of concentrations.

As antigen presenting cells, macrophages have a constitutive expression of MHC-II molecules that is however increased upon activation (Ma et al., 2003). The results shown in Fig. 2B are normalized taking as reference (100%) the MHC-II expression by cells treated only with LPS and INF-γ (positive control of macrophage activation). Treating cells with rIL-10 reduces MHC-II expression of the stimulated macrophages to the level detected in the negative control (Fig. 2B). In the range of 1.0–50.0 ng/ml the effect of cIL-10 and rIL-10, on the expression of MHC-II molecules, is identical.

The two fractions eluted from Mono S column were tested and presented similar results.

To further test the specificity of rIL-10 bioactivity, a blocking IL-10R antibody was used. IL-10 exhibits a wide variety of activities when it binds specifically to its cellular receptor, signaling through the IL-10R results not only in the inhibition of the synthesis of several cytokine genes, but also prevents several cytokines from inducing their biological activities on their target cells (Moore et al., 2001; Pestka et al., 2004). The information about the interaction of rIL-10 with IL-10R is important to determine whether rIL-10 acts as "genuine" IL-10 or if it mimics its function indirectly by, for instance, facilitating endogenous IL-10 expression or release.

As shown in Fig. 3A, in the presence of AntiIL-10R, TNF-α production by stimulated macrophages is high meaning that rIL-10 activity was thus exerting its activity through binding to the IL-10R. The effect of rIL-10 on MHC-II expression was similarly blocked (Fig. 3B). It should be noticed that, in the absence of the blocking antibody, rIL-10 presented biological activity, decreasing TNF-α

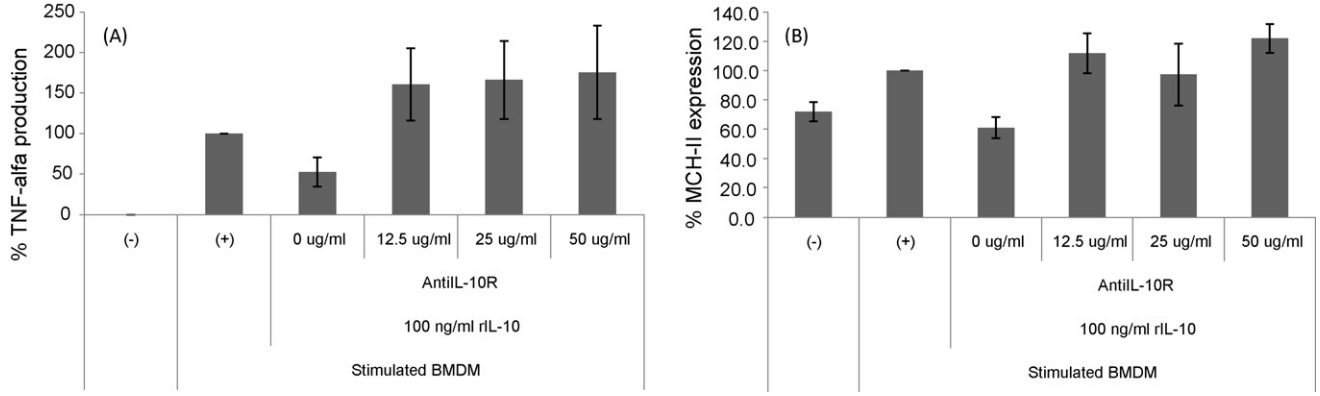


Fig. 3. Anti-IL-10R activity. (A) Percentage of TNF-α production, by 0.1 ng/ml LPS and 1.0 ng/ml INF-γ stimulated BMDM. (B) Percentage of MHC-II expression induced by AntiIL-10R and rIL-10 treatment of stimulated BMDM. Data points are the means ± SD of triplicate independent assays with triplicate cell incubations each. (+) Positive control of macrophage activation; (−) negative control of macrophage activation.

production and MHC-II expression. It was also observed that, in the presence of the blocking antibody, TNF-α production and MCH-II expression were higher than in the positive control (+) meaning that even the activity of endogenous IL-10, expressed by the macrophages in response to LPS and INF-γ, was blocked.

Taken together, these results demonstrate that the rIL-10 is biologically active and exerts its activity specifically through IL-10 receptor binding, acting thus like endogenous IL-10.

3.3. Nanogel/rIL-10 complex formation and stability

Polymer formulations, including polymer–protein conjugates, are finding increasing clinical use. Synthetic and natural polymers have been explored as drug carriers, but most polymers used clinically are still non-biodegradable synthetic ones (Hreczuk-Hirst et al., 2001). The proven clinical tolerability of dextrin and its efficient absorption due to degradation by amylases, suggest that this polymer might be ideal for development as a drug carrier (Hreczuk-Hirst et al., 2001). Furthermore, dextrin is readily excreted due to its low molecular weight, hence accumulation in the tissues is unlikely.

Self-assembled dextrin nanogel, dispersed in water at a concentration of 1.0 mg/ml, was observed using dynamic light scattering (DLS) after filtration through a 0.45 μm membrane, as comprehensively described in previous work (Gonçalves et al., 2007; Gonçalves and Gama, 2008; Gonçalves et al., 2009), and illustrated by Scheme 1.

The DLS analysis, in the intensity distribution, revealed two populations with roughly 25 and 150 nm. The conversion to number distribution highlighted only the smaller population of particles, the predominant one. Thus, the nanogel used in this study present a diameter of about 25 nm, in water, upon self-aggregation. The *z*-value obtained was 23.6 nm, representative of the average size of polydisperse colloids. The polysdispersity index (PdI) provides information on the homogeneity of the dispersion and the obtained PdI for the nanogel was low (<0.5), meaning the sample may be considered homogeneous, consisting of one main population with 23.6 nm. Additionally, the variation of the hydrodynamic diameter (*z*-value) and zeta-potential of the nanogel with pH was evaluated (Gonçalves and Gama, 2008). In the pH range studied, the zeta potential was almost constant and close to zero. Although the low zeta potential, the nanogel is stable. The stability can be attributed to the solvation forces, as discussed by Gonçalves and Gama (2008).

Cytokines are proteins bearing a short half-live, *in vivo*. The encapsulation of rIL-10 in the dextrin nanogel was attempted as a mean to augment its bioavailability and stability. By trapping rIL-10 in a hydrated polymer-network, the nanogel is expected to minimize denaturation and enable a slow release profile, hence maintaining an effective concentration.

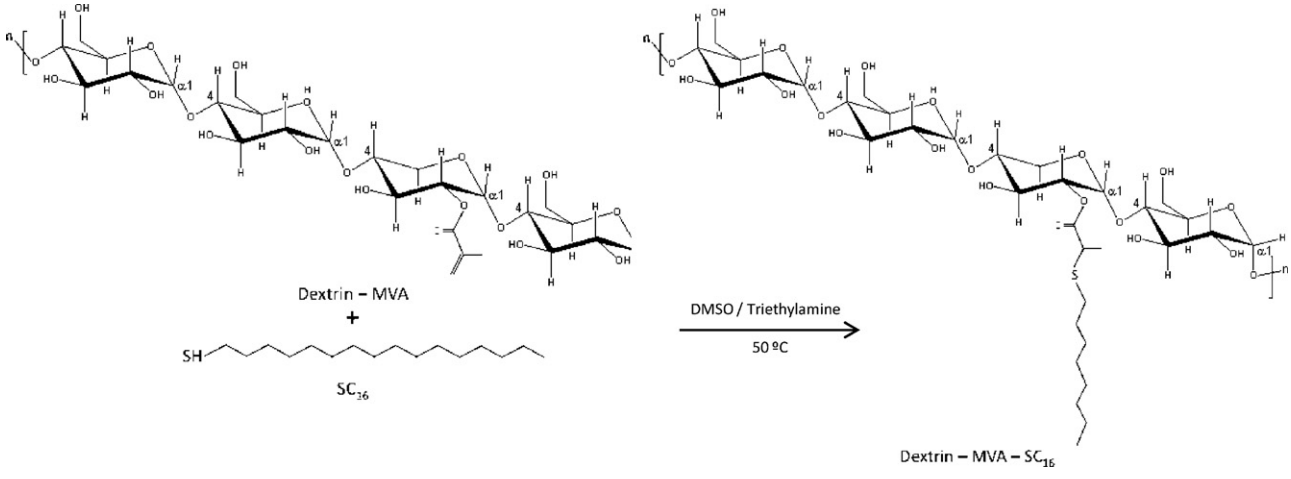
In order to confirm the incorporation of rIL-10 by the nanogel, the cytokine detectable using the ELISA was quantified after formation of the complexes nanogel/rIL-10. The encapsulation was achieved simply mixing the cytokine and nanogel, as described in the methods section. Irrespective of the concentration of rIL-10 incubated with the nanogel (10, 100, 1000 and 10,000 ng/ml), rather low amounts (1.5–2.0 ng/ml) were detected in each case. Thus, even when 10,000 ng/ml of rIL-10 is used, only 0.02% of the rIL-10 remains detectable in solution. Indeed, even lower amounts of rIL-10 (<1 ng/ml) were detected in permeates collected after ultrafiltration of the complex nanogel/rIL-10 using a membrane with a cutoff of 100 kDa, thus allowing the filtration only of the free cytokine. Altogether, these results show that rIL-10 is efficiently and spontaneously incorporated in the nanogel complex. The difference in the concentrations detected in the ultrafiltration permeate (<1 ng/ml) and in the mixture nanogel/rIL-10 (1.5–2.0 ng/ml) is assigned to protein on the surface of the nanogel, allowing its detection by ELISA.

The CD spectra of soluble rIL-10 (0.5 mg/ml) and nanogel/rIL-10 (1 mg/ml nanogel, 0.5 mg/ml rIL-10) are shown in Fig. 4A.

The secondary structure of free soluble rIL-10 is mainly helical, as expected (Zdanov et al., 1995, 1996; Josephson et al., 2000), and the complexation with the nanogel did not induce any conformational change. Similar results were obtained using other concentrations of rIL-10. Furthermore, the nanogel (1 mg/ml) did not interfere in the CD observations, under the conditions employed.

The stability of the free and complexed rIL-10 was accessed at the physiological temperature, 37 °C, by recording the CD spectra over time. Samples of rIL-10 and nanogel/rIL-10 complex were incubated at 37 °C, in PBS, and CD spectra obtained at determined intervals of time (Fig. 4B and C, thick grey lines represent the CD spectra of the samples at beginning of the experiment). After 6 days incubation, CD spectrum of free rIL-10 completely looses its characteristic pattern (thick black line in Fig. 4B) while rIL-10 complexed with the nanogel still presents a typical CD spectrum of a helical protein even after 16 days incubation at 37 °C (thick black line in Fig. 4C). The mean residual ellipticity (θ) variation, at 222 nm, shows that rIL-10 stability is significantly increased when rIL-10 is complexed with the dextrin nanogel.

These results suggest that the nanogel stabilize rIL-10, avoiding its denaturation and precipitation and thus preserving bioavailability for longer periods of time, at physiological temperature. In addition, the stability improvement of the formulation during stor-



Scheme 1. Synthesis of Dex-VMS-SC16.

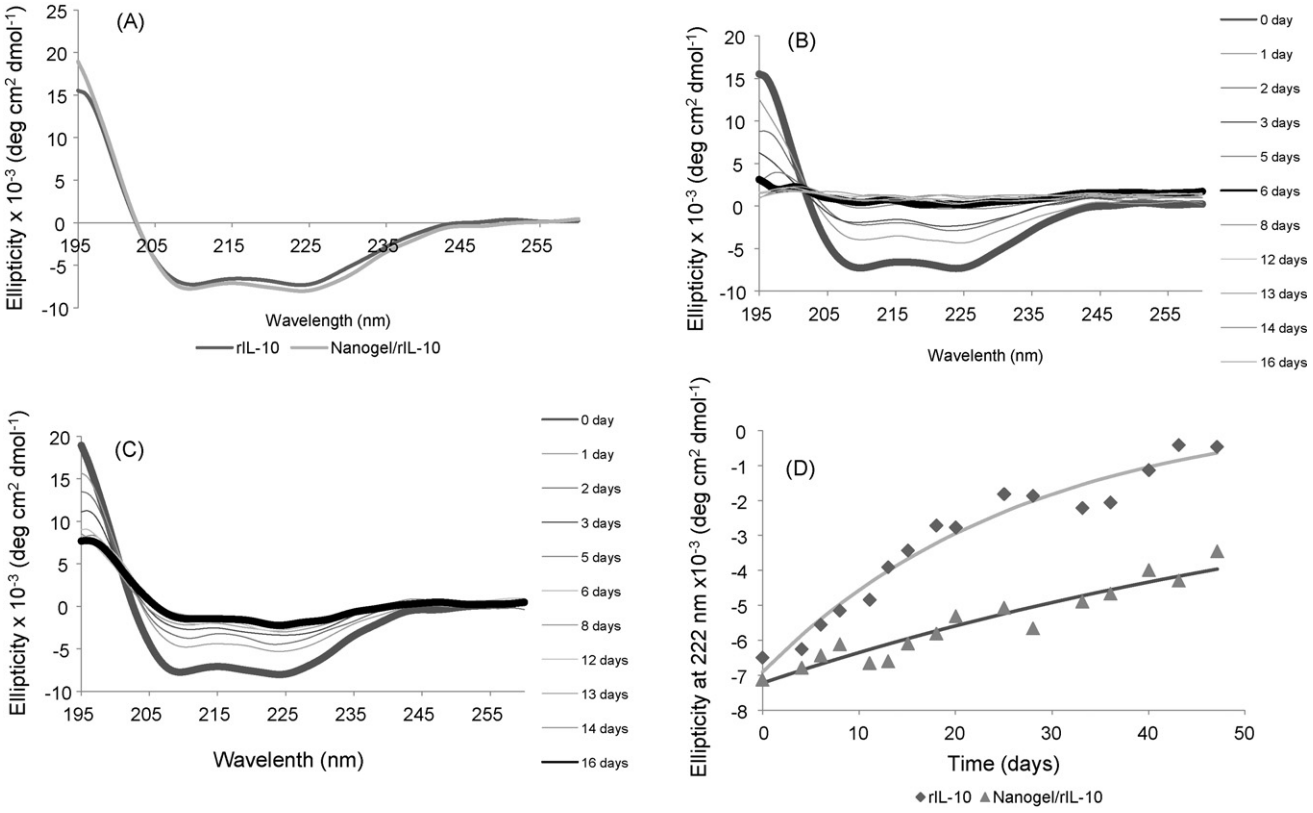


Fig. 4. CD spectra of (A) free rIL-10 (0.25 mg/ml) and complex nanogel/rIL-10 (1 mg/ml nanogel and 0.25 mg/ml rIL-10) at 37 °C in PBS; (B) free rIL-10 (0.25 mg/ml) and, (C) complex nanogel/rIL-10 (1 mg/ml nanogel and 0.25 mg/ml rIL-10) incubated at 37 °C, in PBS for several days; and, (D) variation of the mean residual ellipticity at 222 nm of rIL-10 and nanogel/rIL-10 incubated at 4 °C for several days.

age, at lower temperatures, is also significant (Fig. 4D). The dextrin nanogel may thus effectively perform as stability enhancer, not only as a protein carrier.

3.4. Biological activities of rIL-10 released from the nanogel/rIL-10 complex

The complex nanogel/rIL-10 suspension was added to BMDM cultures to evaluate the release of the cytokine, measured by ELISA, to the culture medium in the presence of serum proteins (Fig. 5). The spontaneous dissociation of rIL-10 from the complex was barely observed in culture medium without serum. On the other hand, rIL-10 was released from the nanogel/rIL-10 complex when FBS was added to the system. Worth noticing that, the BMDM culture (without the nanogel/rIL-10) did not produce IL-10 in significant amounts. After 2 h of incubation in the presence of 20% FBS, the released rIL-10 reaches a maximum, stable concentration of 35 ng/ml, which is still constant after 24 h. A similar release profile is observed in culture medium without BMDM (Fig. 5).

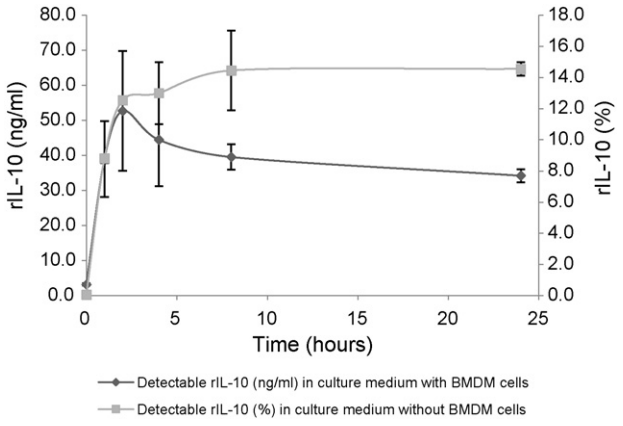


Fig. 5. IL-10 release profile on a BMDM culture and on culture medium alone. Data points are the means ± SD of duplicate independent assays with triplicate incubations each.

The rIL-10 release is probably due to the exchange of rIL-10 with serum proteins. Indeed, when the nanogel/rIL-10 complex is placed in medium containing serum proteins, a partition equilibrium (incorporated versus free rIL-10) is established in approximately 2 h, with or without BMDM cells. However, it must be remarked that the rIL-10 released correspond only to approximately 15% of the protein incorporated initially in the nanogel (Fig. 5). The protein released in this experimental set-up probably corresponds to the commonly observed – in drug release systems – initial burst release of the less tightly adsorbed proteins, less stabilized by the hydrophobic cores present inside the nanogel, as previously discussed (Gonçalves et al., 2007; Gonçalves and Gama, 2008). Theoretically, the constant concentration of IL-10 in the culture medium may be due to (1) a partition equilibrium of the cytokine between the nanogel and the culture medium, or, (2) to a strong interaction of the cytokine, more likely a denatured fraction, with the hydrophobic domains inside the nanogel. Along time, in sink conditions, the release rate is expected to slow-down, as protein stabilized deeper within the nanogel and interacting more tightly with the hydrophobic cores is being exchanged. Furthermore, in the dynamic situation *in vivo*, with continuous depletion of rIL-10 (at different rates, depending on the administration route), the protein is expected to be fully released. Probably, a different release profile will be obtained with different proteins. This release profile is likely to depend on the hydrophobicity of the protein and

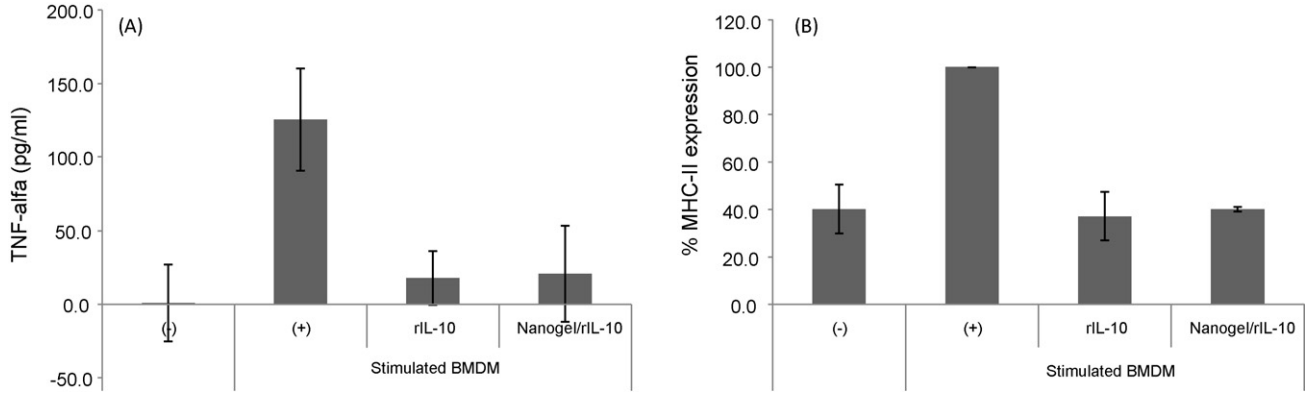


Fig. 6. Biological activity of rIL-10 released from nanogel/rIL-10 complex. (A) TNF-α concentration (pg/ml), produced by 0.1 ng/ml LPS and 1.0 ng/ml INF-γ stimulated BMDM, treated with rIL-10 and nanogel/rIL-10. (B) MHC-II induced, in stimulated BMDM, by 50 ng/ml rIL-10 and nanogel/rIL-10. Data points are the means ± SD of duplicate independent assays with triplicate cell incubations each. (+) Positive control of macrophage activation; (−) negative control of macrophage activation.

affinity for the hydrophobic domains in the nanogel and on its stability. A more comprehensive characterization of the interaction protein–nanogel – using different proteins – and of the release kinetics in sink conditions will be performed in future work.

The bioactivity of the rIL-10 released from the nanogel was also analyzed. A suspension of nanogel/rIL-10 was added to a BMDM culture and incubated for 2 h, such that enough rIL-10 could be released from the complex. Then, LPS and INF-γ was added to activate the macrophages. After 24 h incubation, the amount of TNF-α produced and the expression of MHC-II were evaluated. The rIL-10 released from the nanogel/rIL-10 complex was able to inhibit TNF-α production and MHC-II expression at the same level as the soluble rIL-10, as Fig. 6 shows.

So, the results demonstrate that the rIL-10 released from the nanogel/rIL-10 complex is able to inhibit macrophages at the same level as the soluble rIL-10. We can also conclude that the nanogel encapsulation, besides not inducing any alteration on the rIL-10 secondary conformation (as seen in Fig. 4A), does not alter the biological properties of the protein.

4. Conclusions

A mutated form of murine IL-10 was successfully expressed in *E. coli*. This recombinant protein was recovered and purified from inclusion bodies and demonstrated biological activity similar to a commercially available IL-10. Dextrin self-assembled nanogel was able to efficiently encapsulate and protect rIL-10 from denaturation at 37 °C, and also enables rIL-10 to be released in biologically significant amounts over time. The biological activity of the released rIL-10 was confirmed by the evaluation of the production of TNF-α and expression of MHC-II on endotoxin-stimulated BMDM. The simplicity of the preparation of the nanogel/rIL-10 complex, associated to the enhancement of protein stability and controlled release, makes this a very promising system.

IL-10 has potential application in various medical fields, such as in acute inflammatory diseases, namely rheumatoid arthritis, and these results point to dextrin nanogel as a promising carrier of IL-10, which enables the protein sustained release.

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