Recombinant production of human ICAM-1 chimeras by single step on column refolding and purification.

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Summary

The interaction of the adhesion molecule of the immunoglobulin family Intercellular Adhesion Molecule 1 (ICAM-1) with its ligands such the integrins LFA-1 and Mac-1 is crucial for the regulation of several physiological and pathophysiological processes like cell mediated-elimination of tumor or virus infected cells, cancer metastasis or inflammatory autoimmune processes. Thus, production of milligrams of protein is required to perform structural and functional studies as well as design novel approaches to find out new inhibitors of ICAM-1/LFA-1 interaction. Here we report on the production of a recombinant human ICAM-1 chimera comprising the first two extracellular domains of ICAM-1 linked to the Fc fraction of a human IgG1. To this aim we have used a cost-effective method based on the expression of a His-tagged protein in *E. coli* followed by a single step of refolding and purification on immobilized metal affinity columns. This method is able to produce 3mg/liter of bacterial culture in just 72 hours with purity greater than 95%. The identity and the native structure of refolded human ICAM-1 chimera were confirmed by biochemical and biophysical studies including SDS-electrophoresis, immunoblot, circular dichroism, isothermal titration calorimetry and fluorescence spectroscopy. Native folding and functional activity of the chimera were further confirmed by different cell biology studies, including B cell adhesion, T cell binding and inhibition of NK cell function. These studies indicate a high biological activity of the protein since it induces a 200-fold increase/mg of protein in B cell adhesion and the Inhibitory Dose 50 to block cell-mediated cytotoxicity is 10 pg/effector cell. These analyses show that our protocol is able to produce a recombinant human ICAM-1 chimera fully active and useful to analyse the biological processes in which ICAM-1/LFA-1 interaction is critically involved.

Introduction

The intercellular adhesion molecule 1 (ICAM-1) is a membrane glycoprotein, member of the immunoglobulin superfamily (IgSF) and responsible for mediating cell-cell and cell-extracellular matrix interactions [1-3]. In addition, ICAM-1 is the receptor for different human viruses like rhinovirus [4] and coxsackievirus A21 as well as for the malarial parasite *Plasmodium falciparum* [5-7]. ICAM-1 is formed by 5 extracellular immunoglobulin domains, a hydrophobic transmembrane domain and a short cytoplasmic tail and expressed by several cell types including endothelial and epithelial cells and most types of leukocytes including B and T lymphocytes, NK cells and monocytes [3] [1]. Although the protein is heavily glycosylated, glycosylation does not affect the interaction between ICAM-1 and the integrin LFA-1 [8, 9]. This interaction occurs between domain 1 of ICAM-1 [10] and a metal ion-dependent adhesion site (MIDAS) motif located in the integrin α subunit I domain [11, 12]. Domain 1 of ICAM-1 also contains the interaction site with human rhinovirus [13] and *Plasmodium falciparum* [5] as well as for the extracellular matrix protein fibrinogen [14]. The integrin Mac1 binds to domain 3 of ICAM-1 [8]. ICAM-1 is expressed as a dimer in the plasma membrane of cells and this form binds more efficiently to LFA-1 than monomeric ICAM-1 [15, 16]. However, the crystal structure of dimeric ICAM-1 suggests that the dimer is not required for binding of LFA-1 and rhinovirus [17], which occurs by a single molecule of ICAM-1 [18]. In fact monomeric recombinant ICAM-1 prevents development of insulinitis during autoimmune diabetes [19]. Finally, it has been recently reported that the equilibrium between monomeric and dimeric state regulates the binding to LFA-1 [20].

The interaction of ICAM-1 with LFA-1 regulates several physiological and pathophysiological processes related with lymphocyte extravasation to inflammatory sites and selective entry into lymphoid organs (lymphocyte homing) [3, 21, 22]. In addition, this interaction is critically involved in cell-cell adhesion during antigen presentation and during the recognition and elimination of tumor or virus-infected cells [23]. Apart from its beneficial effect in the elimination of tumor cells and pathogens, ICAM-1/LFA-1 interaction is also involved in diverse pathologies involving inflammation and/or cellular extravasation [24]. It has been shown that specific tumour cells use this interaction to disseminate and spread to other tissues or organs [25-27] and that ICAM-1 expression contributes to drug resistance in multiple myeloma [28]. In

86 addition, lymphocyte extravasation and accumulation in diverse tissues mediated by
 87 LFA-1 is associated with inflammatory diseases like Rheumatoid Arthritis [29],
 88 Atherosclerosis [30] and Inflammatory Bowel Disease [31, 32] among others.
 89 Altogether, these findings suggest that targeting ICAM-1/LFA-1 interaction may help to
 90 inhibit tumor spreading [33] or to treat inflammatory disorders [33, 34] [35].

91 Several chromatographic approaches have been previously developed in order to purify
 92 and refold recombinant mammal proteins produced in bacterial systems. Among them
 93 Immobilised Metal Affinity Chromatography (IMAC) has been proved to be especially
 94 useful to purify proteins genetically modified to contain an amino- or carboxy-terminal
 95 polyhistidine sequences (His-Tag). This type of affinity chromatography is based on the
 96 ability of divalent cations like Cu^{2+} , Co^{2+} or Ni^{2+} to bind imidazole groups from
 97 histidine residues [36, 37]. In order to bind efficiently the protein multiple histidine
 98 groups must be located in close proximity. Very few proteins fulfil this prerequisite and,
 99 thus, this type of chromatography simplifies the whole purification process. This is
 100 especially true if purification is performed under denatured conditions, since the
 101 possibility of histidine groups closely located due to the native three-dimensional
 102 structure is eliminated.

103 One of the main problems of producing mammal proteins in prokaryotic systems is that
 104 proteins are usually expressed as a type of insoluble material known as inclusion bodies.
 105 In order to circumvent this problem and get functional proteins in native form *in vitro*
 106 refolding must be performed.

107 Among the different protocols used to refold proteins *in vitro*, [38], it seems that
 108 addition of detergents to refolding buffer helps to minimise the formation of aggregates
 109 and improves the refold efficiency [39]. A singular advance to avoid the use of large
 110 amounts of buffers and time invested in refolding by dilution was the introduction of the
 111 on column refolding [40]. Most types of chromatographic methods have been used to
 112 refold proteins on columns including size exclusion chromatography [41, 42]
 113 immobilization on gel matrices [43], hydrophobic interaction chromatography [44],
 114 affinity chromatography [45], immobilized liposome chromatography [46] and IMAC
 115 [40, 47-50].

116 Generation of purified active forms of ICAM-1 is very important to study the above-
 117 mentioned functions. Specially, the availability of large amounts of active ICAM-1 is

118 crucial to design efficient and specific approaches to find new inhibitors of ICAM-
119 1/LFA-1 interaction.

120 To this aim we have established a simple and fast protocol to generate large quantities
121 of active ICAM-1 chimera in *Escherichia coli* by reducing the timing and reagents and,
122 thus, improving the efficiency of the process. His-tagged protein is expressed in bacteria
123 as inclusion bodies and refolded and purified in a single step by using Immobilised
124 Metal Affinity Chromatography (IMAC). Our method shows a reduction in total
125 process time of up to 500% and decreases the cost of up to 150% in comparison with
126 refolding by dilution, the most common protocol to purify ICAM-1 in *E. coli* [9]. The
127 purity of ICAM-1 is greater than 95% and the yield is 3 mg/liter of bacterial culture.
128 Moreover, the activity of ICAM-1 has been proven by using biochemical, biophysical
129 and cell biology models.

130

131 **Materials and methods**

132 *Plasmids and bacterial strains*

133 Two synthetic cDNAs corresponding to the first two domains (D₁D₂) of human ICAM1
134 or D₁D₂ linked to human IgG1 Fc region cloned into pET28a(+) plasmid at NdeI/EcoRI
135 sites (Figure 1) were purchased from GenScript (Piscataway, NJ, USA). Plasmids were
136 transformed into chemically competent BL21 *Escherichia coli* strain BL21 CodonPlus
137 (Novagen) for high-level protein expression. The proteins expressed by using these
138 constructs contain a 6 histidine-tag at the N-terminal site.

139

140 *Expression and Purification of D₁D₂Fc and D₁D₂*

141 Bacterial cultures containing pET28a-D₁D₂Fc or pET28a-D₁D₂ were grown at 37°C
142 until the optical density (OD) at 600 nm reached 0.6-0.8. Subsequently, protein
143 expression was induced by adding isopropyl-beta-D-1-galactopyranoside (IPTG) to a
144 final concentration of 1mM for 3 h at 37°C. After protein induction cells were recovered
145 by centrifugation at 17,700 g for 8 min at 4°C. Proteins were solubilised from inclusion
146 bodies by resuspending the cell pellets in 6 M guanidine hydrochloride (GdmCl) at
147 room temperature with gently shaking overnight. Subsequently, cell suspension was
148 centrifuged at 48,400 g for 30 min at 4°C to pellet cellular debris. Supernatants
149 containing the soluble proteins were then recovered and incubated for 1 hour at room
150 temperature with Ni-NTA resin (Qiagen, Hilden, Germany) to allow the polyhistidine
151 tag to bind to the resin. After centrifugation at 157 g for 2 min, most of the supernatant
152 was carefully removed and then resin was resuspended in the remaining supernatant,
153 transferred to a gravity-flow column and allowed to settle.

154 On-column renaturation and purification was performed by several changes of buffers
155 following a modified protocol based on that described by Oganessian [47]. First, the
156 column was washed using the denaturing buffer containing 20mM imidazole to remove
157 non-specifically-bound contaminants and mercaptoethanol 10mM to reduce disulfide
158 bonds. Then, renaturation was carried on using buffer A (20mM Tris-HCl pH 8.0, 0.1M
159 NaCl). In the first step, the column was washed with 10-column volumes (CV) of buffer
160 A containing 0.1% Triton X-100 and mercaptoethanol 10mM. Next, resin was washed
161 with 10 CV of buffer A containing 5mM Methyl-β-cyclodextrin (Sigma, St.Louis,
162 Missouri, USA) to remove detergent from the protein-detergent complex and to allow
163 the protein to refold. The last wash before elution was performed with 20mM Tris-HCl
164 pH 8.0, 0.5M NaCl to remove remaining impurities and Methyl-β-cyclodextrin. All

steps were performed in the presence of a cocktail of protease inhibitors (Roche, Basel, Switzerland). Refolded protein was eluted with buffer A containing 1 M imidazole and protein concentration was measured using a spectrophotometer (NanoView, Healthcare, Waukesha, WI, USA). Fractions containing the highest levels of protein were passed through a desalting column (PD-10 Desalting Columns, GE Healthcare, Waukesha, WI, USA) following manufacturer's instructions and the protein was recovered in phosphate buffered saline (PBS). Protein concentration was adjusted to 500µg/ml by ultrafiltration using 10,000 MWCO Centrifugal Filter Units (Amicon, Millipore, Billerica, MA, USA) and stored at -20°C. The level of protein expression after IPTG induction and the quality of purified proteins was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and staining with Coomassie Blue.

Western blot analysis

Purified D₁D₂Fc and D₁D₂ was analysed by western blotting under reducing conditions by using a mouse mAb against human CD54/ICAM1 (clone 28/CD54; BD bioscience, San Jose, CA, USA; dilution 1:1000) or a mouse mAb against HisTag (Novagen, Madison, WI, USA; dilution 1:1000). Then, the blot was stained with peroxidase-conjugated secondary antibodies, rabbit anti-mouse (Amersham, Piscataway, NJ, USA; dilution 1:10000). Subsequently, a peroxidase substrate for enhanced chemiluminescence (ECL) from Pierce (Rockford, IL, USA) was used for detection.

Circular dichroism spectroscopy

Secondary structure of proteins was examined using circular dichroism (CD) in a Chirascan spectropolarimeter (Applied Photophysics). Spectra were recorded in the far ultraviolet region (200nm-250nm) with a protein concentration of 6µM in PBS (Phosphate buffered saline) and a scan rate of 1 nm/s. The temperature was controlled by a Peltier controller. It was employed a quartz cuvette 0.1cm path length.

Fluorescence Spectroscopy

Protein samples were characterized by fluorescence spectroscopy using a Cary Eclipse spectrofluorometer (Varian, Palo Alto, CA, USA) thermostated by a Peltier temperature control unit, employing either the fluorescence emission from the internal tryptophan residues or from the ANS (8-anilino-1-naphthalenesulfonic acid) extrinsic probe. When

convenient, the fluorescence intensity at a certain wavelength, I_λ , or the average energy of the spectrum:

201

$$\text{Average Energy} = \frac{\sum I_i E_i}{\sum I_i}$$

203

was considered as the reporting signal. Protein samples were excited at 290 nm and 390 nm when tryptophan and ANS spectra were recorded, respectively, with a slit width of 5 nm.

Thermal unfolding assays were conducted in order to assess the structural stability of the proteins. An unfolding model considering an intermediate partially folded state was employed:

210

$$S(T) = F_N(T)I_N(T) + F_I(T)I_I(T) + F_U(T)I_U(T)$$

212

where T is the absolute temperature; $S(T)$ is either the fluorescence intensity at a certain wavelength or the average energy of the spectrum; I_N , I_I and I_U are the intrinsic contributions to the fluorescence of each conformational state, that are assumed to exhibit a linear dependency with the temperature:

217

$$I_X(T) = a_X + b_X T$$

219

and $F_N(T)$, $F_I(T)$ and $F_U(T)$ are the fraction population of each conformational state (native, intermediate and unfolded) and can be expressed in terms of the equilibrium constants associated with each conformational transition, $K_1(T)$ and $K_2(T)$:

223

$$F_N(T) = \frac{1}{1 + K_1(T) + K_1(T)K_2(T)}$$

$$F_I(T) = \frac{K_1(T)}{1 + K_1(T) + K_1(T)K_2(T)}$$

$$F_U(T) = \frac{K_1(T)K_2(T)}{1 + K_1(T) + K_1(T)K_2(T)}$$

225

The equilibrium constants are also temperature dependent:

227

$$K_i(T) = \exp\left\{-\frac{H_i}{RT} + \frac{S_i}{R} - \frac{C_{pi}}{T} + \ln\left(\frac{T}{T_{mi}}\right)\right\}$$

229

230 where R is the ideal gas constant, T_{mi} is the unfolding (or mid-transition) temperature,
 231 ΔH_i is the unfolding enthalpy, and ΔC_{Pi} is the unfolding heat capacity for each transition
 232 i. Non-linear square regression data analysis implemented in Origin (OriginLab)
 233 provides the unfolding parameters (T_{mi} , ΔH_i , and ΔC_{Pi}) for both proteins, D_1D_2Fc and
 234 D_1D_2 .

235

236 *Isothermal titration calorimetry*

237 The ability of D_1D_2Fc to interact with a peptide derived from its natural ligand LFA-1
 238 was analysed by isothermal titration calorimetry (ITC) on a VP-ITC calorimeter
 239 (MicroCal) at 25 °C. The peptide sequence was DSGNIDAAKD corresponding to
 240 aminoacids 244-253 from LFA-1 [51]. 2.2 ml of a 12 μ M solution of D_1D_2Fc and 0.6 ml
 241 of a 150 mM solution of peptide were prepared in PBS and degassed with a vacuum
 242 pump for 10 min. Protein solution was carefully injected into the cell previously washed
 243 with PBS. Each assay consisted of a series of 28 injections of peptide solution of 10 μ l
 244 each (with a 4- μ l first onjection) at 400 s intervals under constant stirring (459 rpm).
 245 The thermal power required to keep the cell at a constant temperature is measured, so
 246 that it provides the heat associated with each ligand injection after integrating the signal
 247 over time. The thermodynamic parameters of protein-peptide interactions (affinity,
 248 enthalpy and entropy changes) as well as the stoichiometry were estimated by using
 249 nonlinear regression analysis. Data were analyzed using the software developed and
 250 implemented in Origin 7.0 (OriginLab).

251

252 *Cell Adhesion assay*

253 The ability of ICAM1 chimera (D_1D_2Fc) to induce cell adhesion of non-adherent cells
 254 expressing LFA-1 was analysed by using the EBV transformed human B
 255 lymphoblastoid cell line R69, a generous gift from José A. López de Castro [52].
 256 Adhesion assay was performed in flat bottom 96-well plates in which different amounts
 257 of ICAM-1 chimera or human IgG1 (Fc control) had been previously immobilized for
 258 18h at 4°C. R69 human cells were washed twice in DMEM medium supplemented with
 259 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (penicillin 100 U/ml,
 260 streptomycin 100 μ g/ml) and resuspended at a final concentration of 10^6 cells/ml. Then,
 261 LFA-1 expression was induced by incubating cells with 10 ng/ml PMA for 2 hours at
 262 37°C [53]. Subsequently, 1×10^5 cells were added to each well and incubated at 37°C for

263 4 hours. To quantify the number of adherent cells a modification of the MTT
 264 colorimetric test designed by Mosmann [54] was used.

265

266 *Flow cytometry (FACS)*

267 The ability of D₁D₂Fc to quantify the expression of LFA-1 on cells was analysed by
 268 flow cytometry in the human T leukemia cell line Jurkat (clon E6-1, ATCC). Jurkat
 269 cells were incubated with different concentrations of D₁D₂Fc or human IgG1 control at
 270 4°C for 30 min, washed twice with FACS buffer (PBS, 5% FCS, 0,1% NaN₃) and then
 271 incubated with PE-conjugated goat anti-human IgG (Fcγ fragment specific; Jackson
 272 ImmunoResearch). After a washing step with FACS buffer, cells were analysed by
 273 FACS using a FACSCalibur with CellQuest Pro software (BD).

274

275 *Cytotoxicity assay*

276 The effect of D₁D₂Fc and D₁D₂ on ICAM-1/LFA-1 dependent cell-cell contact was
 277 analysed by performing a cell cytotoxicity assay using human primary NK cells and the
 278 NK-cell sensitive human leukemia K562. Primary human NK cells were generated by
 279 culturing human peripheral blood mononuclear cells (PBMCs) with mitomycin C
 280 inactivated R69 cells for 5 days as previously described [55]. Then, NK cell fraction
 281 was enriched by MACS using anti-CD56 antibodies as previously described [56]. Cell
 282 cytotoxicity induced by NK cells on K562 was analyzed by flow cytometry as
 283 previously described [56]. Briefly, NK cells were preincubated with medium alone or in
 284 the presence of different amounts of D₁D₂Fc , D₁D₂ or human IgG1 control for 15 min
 285 at 37°C. Then, they were added to K562 cells at different e:t cell ratio for 4h at 37°C,
 286 5% CO₂. Subsequently, phosphatidylserine exposure and AAD uptake were analyzed
 287 by FACS using the annexin-V/AAD kit from BD Pharmingen.

288 **Results and discussion**

289

290 *Expression of recombinant proteins*

291 First we have analysed that *E. coli* BL21-Codon plus cells transformed with pET28a-
 292 D₁D₂Fc or pET28a-D₁D₂ overexpressed the respective proteins after induction with
 293 IPTG. As shown in Figure 2A, the expression of two proteins was clearly increased
 294 after 2 and 3 hours of induction. The approximate MW of these proteins was 50 kDa
 295 (lanes 2 and 3) and 25 kDa (lanes 5 and 6), which matched well with the theoretical
 296 MWs of D₁D₂Fc and D₁D₂, respectively. In contrast, non-induced cells did not express
 297 the proteins (lanes 1 and 4). The highest level of expression was found at 3 hours and
 298 longer induction times (4 and 24h) did not increase that level. Thus, we chose this time-
 299 point in the subsequent experiments.

300

301 *Purification of recombinant proteins*

302 Ni-NTA affinity chromatography was applied for the purification of D₁D₂ and D₁D₂Fc
 303 poly-histidine tagged proteins. Poly-histidine tags form high affinity complexes with
 304 immobilized divalent metal ions (such as Ni²⁺ or Co²⁺), even in the presence of high
 305 concentrations of chaotropic agents (e.g., urea or GdmCl), thereby allowing isolation of
 306 tagged protein from a crude cellular extract [36, 37, 57]. In addition, these properties
 307 allow to refold denatured proteins by changing the buffer composition flowing through
 308 the column [40, 47-50]. Most of D₁D₂Fc and D₁D₂ exist in the bacterial cells in the form
 309 of inclusion bodies that were solubilized by resuspending bacterial pellets in 6M
 310 GdmCl. Soluble proteins were incubated with the Ni-NTA resin slurry to allow binding
 311 of the poly-histidine-tagged proteins and folded as indicated in methodological section.
 312 As shown in Figure 2B, non-bound proteins were washed out during the first two steps
 313 of purification (lanes 3 and 4). These fractions also contained some D₁D₂Fc protein,
 314 although at very low levels. Unbound proteins were not detected anymore in fractions
 315 corresponding to the folding steps (lanes 5 and 6). After folding the bound proteins,
 316 they were eluted by using imidazole. Most of D₁D₂Fc protein eluted in the early
 317 fractions (Figure 2B, lanes 8-12) which contained a small amount of proteins of lower
 318 MW, suggesting that either contaminating proteins co-eluted with D₁D₂Fc or some
 319 protein degradation occurred during the purification process. In order to confirm the
 320 identity of the purified protein and of the low MW forms, we performed immunoblot
 321 using mAbs specific for ICAM-1 (Figure 2C, lane 1) or for the poly-histidine tag

(Figure 2C, lane 2). As shown in the blots a single band corresponding to D₁D₂Fc was detected by using anti-ICAM-1 mAb confirming the identity of the purified protein. In contrast, several bands were detected with the anti-HisTag antibody. The pattern of bands detected by this mAb matched those observed by Coomassie Blue staining (Figure 2B), confirming that the low MW proteins contains a poly-histidine tag and, thus, correspond to short forms of our fusion protein. Since poly-histidine tag is placed at the N-terminal site, this result indicates that degradation of the protein occurs at the C-terminal site where the Fc fraction is located. Moreover, it suggests that the epitope recognised by the anti-ICAM-1 mAb is missed in the degraded proteins. Anyway, the level of degradation is minimal since the intensity of the 50 KDa band corresponding to D₁D₂Fc was much stronger than that of the lower bands.

Similar results were found when D₁D₂ protein was purified (Figure 2D and 2E). However, no degradation products were observed in the purified fraction, confirming that the residual degradation of the D₁D₂Fc chimera occurred by the Fc fraction. This result is not surprising since it is known that Fc regions are especially susceptible to the action of specific proteases like pepsin-like proteases. However, this effect has been minimised by including general protease inhibitors during the purification process.

Altogether, the results indicate that the purified protein corresponds to the recombinant ICAM-1 D₁D₂Fc chimera and degradation is hardly observed. The yield obtained by using this protocol is around 3 mg per liter of bacterial culture and the entire process took just 72 hours from bacterial cultures to purified protein. Of note, the purification of other recombinant proteins using refolding by dilution takes about 14 days in our laboratory.

345

346 *Circular dichroism*

347 Next we have analysed by circular dichroism (CD) whether purified ICAM-1 D₁D₂Fc
348 chimera is properly folded and presents the expected secondary structure. ICAM-1
349 belongs to the IgSF and each of its domains presents a typical Ig-like structure
350 characterised by the presence of β -sheets [58]. Dichroism spectra in the far ultraviolet
351 region are primarily due to the amide bonds linking the amino acid residues. The
352 asymmetry of these chromophores is due to the spatial arrangement of the main chain of
353 the protein, thus, the circular dichroism signals can be interpreted in terms of the
354 content of secondary structure present, i.e. the percentage of residues found in some
355 structural conformation (α helix, β sheets, turns and other structural types).

356 As shown in Figure 3A the CD spectrum of D₁D₂Fc shows a single broad negative
 357 ellipticity centered at approximately 205 nm. This result indicates that the protein
 358 predominately contains β sheets or turns as secondary structures and, thus, that the
 359 folding protocol renders a protein with the expected secondary structure.

360

361 *Fluorescence spectroscopy*

362 Both D₁D₂Fc and D₁D₂ show a fluorescence spectrum with two maxima around 330 nm
 363 and 375 nm, in agreement with these two proteins containing several tryptophan
 364 residues (Figure 3B, i).

365 In order to test the structural integrity of the proteins, fluorescence thermal
 366 denaturations were performed (Figures 3B, ii-vi). Unfolding parameters for D₁D₂Fc
 367 following the thermal denaturation either by intrinsic tryptophan fluorescence or
 368 extrinsic ANS fluorescence provided similar results (Figures 3B, ii and iv, Table 1).
 369 However, for D₁D₂ only ANS fluorescence data showed a clear unfolding curve (Figure
 370 3B, vi). Two transitions can be clearly observed for the unfolding behavior of D₁D₂Fc,
 371 whereas for D₁D₂ was not so evident. However, analysis of D₁D₂ unfolding employing a
 372 model considering a single transition provided illogical results (e.g. negative unfolding
 373 enthalpy, which is impossible for a temperature-driven process). Using the average
 374 energy of the spectrum presents two advantages: 1) signal is concentration-independent;
 375 and 2) global changes in the spectrum are taken into account, that could be overlooked
 376 when just paying attention to a certain wavelength. Because both proteins, D₁D₂Fc and
 377 D₁D₂, contain several tryptophan residues, interpretation of the results obtained when
 378 the intrinsic tryptophan fluorescence was employed as a reporter signal was somewhat
 379 difficult.

380 According to the data analysis, D₁D₂Fc showed two transitions with T_m 's at 40°C and
 381 70°C, and unfolding enthalpies of 20 and 50 kcal/mol, respectively. Deletion of the Fc
 382 domain leads to a slight stabilization of the less stable region and a destabilization of the
 383 more stable region (Table 1).

384

385 *Isothermal titration calorimetry*

386 CD and thermal denaturation data indicate that ICAM-1 chimera presents a proper
 387 secondary and tertiary structure folding. However, this does not mean that the protein is
 388 functionally active and able to recognise its natural ligands. To test D₁D₂Fc activity we

389 have analysed the interaction of this protein with a peptide derived from its natural
390 ligand LFA-1 (DSGNIDAAKD) by isothermal titration calorimetry.

391 As shown in Figure 4 this peptide presents a high affinity for D₁D₂Fc (dissociation
392 constant K_d of 0.090 μ M) as expected since its sequence is derived from the integrin I
393 domain contained in the α subunit of LFA-1, the principal binding site for D1 of ICAM-
394 1. This result agrees with previous findings showing that this peptide inhibits the
395 homotypic T cell adhesion mediated by ICAM-1/LFA-1 interaction [51]. In addition, it
396 indicates that D₁D₂Fc is fully functional in cell-free models and able to bind a peptide
397 derived from LFA-1.

398 Moreover, our data provide for first time the dissociation constant for ICAM-1 and a
399 peptide agonist. The value obtained (90 nM) is lower than that previously found for the
400 interaction between soluble monomeric ICAM-1 and the high affinity form of its natural
401 ligand LFA-1 (K_d : 500 nM) [59] and higher than that found for dimeric ICAM-1 (K_d : 1-
402 10 nM) [15, 16]. This result provides the biophysical explanation to the capacity of the
403 peptide to block lymphocyte intercellular adhesion as previously reported [51]. In
404 addition, it may help to design novel peptide inhibitors to target this interaction and
405 modulate the immune response [60].

406

407 *Binding of ICAM-1 chimera to B and T cell-associated LFA-1*

408 ITC experiments indicate that D₁D₂Fc contains the proper folded domains involved
409 in the interaction with a specific sequence derived from integrin I domain of LFA-1 α .
410 However, short peptides are linear aminoacid sequences that do not completely mimic
411 the interaction among proteins containing a three-dimensional spatial structure. Thus, to
412 prove that D₁D₂Fc is active and useful to analyse processes in which ICAM-1/LFA-1
413 interaction is involved, we have analysed the ability of the purified protein to bind the
414 native form of LFA-1 expressed on the plasma membrane of lymphoid cells. To this
415 aim we tested the ability of immobilised D₁D₂Fc to adhere LFA-1 expressing B cells
416 and the interaction of D₁D₂Fc and LFA-1 in T cells by flow cytometry (Figure 5).

417 As showed in Figure 5A, D₁D₂Fc is able to mediate the adhesion of activated B
418 lymphocytes showing the highest activity at a concentration of 50 μ g/well. Cells
419 incubated with medium alone or in wells coated with IgG1 show extensive clumping
420 and no adherent cells are seen. In contrast, cell clumping is much more reduced in wells
421 coated with D₁D₂Fc and several cells with fibroblast-like morphology can be detected.

422 The quantification of cell adhesion results in a 10-fold increase related to the negative
423 IgG control.

424 The activity of this chimera to promote B cell adhesion was lower than that found
425 by using a chimera expressing the 5 domains of ICAM-1 (data not shown). It has been
426 previously reported that D1 presence on a single molecule of ICAM-1 is enough to bind
427 LFA-1 [17] [18]. However, the presence of the 5 domains of ICAM-1 provides a better
428 adhesion as previously suggested [15, 16]. Another explanation could be that the B cells
429 used in this assay expresses Mac-1 that would bind through the D3 domain absent in
430 D₁D₂Fc.

431 Next, we tested if this chimera was also useful to analyse the expression of LFA-1
432 in the cell membrane by flow cytometry. To this aim we have used the human T
433 leukemia Jurkat. As shown in Figure 5B the percentage of positive cells increases as it
434 increases the amount of chimera added. The performance of this chimera (1 µg/10⁵
435 cells) to recognise its specific ligand (LFA-1) is similar to that of other chimeras
436 expressed and purified in native form in eukaryotic cells and used to analyse specific
437 ligands in Jurkat cells like NKG2D-Fc (data not shown). Incubation with an antiCD54
438 (ICAM-1) antibody blocked binding of the chimera (data not shown) showing that
439 D₁D₂Fc is able to specifically bind to Jurkat cells through its natural ligand LFA-1.

440 As indicated above this result is supported by previous findings indicating that the
441 presence of D₁ is enough to bind LFA-1.

442

443 *Blocking of NK-cell mediated cytotoxicity*

444 The interaction of ICAM-1, expressed on tumor or virus infected cells, with LFA-1,
445 expressed on the membrane of Natural Killer (NK) cells, modulates the formation and
446 signalling of the NK immunological synapse [61]. This process is critically involved in
447 the elimination of tumor and/or virus-infected cells by NK cells [62]. In order to analyse
448 whether D₁D₂Fc was able to interfere with the cytotoxic function of NK cells, we have
449 tested the ability of this chimera to inhibit the elimination of tumor cells by human NK
450 cells. As can be seen in Figure 6 incubation with increasing amounts of D₁D₂Fc
451 completely blocked cell death induced by human NK wells on the human leukemia
452 K562. Control human IgG1 had no effect on this process (data not shown). Importantly,
453 inhibition was not due to the presence of the Fc region, which is known to bind and
454 activate specific receptors in NK cells, since purified D₁D₂ showed a similar activity.
455 Similar results were found by using the leukemic target cells Raji, Jurkat or U937 (data

not shown). These results indicate that this chimera can also be used to study the processes in which NK cells maybe involved. Moreover, it indicates a good biological activity of the chimera since 100 pg of protein is able to completely block cell death exerted by one NK cell.

460

461 **Conclusions**

462 To our knowledge, this is the first report on the purification of an active form of
463 ICAM-1 by using a simple and fast bacterial-based method more advantageous than
464 mammalian models, which are very expensive and often difficult to set-up, or than other
465 traditional folding protocols, which are time consuming and require large amounts of
466 reagent such a refolding by dilution. In comparison with refolding by dilution, the most
467 common protocol to purify ICAM-1 in *E. coli* [9], our method shows a reduction in total
468 process time of up to 500% and decreases the cost of up to 150%. If we compare it with
469 mammalian models like HEK or COS cells, the reduction in time and money is much
470 higher. These chimeras are of special interest to study cell adhesion assays, expression
471 of functional ligands on cells by flow cytometry, blocking cell-cell adhesion mediated
472 processes as well as quantification of soluble ligands by ELISA. Our protocol has
473 proven useful in order to analyse several physiological processes in which ICAM-
474 1/LFA-1 interaction is critically involved. Moreover, it is fast and cheap, providing a
475 perfect platform in order to develop large-scale inhibitor screenings or *in vivo* models of
476 ICAM-1 associated pathologies, which are hard to set-up by using commercial purified
477 proteins or other expression systems.

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489 purification protocol.

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Table 1. Thermodynamic parameters for the unfolding of D1D2Fc and D1D2 obtained by following the thermal unfolding by intrinsic tryptophan or ANS fluorescence.

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	T_{m1} (°C)	ΔH_1 (kcal/mol)	T_{m2} (°C)	ΔH_2 (kcal/mol)
D1D2Fc (Trp)	39.1 ± 0.2	20.0 ± 0.3	69 ± 1	51 ± 1
D1D2Fc (ANS)	40.0 ± 0.4	19.5 ± 0.4	70 ± 1	49 ± 1
D1D2 (ANS)	46 ± 1	23 ± 1	65 ± 1	27 ± 1

496 **Figure legends**

497 **Figure 1.** Schematic representation of the aminoacid sequences of the proteins
 498 expressed by using the constructs pET28a-D₁D₂Fc and pET28a-D₁D₂. Numbers indicate
 499 the corresponding aminoacids in human ICAM-1 sequence. Histidine tag (H), domain 1
 500 (D₁), domain 2 (D₂), Linker (L), human IgG1 Fc region (Fc).

501 **Figure 2. Analyses of the expression and purification of D₁D₂Fc D₁D₂ in *E. coli* by**
 502 **SDS-PAGE electrophoresis and western blot.** Bacteria were transformed by using
 503 pET28a-D₁D₂Fc or pET28a-D₁D₂ constructions and proteins were expressed and
 504 purified as described in materials and methods. **A** analysis of protein overexpression in
 505 bacteria by Coomassie Blue staining after SDS-PAGE. M: MW markers; 1,4: non-
 506 induced transformed bacteria; 2, 3, 5, 6: bacteria induced with IPTG for 2h (2 and 5) or
 507 3h (3 and 6). **B and D**, analysis of protein refolding and purification by Coomassie Blue
 508 staining after SDS-PAGE. M: MW markers; 1: induction with IPTG; 2-4 non-bound
 509 proteins; 5-6: folding steps; 7-15: elution with Imidazole. **C and E**, purified proteins
 510 were separated by SDS-PAGE and analysed by western blot using antibodies specific
 511 against ICAM-1 (lane 1) or poly-histidine tag (lane 2).

512 **Figure 3. Spectroscopic structural characterization.** **A**, Circular Dichroism spectra of
 513 D₁D₂Fc recorded in the far ultraviolet region (200nm-250nm). **B**, Thermal denaturation.
 514 i) Tryptophan fluorescence emission spectra of D₁D₂Fc (protein 2 μ M in PBS buffer).
 515 Inset: Tryptophan fluorescence emission spectra of D₁D₂ under the same conditions; ii),
 516 Thermal unfolding of D₁D₂Fc followed by tryptophan intrinsic fluorescence emission at
 517 330 nm. The temperature dependence of the fluorescence intensity at 330 nm was fitted
 518 to a 2-transition unfolding model; iii) ANS fluorescence emission spectra of D₁D₂Fc
 519 (protein 2 μ M and ANS 100 μ M in PBS buffer); iv) Thermal unfolding of D₁D₂Fc
 520 followed by ANS fluorescence. The temperature dependence of the fluorescence
 521 average energy of the ANS spectrum was fitted to a 2-transition unfolding model. A
 522 similar result was obtained when the temperature dependence of the ANS fluorescence
 523 emission at 470 nm was fitted to a 2-transition unfolding model; v) ANS fluorescence
 524 emission spectra of D₁D₂. Protein 2 μ M and ANS 100 μ M in PBS buffer; vi) Thermal
 525 unfolding of D₁D₂ followed by ANS fluorescence. The temperature dependence of the
 526 fluorescence average energy of the ANS spectrum was fitted to a 2-transition unfolding
 527 model. A similar result was obtained when the temperature dependence of the ANS
 528 fluorescence emission at 470 nm was fitted to a 2-transition unfolding model.

529 **Figure 4. Interaction of LFA-1 peptide with D₁D₂Fc by isothermal titration**
 530 **calorimetry (ITC).** Purified D₁D₂Fc (12 μ M) was titrated with a peptide derived from
 531 the binding site of LFA-1 (150 μ M). The assay was performed in PBS buffer at 25°C.
 532 The non-linear regression analysis provided a dissociation constant of 91 nM.

533 **Figure 5. Binding of D₁D₂Fc to cell-associated LFA-1.** **A,** B cell adhesion assay.
 534 Different amounts of D₁D₂Fc or human IgG1 control were immobilised in 96 well
 535 plates o.n. at 4°C. After washing out non-bound protein, the PMA-activated R69 B cell
 536 line was added by triplicates and cell adhesion was analysed by MTT as described in
 537 materials and methods. Quantification of cell binding was represented as fold increase
 538 with respect to the human IgG1 control. **B,** analysis of D₁D₂Fc binding to LFA-1 in
 539 Jurkat cells by flow cytometry. Different amounts of D₁D₂Fc or human IgG1 control
 540 were incubated with Jurkat cells. After washing out non non-bound proteins cells were
 541 incubated with PE-conjugated goat anti-human IgG Fc γ Ab and analysed by flow
 542 cytometry. A representative histogram is shown. Numbers correspond to the % of cells
 543 as gated by the vertical bars. Black: goat anti-human IgG; Grey: human IgG1; Colour:
 544 D₁D₂Fc (red: 5 μ g; green: 10 μ g; blue: 15 μ g). Values in the graph are represented as
 545 mean $\pm$ SEM of 3 independent experiments performed by duplicate. Statistical analyses
 546 were done with two-way ANOVA with Bonferroni post-test by comparing IgG with
 547 D₁D₂Fc. ns: not statistically significant; *** p<0.001.

548 **Figure 6. D₁D₂Fc inhibits NK-cell mediated cytotoxicity on tumor cells.** MACS-
 549 enriched human NK cells, previously stained with the fluorescence dye CFSE, were
 550 incubated with K562 cells at 1:1 effector:target ratio (1×10^5 cells) for 4h in the presence
 551 or absence of different amounts of D₁D₂Fc, D₁D₂ or human IgG1 control. Subsequently,
 552 cell death was analysed by measuring PS translocation by flow cytometry in the CFSE
 553 negative cell population (K562) as described in materials and methods. Values are
 554 represented as mean $\pm$ SEM of 2 different experiments. Statistical analyses were done
 555 with two-way ANOVA with Bonferroni post-test by comparing IgG, D₁D₂ and D₁D₂Fc
 556 with control. ns: not statistically significant; *** p<0.001.

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- we have established a fast and cost-effective method to produce recombinant human ICAM-1
- ICAM-1 presents a proper folding as revealed by analyses of secondary and tertiary structure
- purified ICAM-1 is highly active and proves useful for biochemical, immunological and cell biology studies.
- ICAM-1 has been used to calculate the dissociation constant of peptide inhibitors used to treat inflammatory disorders

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Figure 1

Figure 2

Figure 3

A

B

Figure 4

Figure 5

Figure 6