The extradomain A of fibronectin (EDA) combined with poly(I:C) enhances the immune response to HIV-1 p24 protein and the protection against recombinant *Listeria monocytogenes*-Gag infection in the mouse model

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ABSTRACT

The development of effective vaccines against HIV-1 infection constitutes one of the major challenges in viral immunology. One of the protein candidates in vaccination against this virus is p24, since it is a conserved HIV antigen that has cytotoxic and helper T cell epitopes as well as B cell epitopes that may jointly confer enhanced protection against infection when used in immunization-challenge approaches. In this context, the adjuvant effect of EDA (used as EDAP24 fusion protein) and poly(I:C), as agonists of TLR4 and TLR3, respectively, was assessed in p24 immunizations using a recombinant *Listeria monocytogenes* HIV-1 Gag proteins (Lm-Gag, where p24 is the major antigen) for challenge in mice. Immunization with EDAP24 fusion protein together with poly(I:C) adjuvant induced a specific p24 IFN- γ production (Th1 profile) as well as protection against a Lm-Gag challenge, suggesting an additive or synergistic effect between both adjuvants. The combination of EDA (as a fusion protein with the antigen, which may favor antigen targeting to dendritic cells through TLR4) and poly(I:C) could thus be a good adjuvant candidate to enhance the immune response against HIV-1 proteins and its use may open new ways in vaccine investigations on this virus.

1. INTRODUCTION

The worldwide distribution of human immunodeficiency virus type 1 (HIV-1) infection and associated functional impairment and mortality point out the urgent need for an effective vaccine, which should induce both neutralizing antibodies and a potent cell mediated immunity. This would allow both the neutralization of free virus and the elimination of infected cells [1, 2]. In HIV-1, p24 is the major structural capsid protein encoded by the *gag* gene, which forms the virus core by homodimerization. HIV p24 protein has a variety of CTL/CD8 epitopes (www.hiv.lanl.gov/content/immunology/maps/ctl/p24.html), triggering cytotoxic T-cell immune responses and constituting an important target for cell mediated immunity (CMI) against the virus. It has also highly conserved T helper cell epitopes useful for priming a broadly reactive immune response to HIV-1 [3]. T helper function may enhance the production of neutralizing antibodies directed to envelope proteins [4]. Therefore, the p24 antigen is an attractive candidate for the development of an effective anti-HIV-1 vaccine or therapeutical drug. The p24 protein has also B cell epitopes, leading to the production of antibodies to this protein that are frequently used in lentiviral diagnosis and as markers of disease progression, since asymptomatic seropositive individuals display high p24-specific antibody titers, whereas acute HIV-1 infection and progression is associated with the reduction or absence of p24 specific antibodies [5].

Aluminum salts (Alum) have been approved as the only adjuvant of human application for many decades since the experiments by Glenny and collaborators [6]. However, this adjuvant does not enhance CTL responses [7] and may induce undesirable side-effects. Thus, the development of new adjuvants to enhance cell and antibody mediated immunity when administered with recombinant proteins is crucial to obtain effective vaccines. In this context, pathogen-associated molecular patterns (PAMP) have been confirmed as potent immunomodulators due to their interaction with dendritic cells. Their signaling pathways starts in pattern recognition receptors (PPRs)

1 such as the Toll-like receptors (TLR) specific for each PAMP and ends triggering both,
2 innate and adaptive immune responses [8].
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4 The cellular glycoprotein fibronectin (FN) has multiple isoforms generated from a single
5 gene by alternative splicing of three exons. One of these isoforms is the extra domain
6 A (EDA) presented during embryonic development and produced in response to tissue
7 injury. EDA-containing fibronectin fragments or specific domains of fibronectin are also
8 believed to play important roles in physiological and pathological processes such as
9 wound healing in adults and rheumatoid arthritis [9, 10]. Although information on the
10 biological properties of EDA is limited, it is known that EDA induces NF- κ B factor
11 activation, which is involved in connective tissue destruction, tissue remodeling in
12 response to chronic inflammatory lesion development, and in monocyte and dendritic
13 cell migration throughout their basal membrane. These EDA associated effects are
14 triggered in response to the interaction between EDA and its specific TLR-4, a receptor
15 that also interacts with LPS of Gram negative bacteria [11, 12]. Altogether, these
16 observations suggest that the expression of EDA in response to tissue injury, or other
17 cellular warning signals, might induce recruitment of dendritic cells to the site of injury
18 and trigger their subsequent maturation. Indeed, previous work demonstrates that
19 EDA, as fusion protein, may constitute a strategy to target viral or tumoral antigens to
20 dendritic cells *in vivo* [11, 13, 14]. Therefore, the capacity of EDA to induce dendritic
21 cell maturation through TLR4 activation may also favor antigen uptake, expression of
22 co-stimulatory signals, antigen presentation and the induction of anti-viral or anti-
23 tumoral T cell responses [11, 13].
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48 Double stranded RNA is regarded as a PAMP and may be found in some RNA viruses
49 and also in dsRNA intermediates often produced during viral replication, activating the
50 immune system. Poly(I:C), a synthetic dsRNA that mimics the effects of naturally
51 occurring dsRNA, is thus frequently used as an adjuvant in both, antitumor treatment
52 and vaccine development, mainly because of its effects activating CD8 T cells and NK
53 cells. Poly(I:C) binds TLR3 and this interaction is thought to be crucial for driving cell-
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mediated immune responses. A synergistic activity between different TLR agonists may trigger more vigorous T cell responses [15, 16].

On the basis of the characteristics and immunogenic properties of HIV-1 p24 and adjuvant characteristics of EDA and poly(I:C), this work aims to determine whether EDA and poly(I:C) could be used as HIV p24 immunopotentiators.

2. MATERIALS AND METHODS

2.1. Production and purification of EDAP24 and p24 recombinant proteins

Plasmid pET20b-EDA [11] was used for the construction pET20b-EDAp24, which expressed a fusion protein containing HIV-1 p24 linked to the carboxy-terminus of EDA and carrying six histidines at the carboxy-terminus. Plasmid pET20b-p24 expressing p24 alone and linked to a six-histidine tag at the carboxy-terminus was constructed using conventional cloning techniques and a plasmid (kindly provided by Prof. I. Jones, University of Reading, UK) containing HIV-1 p24 gene as template. The resulting PCR product was subcloned in pET20b commercial plasmid (Novagen). Plasmids pET20b-EDAp24 and pET20b-p24 were sequenced and then transfected into BL21(DE3) cells (Amersham Pharmacia Biosciences) for the expression of the corresponding recombinant proteins.

EDAp24 was expressed using an auto-inducible medium, as previously described [17] except for the bacteria disruption by sonication and incubation with lysozyme, anti-metalloproteases, Triton, DNase and RNase solution. The p24 single recombinant protein was expressed as previously described [18] except for a 6M guanidine hydrochloride treatment to unfold p24 from bacterial inclusion bodies, followed by a refolding by a guanidine hydrochloride decreasing gradient (6-0M) in PBS. Both protein solutions were stored at 4°C until protein purification in a pre-charged nickel-sepharose column (HisTrap™ HP, GE Healthcare). His-tagged recombinant proteins were recovered by a binding buffer with an increasing gradient of imidazole (0-500 mM) and dialyzed for 2 days at 4°C. Additionally, to avoid the presence of eventual bacterial

contaminants, the protein preparations were filtered through a 0.2 µm membrane (Millipore®). Residual endotoxins were removed using Profos EndoTrap® columns (Hyglos GmbH), as previously described [11, 12]. The resulting proteins were stored at -80°C in PBS until use.

2.2. Characterization of protein preparations

After confirming the absence of bacterial contaminants (by plating in agar and incubation of plates at 37°C for 5 days) and endotoxins (by Quantitative Chromogenic Limulus Amebocyte Lysate assay; Cambrex), the protein concentration was determined by the standard Bradford method (Bio-Rad Laboratories Inc.). Purity and size of recombinant proteins were assessed by SDS-PAGE [19] followed by Coomassie Brilliant Blue R-250 staining [20]. Since both proteins carried histidine groups, their electrophoretic profiles were assessed by Western-Blot (WB) with an anti-polyhistidine serum-peroxidase conjugate (1:2,500; Sigma). For individual detection of EDAP24 and p24, WB was performed with a mouse monoclonal antibody anti-p24 (1:250) as primary antibody (NIBSC, WHO-UNAIDS HIV Vaccine Initiative-Health Protection Agency) and an anti-IgG horseradish peroxidase conjugate (1:2,500) as secondary antibody (Nordic Immunological Laboratories). Reactions were developed with diaminobenzidine and hydrogen peroxide. A recombinant EDA protein and a rabbit anti-EDA antibody, used as control in this and subsequent assays, were obtained as previously described [11, 13].

2.3. Evaluation of in vitro biological activity of EDAP24 and p24 proteins

The epitopic conformation of both EDAP24 and p24 was determined by a sandwich ELISA in 96-well microtiter plates (Thermo Scientific Nunc®) coated overnight at 4°C with 1 µg/well of a sheep affinity purified anti-HIV-1 p24 antibody (NIBSC, WHO-UNAIDS HIV Vaccine Initiative-Health Protection Agency) in carbonate-bicarbonate buffer (pH 8.6). Then, the recombinant protein (100 µL/well) and the mouse anti-p24 biotinylated antibody (NIBSC, WHO-UNAIDS HIV Vaccine Initiative-Health Protection Agency) diluted 1:100 were added to plates and plates were incubated (overnight at RT

for the protein, and 2 h at RT for the antibody). Finally, the reaction was developed by adding streptavidine-peroxidase conjugate (1:500) and incubating (2 h at 37°C) with 3,3',5,5'-Tetramethylbenzidine substrate solution. Peroxidase activity was stopped with 2M H₂SO₄ and absorbance measured at 450 nm in a MultiSkan EX apparatus (Labsystem).

2.4. Immunological studies in mice

Mice from Charles River International (France) were accommodated in the animal facilities of the Universidad Pública de Navarra (UPNA; registration code ES/31-2016-000002-CR-SU-US). Mice experiments were performed in compliance with the current European and national regulations, and with the approval of the Comité de Ética, Experimentación Animal y Bioseguridad (CEEAB) of UPNA and Navarra Government. Groups of 8-10 week-old female BALB/c mice (n=8) were intraperitoneally (IP) inoculated with 0.1 mL of the following suspensions prepared in PBS: i) 30 µg of p24; ii) 50 µg of EDAP24 (corresponding to 30 µg p24 and 20 µg EDA); iii) 30 µg of p24 mixed with 50 µg poly (I:C) (Amersham Pharmacia Biosciences); iv) 50 µg of EDAP24 mixed with 50 µg poly (I:C); v) 20 µg EDA mixed with 50 µg poly (I:C); or vi) PBS.

2.4.1. Cellular immune response of interferon gamma

Seven days after immunization, 3 mice of each group were killed, their spleens aseptically removed, and splenocytes stimulated with the AMQMLKETI cytotoxic peptide, encompassing amino acids p24 (75-83) from HIV-1 Gag. This peptide, synthesized using Fmoc-chemistry, had above 90% purity as determined by HPLC. The number of specific interferon gamma (IFN-γ) producing spleen cells was determined by ELISPOT (BD™ ELISPOT, BD Biosciences) and the levels of IFN-γ secreted by splenocytes, by a sandwich ELISA (BD Opteia™ Set Mouse IFN-γ, BD Biosciences), using non-stimulated splenocytes of these mice as controls. In both tests 1×10⁶ splenocytes were used per well and procedures were carried out following the manufacturer's instructions. Spots were counted using an automated ELISPOT reader. Both the number of spots/well and the levels of IFN-γ obtained from splenocytes were

expressed as the mean and SE (n=3) of the individual values obtained after AMQMLKETI peptide stimulation minus those obtained in parallel with non-stimulated splenocytes. Results were graphically represented as box-plots.

2.4.2. Humoral response

Just before challenge (4 weeks after immunization), IgG1 and IgG2a isotype titers were determined by an indirect ELISA in sera of immunized mice (n=5). For this, the remaining 5 mice of each experimental group were bled (by retroorbital plexus puncture, under anaesthesia) and serum samples stored at -20°C until its use. IgGs were quantified in 96-well microtiter plates coated by incubation (overnight at 4°C) with 200 ng/well of p24 protein obtained in the methylotrophic yeast *Pichia pastoris* (NIBSC, WHO-UNAIDS HIV Vaccine Initiative-Health Protection Agency). Serially diluted serum samples were added (100 µL/well) and, after incubation (1 h at 37°C), washing and blocking, an anti-mouse IgG1 or IgG2a horseradish peroxidase conjugated antibody (1:2,000 in blocking buffer) was added and incubated (1 h at 37°C). After a final washing, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution (Millipore®) was added. Following incubation (30 min at RT), the Optical Density (O.D.) was measured at 405 nm in the Multiskan EX apparatus. Serum titer was defined as the reciprocal of the highest serum dilution showing a mean O.D. value equal to or greater than that shown by the negative control sera plus 3 times the SD. Results were expressed as the mean ± SD of individual ratio between log₁₀ IgG2a titer and log₁₀ IgG1 titer.

2.5. Protection studies in mice

Groups of 5 BALB/c female mice (8-10 week-old) were inoculated IP with either p24 or EDAP24 plus poly(I:C) as detailed above (see point 2.4). A group of mice (n=5) inoculated with PBS was used as control. In an additional experiment, the protection conferred by EDAP24 plus poly(I:C) was assessed again with respect to PBS control and also compared with EDAP24 administered alone. Four weeks after immunization, all mice were challenged IP with 1-2×10⁶ CFU/animal of the HIV-1 gag recombinant *Listeria monocytogenes* (Lm-Gag; kindly provided by Dr. Paterson, University of

Pennsylvania, USA) and slaughtered 3 days after challenge, in order to determine the number of bacteria in spleens as previously described [21]. Briefly, spleens were aseptically removed, individually weighed, homogenized (1:10; w/v) and serially diluted in PBS. Spleen suspensions were then plated (0.1 mL by triplicate) in LB agar, and plates incubated for 24 h at 37°C (detection limit ≤ 4 CFU/spleen). Results were expressed as the mean $\pm$ SE (n=5) of CFU/spleen, previous logarithmic conversion to normalize the intra-group variance. The number of animals free of infection in spleens per group was also recorded.

2.6. Statistical analysis

The Kolmogorov-Smirnov test was applied to assess the normal distribution of data obtained in each experiment. Thereafter, means were statistically compared by a one-way ANOVA test, followed by the Fisher's Protected Least Significant Difference (PLSD) test. Kruskal-Wallis and Mann-Whitney tests were used for experiments with non-normal data distribution. The Statview®Graphics 5.0 for Windows (SAS Institute Inc®) statistical package was used in all cases.

3. RESULTS

3.1. Characterization of EDAP24 and p24 recombinant proteins

Knowing the immunostimulatory properties of EDA, we produced a fusion protein EDAP24 with the aim of inducing anti-p24 T cell responses. Both the fusion protein EDA-p24 and p24 were produced, quantified by the Bradford assay and characterized by both SDS-PAGE and WB using anti-histidine antibodies. According to the Bradford assay, preparations of purified EDAP24 and p24 had a total protein concentration of 438 and 389 $\mu\text{g/mL}$, respectively. The Coomassie Brilliant Blue staining (Fig. 1A) and WB membranes using antibodies against histidine tags (Fig. 1B) yielded results compatible with those obtained with p24 and EDA (not shown), revealing the existence of bands corresponding to approximately 39, 26 and 13 kDa proteins, the expected molecular weight of EDAP24, p24 and EDA, respectively (Figs. 1A and 1B). Moreover,

1 the electrophoretic profiles of p24 in WB showed high molecular weight forms, likely
2 corresponding to dimeric and trimeric forms of this recombinant protein (Fig. 1B). This
3 was consistent with the protein aggregates observed in p24 but not in EDAP24
4 preparations, both macroscopically (as a transparent aggregate) and also by Scanning
5 Electron Microscopy (SEM) as polyhedral structures (not shown). Thus, both the
6 electrophoretic and SEM results strongly suggest that the conformation of EDAP24
7 fusion protein differs from that of the p24 protein. All preparations were free of
8 contaminants and endotoxins, as assessed by plating in LB agar and Limulus
9 amebocyte lysate assay, respectively. Finally, the antigenicity and conserved antigen
10 conformation of both EDAP24 and p24 was confirmed (data not shown) by the
11 conformational ELISA sandwich with a mouse anti-p24 monoclonal antibody (described
12 in section 2.3).

26 **3.2. Simultaneous administration of EDA and poly(I:C) enhances the p24 specific** 27 **immune responses**

31 The induction of specific IFN- γ producing cells and secretion of IFN- γ was assessed in
32 the spleens of BALB/c mice immunized with p24 or EDAP24, with or without poly(I:C).
33 After stimulation of splenocytes with the cytotoxic AMQMLKETI peptide, IFN- γ
34 producing cells were quantified by ELISPOT (Fig. 2A) and IFN- γ secretion levels, by
35 ELISA sandwich (Fig. 2B). In both assays, administration of p24 or EDAP24
36 recombinant proteins in the absence of poly(I:C) failed to induce a cellular immune
37 response, as PBS controls. However, poly(I:C) combined with EDA (administered as
38 EDAP24 fusion molecule) induced a significant ($p < 0.05$ vs. PBS, p24 or EDAP24
39 groups) p24 specific IFN- γ cellular immune response, while only a statistical trend
40 ($p = 0.054$) was observed when poly(I:C) was administered with p24 alone. A significant
41 increase in both IFN- γ producing cells ($p \leq 0.028$; Fig. 2A) and IFN- γ secretion levels
42 ($p \leq 0.012$; Fig. 2B), was found in mice immunized with EDAP24 mixed with poly(I:C)

1 compared to those immunized either with p24 plus poly(I:C) or EDAP24 alone,
2 indicating a positive adjuvant effect between EDA and poly(I:C) on IFN- γ production.
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4 Specific antibody levels (IgG1 and IgG2a isotypes) were measured at the time of
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6 challenge by an indirect ELISA test, using a commercial p24 protein produced in the
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8 yeast *Pichia pastoris* expression system. Results showed that a single immunization
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10 with EDAP24 or p24, either alone or combined with poly(I:C), was enough to induce
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12 significant levels of p24-specific IgG1 and IgG2a antibodies in sera of BALB/c mice, in
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14 comparison with control mice inoculated with PBS (Table 1). Besides, the ratio
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16 IgG2a/IgG1 indicated slightly higher increased IgG1 levels relative to IgG2a in all the
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18 p24 immunized mice (Table 1). This ratio was similar in the p24 and EDAP24 groups
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20 where the antigen was administered jointly with poly(I:C) (0.74 ± 0.16 and 0.71 ± 0.10 ,
21
22 respectively). The decrease ($p \leq 0.05$), in IgG2a levels observed in the group immunized
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24 with EDAP24 compared to the remaining immunized groups (including p24 group) may
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26 be due to differences delivering of p24 alone and in the form of EDAP24 fusion protein.
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28 However, this decrease disappeared when poly(I:C) was included to the immunization
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30 mixture, suggesting again the possible advantage of using a combination of both
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32 adjuvants rather than EDA alone (as a fusion protein) to induce antibody production.
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37 **3.3. Simultaneous administration of EDA and poly(I:C) conferred protection** 38 **against Lm-Gag challenge in mice** 39 40 41

42 To determine the potential of EDA and/or poly(I:C) as an adjuvant system, in the
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44 context of HIV-1 p24 vaccination, a protection experiment was conducted in BALB/c
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46 mice (n=5) by administering a single immunization dose with either EDAP24 or p24
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48 recombinant proteins combined with poly(I:C), followed by challenge with Lm-Gag.
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50 Protection was expressed as the mean (n=5) log CFU/spleen of the Lm-Gag challenge
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52 infection in spleens and as the number of mice found with spleens free of infection
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54 (Table 2). All the mice immunized simultaneously with recombinant p24 and poly(I:C)
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56 were infected by Lm-Gag, at the levels of unvaccinated controls (Table 2). However,
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58 the simultaneous immunization with EDAP24 and poly(I:C) prevented mice from
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1 virulent infection significantly ($p < 0.05$) compared to both unvaccinated and p24 plus
2 poly(I:C) immunized mice. In fact, 3 out the 5 mice immunized with EDAP24 combined
3 with poly(I:C) were completely free of infection, whereas most of the unvaccinated mice
4 were found infected at levels above 4 logs of Lm-Gag. In an additional experiment,
5 results of EDAP24 + poly(I:C) and the PBS control were confirmed and also no
6 protection was conferred by EDAP24 in the absence of poly(I:C) (Table 2). Overall,
7 these results indicate that the positive immunostimulatory effect of EDAP24 fusion
8 protein combined with poly(I:C) encompasses a significant effective protection against
9 challenge in mice, and opens a way towards the study and development of a new type
10 of HIV vaccines.

21 4. DISCUSSION

22 The main goal of this work was to investigate the potential interest of EDA and poly(I:C)
23 as immunostimulators in anti-viral vaccine development, using the HIV-1 protein p24 as
24 antigen. This protein is one of the main Gag molecules reported in the study of HIV
25 vaccines. HIV Gag-specific T-cell responses appear to dominate in rectal mucosa of
26 HIV controllers and, particularly, CD8+ T-cells from HIV-1-infected individuals may
27 target a single immunodominant Gag p24 determinant (reviewed by [22]). Thus, the
28 enhancement of immune responses to p24 is an important challenge towards the
29 development of efficient multicomponent anti-HIV compounds. A number of adjuvants
30 have been investigated in order to enhance the immune response to HIV p24 but, to
31 our knowledge, few strategies based on protein-adjuvant fusion molecules have been
32 explored. Examples of adjuvants used as fusion molecules are those based on cholera
33 toxin subunit B [23], hepatitis B core antigen [24], hsp70 from *Mycobacterium*
34 *tuberculosis* [25] and Ricin subunit B [26]. In previous work, we demonstrated the
35 adjuvant effect of EDA when combined with the SIINFEKL ovalbumin peptide [11], NS3
36 hepatitis C virus peptide [13] or Human Papillomavirus E7 protein [14]. We have also
37 observed that EDA exerts its adjuvant effect more efficiently when delivered not far

1 apart but in close contact with the antigen (San Román et al., submitted for
2 publication). Accordingly, the fusion between EDA and p24 would be expected to
3 enhance the interaction of p24 with dendritic cells and the antigen-specific immune
4 response via TLR4 stimulation. Unlike other adjuvants, EDA has the advantage of its
5 innocuousness, avoiding undesirable side effects observed with other adjuvants such
6 as Freund's complete adjuvant or cholera toxin, although to date the use of EDA has
7 not been approved. Thus, two recombinant p24 molecules (i.e. p24 and EDAP24 fusion
8 protein) were genetically engineered, cloned and individually produced in the *E. coli*
9 BL21-pET20b expression system. EDAP24 was purified from the soluble bacterial
10 fraction whereas the p24 protein (produced in the absence of EDA) accumulated in
11 inclusion bodies, when expressed in either the auto-inducible or the IPTG induction
12 system. This is in contrast with previous studies where p24 was extracted mainly from
13 the soluble fraction [27], likely due to the different bacterial expression system
14 employed in that study. In any case, conserved protein conformation and maintenance
15 of p24 antigen properties were confirmed in both p24 and EDAP24 recombinant
16 proteins, as both molecules had the expected molecular weight in WB and p24
17 epitopes were recognized with monoclonal antibodies in ELISA sandwich assays.
18 Moreover, considering that the combination of two different adjuvants may lead to
19 enhancement of the immune response to recombinant proteins [24, 26, 28], we
20 hypothesized that the combination of EDA with poly(I:C) would activate different TLRs
21 (EDA and poly(I:C) are agonists of TLR-4 and TLR-3, respectively) in the context of
22 HIV-1 p24 immunization and would be advantageous through the enhancement of the
23 immune response and protection against infection.

24 Humoral (IgG isotypes) and cellular (IFN- γ production) immune responses as well as
25 protection against infection, using p24 and EDAP24 recombinant proteins administered
26 alone or along with poly(I:C), were assessed in a preclinical mouse model. Since mice
27 cannot be infected with HIV-1, a murine model based on challenging with a
28 recombinant *Listeria monocytogenes* expressing HIV-1 Gag proteins (Lm-Gag strain)
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was used to evaluate the efficacy of anti-HIV vaccines [29, 30]. Protection against *Listeria* involves first the rapid clearance of the majority of bacteria within 12 h of infection by resident spleen and liver macrophages. Innate mechanisms including neutrophil recruitment, macrophage activation, and secretion of IFN- γ by NK and $\gamma\delta$ T cells are critical in controlling the infection and preventing systemic dissemination during the first few days. Thereafter, both CD4 and CD8 T cells contribute to the anti-listerial cell mediated response [29, 30]. In the mouse model used, IgG antibodies produced upon immunization against the p24 may have allowed effective phagocytosis against Gag-recombinant *Listeria* but the antibody levels observed would not explain the differences in bacterial clearance between groups. The increased IFN- γ production triggered by the presence of both EDA (as EDAP24 fusion protein) and poly(I:C) may be due to increased T cell secretors of this cytokine helping CTLs to clear Gag-expressing infected cells [30]. Also, Gag-specific T cell activation and lymphokine release may have activated macrophages making them competent to kill the intracellular bacteria. Furthermore, the complete clearance of p24 recombinant *Listeria* in three of the animals inoculated with both adjuvants strongly suggests the presence of a potent CTL response against p24 epitopes. Thus, antibody production in conjunction with innate and specific T-cell immunity, may explain the positive effect of both adjuvants jointly administered, on protection of p24 immunized mice against p24 recombinant *Listeria*.

The development of an HIV-1 vaccine that elicits cellular and humoral immunity and confers a protective effect against HIV-1 infection is still a challenge. The results obtained here on IFN- γ , specific IgG levels and clearance of p24 recombinant *Listeria* (likely via CTL) are encouraging for their application in vaccination against HIV infections, knowing that a protective immune response against this virus involves ideally the production of neutralizing antibodies, cytotoxic T-cell and T helper immune responses [4], activation of NK cells, production of antibody dependent cell cytotoxicity

(ADCC) [31] and inhibition of viral replication, referred to as “antibody-dependent cell-mediated virus inhibition” (ADCVI) (reviewed in [22]). In the mouse model used, IgG antibodies to HIV p24 were produced, but these would not be able to neutralize HIV, since epitopes for HIV neutralizing antibodies are located in the HIV envelope protein [32]. However T-helper cells, activated by increased IFN- γ , would be expected to help not only the development of IgG antibodies addressed to different viral envelope proteins, involved in neutralizing and/or ADCC functions [4], but also the production of p24-specific CTL responses that, like in the case of recombinant *Listeria* infected cells, would clear the virus infected cells.

In summary, EDA as a fusion protein with HIV antigens or antigenic epitope combinations mixed with poly(I:C) can be proposed as a combined adjuvant candidate in the development of new HIV vaccines.

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Figure captions:

Fig. 1. SDS polyacrylamide gel electrophoretic patterns of EDAp24 and p24 recombinant proteins upon Coomassie Brilliant Blue staining (panel A) and anti-polyhistidine Western-Blot (panel B). Lane 1: Molecular weight marker; lane 2: EDAp24; lane 3: p24; lane 4: EDA. Load was equivalent to 2 µg/lane.

Fig. 2. EDAp24 administered with poly(I:C) induced significant IFN-γ production in BALB/c mice. Animals (n=3) were immunized IP with: i) p24, ii) EDAp24, iii) p24 plus poly(I:C), or iv) EDAp24 plus poly(I:C). (A) Number of IFN-γ producing cells measured in the ELISPOT assay in the presence or absence of peptide AMQMLKETI and (B) levels of IFN-γ determined by the sandwich ELISA, both in cultured spleen cells stimulated or not (controls) with AMQMLKETI peptide. ELISPOT and ELISA assays were carried out with samples obtained from the same animals. Results are expressed as box plot representing the individual values obtained after AMQMLKETI peptide stimulation minus those obtained in parallel with non-stimulated splenocytes.

1 **Table 1.** Titers of anti-p24 IgG1 and IgG2a isotypes measured by indirect
2 ELISA in sera of BALB/c mice (n=5) just before challenge, 4 weeks after
3 intraperitoneal immunization with: *i*) p24; *ii*) EDAP24; *iii*) p24 plus poly(I:C); or *iv*)
4 EDAP24 plus poly(I:C). Results of sera from mice inoculated with PBS (controls)
5 are not presented since all showed undetectable levels of IgG2a and IgG1.
6

Immunization	log ₁₀ IgG1 ^a	log ₁₀ IgG2a ^a	log ₁₀ IgG2a/ log ₁₀ IgG1 ^b
p24	4.11 ± 0.35	3.28 ± 0.75	0.79 ± 0.14
EDAP24	4.23 ± 0.16	2.48 ± 0.46*	0.59 ± 0.12*
p24 + poly(I:C)	4.35 ± 0.13	3.20 ± 0.64	0.74 ± 0.16
EDAP24 + poly(I:C)	4.41 ± 0	3.14 ± 0.45	0.71 ± 0.10

7

8 ^a Mean and SD (n=5) of individual log₁₀ IgG titers.

9 ^b Mean and SD (n=5) of individual log₁₀ IgG2a/ log₁₀ IgG1 ratio

10 * p ≤ 0.05 vs. other groups.

11

Table 2. EDAP24 co-administered with poly(I:C) significantly protects mice against infection by the recombinant *Listeria monocytogenes* carrying the gag gene of HIV-1 (Lm-Gag).

Immunization	log ₁₀ CFU/spleen Lm-Gag (mean ± SE)	No. of mice free of Lm-Gag infection/totals
EDAP24 + poly(I:C)	1.74 ± 0.62 ^A	3/5 ^B
EDAP24	3.74 ± 0.14	0/5
p24 + poly(I:C)	3.18 ± 0.63	0/5
PBS	3.53 ± 0.47	0/5

BALB/c mice (n=5) were inoculated intraperitoneally with: i) EDAP24 plus poly(I:C); ii) EDAP24; iii) p24 plus poly(I:C); or iv) PBS (controls). Four weeks after inoculation, each mouse was infected intraperitoneally with 1-2×10⁶ CFU of Lm-Gag, and the number of viable bacteria in spleens was determined 3 days after challenge. Protection is expressed as mean ± SE (n=5) of log₁₀ CFU/spleen of Lm-Gag. Statistical comparisons of means were performed by ANOVA and Fisher's PLSD test (^Ap ≤ 0.048 vs. other groups), and the no. of animals infected, by chi-square test (^Bp ≤ 0.038 vs. other groups).

Figure(s)