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The Extra Domain A from Fibronectin Targets Antigens to TLR4-Expressing Cells and Induces Cytotoxic T Cell Responses In Vivo¹

Juan J. Lasarte,²* Noelia Casares,* Marta Gorraiz,* Sandra Hervás-Stubs,†† Laura Arribillaga,* Cristina Mansilla,* Maika Durantez,* Diana Llopiz,* Pablo Sarobe,* Francisco Borrás-Cuesta,* Jesús Prieto,* and Claude Leclerc†‡

Vaccination strategies based on the in vivo targeting of Ags to dendritic cells (DCs) are needed to improve the induction of specific T cell immunity against tumors and infectious agents. In this study, we have used a recombinant protein encompassing the extra domain A from fibronectin (EDA), an endogenous ligand for TLR4, to deliver Ags to TLR4-expressing DC. The purified EDA protein was shown to bind to TLR4-expressing HEK293 cells and to activate the TLR4 signaling pathway. EDA also stimulated the production by DC of proinflammatory cytokines such as IL-12 or TNF-α and induced their maturation in vitro and in vivo. A fusion protein between EDA and a cytotoxic T cell epitope from OVA efficiently presented this epitope to specific T cells and induced the in vivo activation of a strong and specific CTL response. Moreover, a fusion protein containing EDA and the full OVA also improved OVA presentation by DC and induced CTL responses in vivo. These EDA recombinant proteins protected mice from a challenge with tumor cells expressing OVA. These results strongly suggest that the fibronectin extra domain A may serve as a suitable Ag carrier for the development of antiviral or antigemorral vaccines. The Journal of Immunology, 2007, 178: 748–756.

Pathogens and cancer remain the leading causes of death worldwide. The development of vaccines to prevent diseases for which no vaccine currently exist, as well as improvement of the efficacy and safety of existing vaccines, remains a high priority. In most cases, the development of such vaccines requires strategies capable of stimulating CD8⁺ CTLs through the presentation to T cell receptors of short peptides associated with MHC class I molecules (1). These peptide-MHC class I complexes are presented on the surface of professional APCs, which are also capable of providing the costimulatory signals required for optimal CTL activation.

Dendritic cells (DCs),³ the most potent APCs with a unique ability to stimulate naive T cells, have the capacity to capture Ags, process them into peptides, and present these peptides in association with MHC class I or II molecules to CTLs or Th cells, respectively (2). Immature DCs can capture Ags but must mature to become capable of stimulating naive T cells. The unique capacity of DCs to elicit immune responses controlling tumor growth or viral spread in animal models has prompted many laboratories to use DCs in clinical trials. In general, these strategies consist in the isolation of DCs or DC precursors from patients to be then loaded with tumoral Ags followed by their in vitro maturation using different stimuli prior to their transfer into the patient. This approach has been demonstrated to be efficient for treating cancer or viral infections in many animal models (3–8). However, clinical trials have shown that several variables (DC lineage, Ag loading to DCs, maturation stage of DCs, migration capacity, route of injection, number of DC injected, etc.), mostly related to in vitro manipulation of DC, are critical to elicit strong immune responses in patients (9). Therefore, a procedure to target Ags in vivo to DCs would greatly facilitate and improve vaccination protocols. The use of chaperone-peptide complexes for their uptake by dendritic cells through heat shock protein (Hsp) receptors such as CD91 (10) or lipoxigenase 1 (LOX-1) (11) have been proposed as a method to trigger the cross-presentation of Ags and the initiation of Ag-specific immunity. A bacterial ligand of CD11b/CD18 (12), the adenylate cyclase from Bordetella pertussis that can bind to and translocate via the cell membrane into the cell cytosol, has also been used to deliver Ags to DCs and has proven to be very effective in priming CTL responses in vivo in the absence of an adjuvant (13, 14). mAbs specific for DC cell surface Ags, such as anti-DEC-205 (15), also provide an efficient means to deliver Ags to DCs in vivo. However, activation of DCs is essential to trigger adaptive immune responses, because the targeting of Ags to immature DCs may lead to tolerance (16). Thus, procedures for targeting Ags to DCs in vivo must, in addition, induce their maturation. Some of the most potent DC maturation stimuli are ligands for TLRs (reviewed in Ref. 17). At present, several ligands for the

¹Responses In Vivo
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Abbreviations used in this paper: DC, dendritic cell; EDA, extra domain A from fibronectin; BM, bone marrow; BMDC, BM-derived DC; CM, conditioned medium; ELAM, endothelial leukocyte adhesion molecule; Hsp, heat shock protein; hTLR4, human TLR4; KO, knockout; PAMP, pathogen-associated molecular pattern; SEAP, secreted embryonic alkaline phosphatase; WT, wild type.

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different TLRs have been identified. Most of these ligands are derived from pathogens, although the TLR family is also critical for the recognition of certain endogenous molecules. Indeed, TLR4, which is able to recognize LPS, can also be activated by Hsp60 (18, 19) and by the spliced exon encoding the type III repeat extra domain A (EDA) from fibronectin (20).

EDA is produced in response to tissue injury in pathologies such as rheumatoid arthritis, wound healing, epithelial fibrosis, vascular intimal proliferation, or inflammation in adults (reviewed in Ref. 21). Although the biological functions of EDA are not well understood, it has been shown that EDA induces NF-κB activation (20), proteoglycan release, and the production of metalloproteinase 1, 2, and 9 (21), which are known to be involved in the destruction of the connective tissue in chronic inflammatory lesions and in the migration of monocytes and DCs through the basement membrane (22). All of these results suggest that the local production of EDA in response to tissue injury or other danger signals might induce the recruitment of DCs to the site of injury and trigger the subsequent maturation of DCs. For these reasons, we speculated that the production of a fusion protein consisting of the recombinant EDA fused to an Ag might constitute a strategy for targeting viral or tumoral Ags to DCs in vivo. The capacity of EDA to induce maturation of DCs through its interaction with TLR4 might also favor Ag uptake, the expression of costimulatory signals, the migration of DCs to draining lymph nodes and, finally, the induction of antiviral or antitumoral T cell responses. In the present work, we have investigated the ability of a recombinant fusion protein between EDA and the CD8α T cell epitope from OVA (OVA 257–264) to target DC, induce CTL responses in vivo and protect mice from OVA-expressing tumor growth.

Materials and Methods

Mice

Female C57Bl/6 mice (6–8 wk old) from Harlan Sprague Dawley and female TLR4−/− mice bred onto a C57Bl/6 background and given by M. Chignard (Pasteur Institute, Paris, France) were housed in appropriate animal care facilities during the experimental period and handled following the international guidelines required for experimentation with animals.

Peptides

The peptide SIINFEKL, an immunodominant H-2b restricted CTL epitope corresponding to aa 257–264 from chicken OVA, was purchased from NeoMPS.

Preparation of the recombinant fusion proteins

Fibronectin EDA was amplified by RT-PCR using specific primers and RNA from hepatocytes obtained from mice treated with Con A to induce liver damage (23). Liver tissue sections were homogenized and lysed in Ultraspec (Biotecux) using an Ultra-Turrax Driver T.25 (Janke and Kunkel, Ika-Labortechnik). RNA was isolated according to the methods of Chomczynski and Sacchi (24) and was reverse transcribed and amplified by PCR as described previously (25) using the upstream primer CCATGACCTATCCGATCTACACT and the downstream primer ACCGCG CGCTGTGGACTGGTATCCAACTACGGG for the construction of an expression vector to produce the EDA protein. For the production of the EDA-SIINFEKL protein, the cDNA was amplified using the same upstream primer combined with the downstream primer ACCGCGCGCCCA TTTCGATCGTTTCAAGGTTGATATTACCTCCAAGCCTGTGGA CTGGATCGTACCACCTACGGG. The resulting PCR products were cloned in pcR2.1–TOPO using the TOPO TA cloning kit (Invitrogen Life Technologies). These plasmids were digested with NdeI and NotI and the obtained DNA fragments were subcloned in the NdeI/NotI-digested plasmid pET20b (Novagen), which enables expression of fusion proteins carrying six histidine residues (His6 tags) at the carboxyl terminus. The resulting plasmid pET20b–26, expressing the fusion protein EDA-SIINFEKL, and plasmid pET20b–1–2, expressing EDA, were transfected into BL21DE3 cells for the expression of the recombinant protein. For the construction of a plasmid expressing EDA-OVA fusion protein, mRNA from EG7-OVA cells was isolated, reverse transcribed as described above, and amplified by PCR using the upstream primer CGGGCGCGCAATGGGCTCCATCGGC GCA and the downstream primer CGGGCGCGGGAAAAACACTCT. The PCR product was cloned in pcR2.1–TOPO using the TOPO TA cloning kit (Invitrogen), digested with the NcoI enzyme, and subcloned in the NotI-digested plasmid pET20b–2. All constructs were verified by DNA sequencing. EDA, EDA-SIINFEKL, and EDA-OVA proteins were purified by affinity chromatography (HisTrap; Pharmacia) using a fast protein liquid chromatography platform (AKTA; Pharmacia). The eluted protein was desalted using HiTrap desalting columns (Pharmacia) and concentrated using an Amicon Ultra 4-5000 MWCO centrifugal filter device (Millipore). The recombinant proteins were purified from endotoxins by using EndoTrap columns (Profos) until the levels of endotoxin were below 0.2 endotoxin units per microgram of protein as tested by Quantitative Chromogenic Limulus Amebocyte Lysate assay (Cambrex).

Binding assays to TLR4-expressing cells

To test whether the recombinant EDA-SIINFEKL protein was able to bind to TLR4-expressing cells, we used HEK293 expressing human TLR4-MD2-CD14 (HEK TLR4; Invigen) or Lacz (HEK Lacz; Invigen) as a negative control. Cells were pulsed with 1 mM EDA-SIINFEKL for 1 h at 4°C, washed with PBS, and fixed with 4% paraformaldehyde in PBS for 10 min. After three washes, cells were incubated with anti-His Abs (Qia- gens) plus anti-CD16 (FcBlock; BD Biosciences), labeled with anti-mouse IgG-FITC Abs, and analyzed by flow cytometry. Alternatively, we measured the capacity of EDA-SIINFEKL to inhibit the binding of the anti-TLR4 mAb HTA125 (Abcam) to HEK TLR4. HEK TLR4 cells were incubated for 2 h at 4°C in the presence or absence of different concentrations of EDA-SIINFEKL. Cells were washed and incubated with anti-TLR4FITC Abs and analyzed by flow cytometry. The percentage of inhibition of binding was plotted.

Cell adhesion assays

EDA-containing proteins (2.5 μg/well) were coated to 96-well plates in 0.1 M CO3Na2 (pH 9.5) by overnight incubation at 4°C. Wells were washed with PBS and blocked with 1% BSA in RPMI 1640 at 37°C for 2 h. HEK TLR4 or HEK Lacz cells were labeled overnight with 2′,4′-dinitrophenyl thymidine (2′,4′-DNT; 3 μM/cm2) and detached using EDTA (20 mM in PBS, pH 7.4) and resuspended in RPMI 1640 containing 250 μM MnCl2. Ten thousand cells per well were plated directly into wells (in a final volume of 100 μl). Plates were centrifuged (top side up) at 10 × g for 5 min followed by incubation for 2 h at 37°C in a humidified incubator with 5% CO2. Nonadherent cells were removed by centrifugation (top side down) at 40 × g for 5 min. Adherent cells were harvested (FilterMate Harvey; PerkinElmer) and the incorporated radioactivity was measured in a TopCount scintillation counter (Packard Instrument). The numbers of cells adherent to each well were calculated with the aid of the corresponding standard curves.

Measurement of activation of TLR4 signaling pathway

HEK293/human TLR4 (hTLR4)-MD2-CD14- or HEK293LacZ-expressing cells were transfected with a plasmid carrying the human secreted embryonic alkaline phosphatase gene (SEAP) accordingly to manufacturer’s instructions (Invigen). SEAP expression is controlled by an NF-κB-inducible endothelial leukocyte adhesion molecule (ELAM)-1 promoter (pNFt5-SEAP; Invigen). Twenty-four hours after transfection, cells were incubated in the presence or absence of different concentrations of LPS or of 100 nM of the EDA-SIINFEKL protein, either untreated or digested with protease K using agarose-protease K beads according to manufacturer’s instructions (Sigma-Aldrich). The recovered supernatant containing the digested EDA-SIINFEKL protein was used directly. After 24 h, the expression of the reporter gene was measured in the culture supernatants by a colorimetric assay (SEAP reporter assay kit; Invigen). Results represent the fold NF-κB induction factor (OD obtained with supernatants from HEK293/hTLR4-MD2-CD14 divided by OD obtained with supernatants from HEK293/LacZ).

In vitro analysis of DC maturation

Bone marrow (BM)-derived DCs (BMDCs) were generated from mouse femur marrow cell cultures. After lysing erythrocytes with ammonium chloride potassium (ACK) lysing buffer, BM cells were washed and subsequently depleted of lymphocytes and granulocytes by incubation with a mixture of Abs against CD4 (OK1; American Type Culture Collection), CD11c (536.72; American Type Culture Collection), Ly-6G/Grl (BD Pharmingen), and CD45RB/2B20 (BD Pharmingen) followed by rabbit complement. Remaining cells were grown at 106 cells/ml in 12-well plates in conditioned medium (CM) (RPMI 1640 supplemented with
10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10⁻⁵ M 2-ME) supplemented with 20 ng/ml murine GM-CSF and 20 mg/ml murine IL-4 (both from Peprotech). Every 2 days, two-thirds of medium was replaced with fresh medium containing cytokines. Nonadherent dendritic cells were harvested at day 7 and cultured in the presence or absence of different stimuli at 37°C and 5% CO₂. After 24 h of culture, supernatants were harvested and IL-12 and TNF-α were measured by ELISA (BD Pharmingen), according to manufacturer’s instructions. Expression of DC maturation markers was measured by flow cytometry as described below. In some experiments, EDA-SIINFEKL protein was digested by incubation with agarose-proteinase K as described above.

In vivo analysis of DC maturation

In vivo induction of the maturation of DCs was evaluated with flow cytometry by measuring the expression of various surface markers. C57BL/6 mice were injected i.v. with 25 μg of EDA-SIINFEKL untreated or digested with proteinase K and 25 μg of LPS or with PBS alone. Fifteen hours after incubation, mice were sacrificed and CD11c+ cells were purified as previously described (26). Briefly, spleen cells from mice were removed and treated for 45 min at 37°C with 400 U/ml collagenase type IV and 0.5% FCS (Invitrogen Life Technologies), a single spleen cell suspension was prepared and incubated with anti-CD16/32 (clone 2.4G2; BD Pharmingen) and with colloidal superparamagnetic microbeads conjugated to an anti-CD11c mAb (MACS-anti-CD11c, N418 clone; Miltenyi Biotec), following the manufacturer’s instructions. CD11c+ cells were purified with high-speed magnetic cell sorting (autoMACS Posseld program; Miltenyi Biotec). The purity of CD11c preparations was always 95–99%. Cells were labeled and analyzed by flow cytometry.

Flow cytometry

Cells were preincubated with a rat anti-CD16/32 mAb (clone 2.4G2; BD Pharmingen) for 15 min to block the nonspecific binding of primary Abs. After this initial incubation, cells were stained with the primary Abs at 4°C for 15 min, washed, acquired on FACScan cytometer (BD Biosciences), and analyzed using Cell Quest software (BD Biosciences). The Abs used were anti-IAPβ (clone 25-9-17), anti-H-2K² (clone AF6-88.5), anti-CD40 (clone 3/23), anti-CD54 (clone 3E2), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), and anti-CD11c (clone HL-3), all from BD Pharmingen.

In vitro assays for Ag presentation

BMDCs were cultured in the presence or absence of different stimuli. In some experiments, chloroquine (3 μM), monensin (1 μl of GolgiStop; BD Pharmingen), brefeldin (1 μl of GolgiPlug; BD Pharmingen), or cycloheximide (4 μg/ml) was added to the BC cultures. Sixteen hours after the initiation of the culture, DCs were collected, fixed with 0.05% glutaraldehyde, plated in 96-well plates at different cell concentrations, and used as APCs in the presence of 10⁵ nonadherent cells per well from OT-1 transgenic mice or in the presence of 10⁵ B2B hybridoma T cells per well. IFN-γ production by T cells from OT-1 mice in the presence of treated DCs was measured by ELISA (BD Pharmingen). In some experiments, 10⁵ TAP-competent EL-4 cells or TAP-deficient T2 cells transfected with the mouse H-2K² allomaint (T2-K²) (provided by M. Del Val, Centro Nacional de Microelectronicà, Madrid, Spain) incubated or not incubated with different concentrations of EDA-OVA or OVA protein, were used as APCs in the presence of B2B hybridoma cells. In other experiments, B2B hybridoma cells (10⁶ cells/well) were cultured in CM for 18 h in the presence of spleen cells (10⁶ cells/well) from C57BL/6 wild-type (WT) mice or TLR4-knockout (KO) mice and different concentrations of EDA-SIINFEKL. The activation of B2B cells in the presence of treated APCs was conducted by measuring either the IL-2 production by a CTL-based bioassay or LacZ activity as previously described (27).

Measurement of in vivo induction of CTL and production of IFN-γ

C57BL/6 mice were immunized i.v. with 4 nmol of EDA-SIINFEKL or SIINFEKL on days 0 and 10. On day 20, mice were sacrificed for analysis of CTL response against SIINFEKL. Splenocytes from immunized animals were cultured in the presence of 0.1 μg/ml SIINFEKL at 5 × 10⁶ cells/ml (10 ml) for 5 days in CM. On day 5, cells were harvested for chromatin release assays. Lytic activity was measured by incubating different numbers of effector cells for 4 h with 1 × 10⁶ EL-4 target cells previously labeled with ⁵¹Cr and loaded or not loaded with SIINFEKL. The percentage of specific lysis was calculated according to the formula: (cpm experimental – cpm spontaneous)/(cpm maximum – cpm spontaneous) × 100, where spontaneous lysis corresponds to target cells incubated in the absence of effectors cells and maximum lysis is obtained by incubating target cells with 5% Triton X-100. To measure the production of IFN-γ in response to SIINFEKL, splenocytes from immunized mice were plated at 96-well plates at 8 × 10⁵ cells/well with CM alone or with 30 μM peptide in a final volume of 0.25 ml of CM. Supernatants (50 μl) were removed 48 h later and IFN-γ was measured by ELISA (BD Pharmingen) according to the manufacturer’s instructions.

Tumor rejection experiments

Mice were injected s.c. on days 0 and 10 with 3 nmol of EDA-SIINFEKL, SIINFEKL, or PBS alone. Twenty days after the second immunization, mice were challenged s.c. with 10⁷ EG7-OVA cells. For the treatment of established tumors, mice received 10⁷ EG7-OVA cells and, once the tumors were palpable (≈125 mm³), received intratumoral injection of PBS or 1 nmol of EDA-OVA. Tumor size was monitored twice a week with a caliper. Average tumor size is expressed in cubic millimeters using the formula V = (length × width²)/2. Mice were sacrificed when tumor size reached a volume greater than 8 cm³.

Results

Binding of EDA-SIINFEKL to TLR4-expressing cells

Although the EDA of fibronectin has been shown to activate TLR-4 (20), the physical binding of EDA to TLR4 has not been studied to date. To address this issue, we thus produced the recombinant protein EDA as well as a recombinant fusion protein, EDA-SIINFEKL, which carries the CD8 + T cell epitope SIINFEKL from OVA (OVA 257–264). The recombinant EDA and EDA-SIINFEKL proteins were expressed in Escherichia coli as a His₆ fusion protein, purified by affinity chromatography and desalted and contaminant endotoxins were removed as described in methods. The resulting proteins were characterized by SDS-PAGE (Fig. 1A) and Western blotting using anti-His Abs (data not shown).

We then used flow cytometry to test whether the EDA-SIINFEKL protein had the capacity to bind to HEK293 cells expressing human TLR4/MD2/CD14 (HEK TLR4) or to control HEK293 cells expressing LacZ (HEK LacZ). As shown in Fig. 1B, after incubation with EDA-SIINFEKL, HEK TLR4 cells presented a higher intensity of fluorescence than HEK LacZ, suggesting that EDA-SIINFEKL is indeed able to bind to TLR4. We also studied the capacity of the EDA-SIINFEKL protein to inhibit the binding of an anti-hTLR4 Ab to HEK-hTLR4 that has been shown to block activation of monocytes with LPS (28). As shown in Fig. 1, C and D, preincubation of HEK TLR4 with EDA-SIINFEKL inhibited the subsequent binding of this anti-hTLR4 Ab to these cells.

The capacity of EDA-SIINFEKL to bind to TLR4 was also measured by means of a cell adhesion assay based on the coating of ELISA plates with EDA-SIINFEKL. As shown in Fig. 1E, only HEK TLR4 cells, but not control cells expressing the reporter gene LacZ, were able to bind to EDA-SIINFEKL-coated plates. Similar results were obtained using plates coated with the EDA protein (not shown).

Activation of TLR4 signaling pathway by the EDA protein

TLR4 signaling leads to the translocation of NF-κB, a transcription factor that binds to consensus elements within the promoters of a variety of genes (29). To determine whether a recombinant EDA-SIINFEKL protein can activate TLR4, we tested the effect of this protein on HEK TLR4 or LacZ cells transfected with a plasmid carrying the human SEAP gene under the control of the NF-κB-inducible ELAM-1 promoter. As shown in Fig. 2A, EDA-SIINFEKL was able to activate the TLR4 signaling pathway as efficiently as LPS. Pretreatment of the EDA protein with proteinase K fully abrogated NF-κB activation (Fig. 2A), suggesting that this effect was not related to LPS contamination.
The EDA-SIINFEKL fusion protein stimulates IL-12 and TNF-α production by BMDCs

We then examined whether the EDA-SIINFEKL recombinant protein was able to stimulate BMDCs to produce proinflammatory cytokines. After 48 h of culture with EDA-SIINFEKL, BMDCs produced high levels of IL-12 p70 and TNF-α (Fig. 2, B and C, respectively). In both cases, the production of these cytokines was completely abrogated by pretreatment of EDA-SIINFEKL with proteinase K. Similar results were obtained when BMDCs were incubated with the EDA protein (data not shown).

EDA-SIINFEKL induces TLR4-dependent in vitro and in vivo maturation of DCs

We then analyzed whether EDA-SIINFEKL induces in vitro maturation of BMDCs. Thus, BMDCs derived from WT or TLR4-KO C57BL/6 mice were incubated with 1 μg/ml LPS, 100 nM EDA-SIINFEKL, or with medium alone and, 24 h later, cell maturation was measured by flow cytometry. As shown in Table I, LPS strongly stimulated the expression of H2-Kb, IAα, CD40, and CD86 molecules on BMDCs. Interestingly, incubation of BMDC with 100 nM EDA-SIINFEKL also induced a marked increase in the expressions of H2-Kb and IAα and, to a lesser extent, of CD40 and CD86 molecules (Table I). This increased expression was completely abrogated by the treatment of EDA-SIINFEKL with proteinase K (data not shown). The capacity of EDA-SIINFEKL to induce DC maturation was totally dependent on the presence of TLR4 molecules as shown by its lack of effect on TLR4-KO-purified DC (Table II).

Formation of Kb-SIINFEKL complexes after EDA-SIINFEKL processing by DCs

We then studied the capacity of EDA-SIINFEKL to deliver the CTL SIINFEKL epitope to the MHC class I pathway for recognition by OT-1 transgenic mice T cells. Thus, BMDCs were cultured in the presence of different concentrations of the synthetic OVA peptide, EDA alone, or with the OVA peptide or of the EDA-SIINFEKL fusion protein. At low doses the OVA peptide alone or with EDA was more efficiently presented to specific OT-1 T cells than EDA-SIINFEKL as shown by IFN-γ production (Fig. 3A). However, at doses >30 nM EDA-SIINFEKL stimulates higher levels of IFN-γ than the OVA peptide alone, suggesting that the maturation of BMDCs induced by EDA-SIINFEKL strengthen epitope presentation. As expected, BMDCs incubated with EDA alone did not activate OT-1 T cells.

To determine whether TLR4 expression on DCs could favor the presentation of EDA-SIINFEKL, B3Z hybridoma cells specific for the OVA epitope were incubated with different concentrations of EDA-SIINFEKL, either untreated or digested with proteinase K and 25 μg of LPS or in PBS alone. Fifteen hours later, CD11c+ DCs purified from mice injected with EDA-SIINFEKL showed an increased expression of the MHC class I and class II molecules CD40 and CD86. This capacity of EDA-SIINFEKL to induce DC maturation was totally abrogated by digestion with proteinase K (Table II), which, in contrast, did not affect the effect of LPS on these maturation markers (data not shown). The capacity of EDA-SIINFEKL to induce DC maturation was TLR4 dependent as shown by its lack of effect on TLR4-KO-purified DC (Table II).
EDA-SIINFEKL in the presence of spleen cells from C57BL/6 WT mice or TLR4-KO mice. B3Z activation by EDA-SIINFEKL was more efficient in the presence of APCs expressing TLR4 (Fig. 3B). Moreover, the presentation of EDA-SIINFEKL to B3Z was fully abrogated by the addition of anti-TLR4 Abs (Fig. 3C), suggesting that TLR4 is implicated in the uptake of EDA-SIINFEKL.

We then studied the effect of different drugs on the processing of the EDA-SIINFEKL fusion protein and found that this presentation was fully inhibited by monensin, brefeldin or cycloheximide, but not by chloroquine, a known inhibitor of acidification of late endosomes and lysosomes. As expected, the presentation of the synthetic OVA peptide to OT-1 cells was not affected by these drugs (Fig. 3D). These data suggest that internalization of EDA-SIINFEKL was not mediated by macropinocytosis and that EDA-SIINFEKL is processed by the MHC class I cytosolic pathway.

EDA-SIINFEKL induces OVA-specific CTL in vivo and protects mice from a challenge with tumor cells expressing the OVA protein

We tested whether mice immunized with the EDA-SIINFEKL fusion protein developed specific OVA-specific T cell responses. C57BL/6 mice were immunized i.v. on days 0 and 10 with 4 nmol of EDA-SIINFEKL or the synthetic OVA peptide in the absence of an adjuvant. As shown in Fig. 4A, EDA-SIINFEKL, but not the synthetic OVA peptide, stimulated a strong and OVA-specific production of IFN-γ. We then tested the capacity of EDA-SIINFEKL to induce OVA-specific CTLs by using the conventional chromium release assay. As shown in Fig. 4B, the immunization of C57BL/6 with EDA-SIINFEKL, but not with the synthetic OVA peptide, induced OVA-specific CTL response. A low CTL activity was also detected in these mice, but not in C57BL/6 TLR4-KO mice, by the in vivo killing assay after one injection of EDA-SIINFEKL (data not shown).

Then, to study the capacity of EDA-SIINFEKL to protect mice against the graft of EG7-OVA tumor cells, mice were injected s.c. on days 0 and 10 with 2 nmol of EDA-SIINFEKL, the synthetic OVA peptide, or PBS, and 20 days after the second immunization

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aBMDCs from WT or TLR4-KO C57BL/6 mice were cultured in the presence of different stimuli at 37°C and 5% CO₂. After 24 h of culture, the expression of DC maturation markers was measured by flow cytometry. Results are expressed as mean relative fluorescence intensity. Results are representative of two independent experiments.

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aC57BL/6 mice were injected i.v. with PBS or EDA. Fifteen hours after immunization, spleen CD11c⁺ cells were purified and DC maturation marker expression was measured by flow cytometry. Results are expressed as mean relative fluorescence intensity. Results are representative of two independent experiments.
they were grafted s.c. with $10^5$ EG7-OVA cells. All mice immunized with the OVA peptide or with PBS developed tumors, whereas 40% of mice immunized with EDA-SIINFEKL remained tumor free ($p < 0.001$) (Fig. 5, A and B).

**EDA as a vehicle for large Ags**

As shown above, the EDA protein may serve as a suitable vehicle for targeting the OVA epitope to TLR4-expressing cells and inducing efficient CTL responses against this epitope. To evaluate the capacity of EDA to improve the immunogenicity of a larger protein, we constructed and purified the recombinant fusion protein EDA-OVA, which contains the full length of OVA (397 aa) at the C terminus of EDA. This purified protein was analyzed by SDS-PAGE, and a band corresponding to the putative molecular mass (55 kDa) was found (Fig. 6A). The capacity of BMDCs to present EDA-OVA to OT-1 T cells was then tested. Interestingly, the amount of IL-2 released to the culture supernatant was measured by a CTLL-based bioassay. D. Effect of different drugs on the presentation of the EDA-SIINFEKL protein to specific T cells. BMDC were incubated for 1 h in the absence or presence of 30 μM chloroquine (Chloroq), monensin (Monen), brefeldin (Brefeld), or 4 μg/ml cycloheximide (Cyclohex) before the addition of EDA-SIINFEKL (filled bars) or the OVA synthetic peptide (open bars). After 10 h of culture, treated BMDCs were fixed with glutaraldehyde and used as APCs ($10^6$ cells/well) in coculture with nonadherent cells from OT-1 mice ($10^6$ cells/well). Twenty-four hours after, the amount of IFN-γ released was measured by ELISA. Results are representative of three independent experiments. No inhib, No inhibitor.

**FIGURE 3.** DC cells capture EDA-SIINFEKL and present the SIINFEKL epitope efficiently. A, IFN-γ production by nonadherent cells from OT-1 mice. BMDCs were cultured in the presence of medium alone or with different concentrations of the synthetic OVA peptide alone (○), the recombinant EDA-SIINFEKL (●), EDA plus OVA peptide (▲), or EDA alone (▲). Twenty-four hours later, DCs were used as APCs in the presence of $10^6$ nonadherent OT-1 cells. B, B3Z hybridoma cells ($10^5$ cells/well) were cultured in the presence of spleen cells from C57BL/6 WT mice (●) or TLR4-KO mice (○) ($10^5$ cells/well) and different concentrations of EDA-SIINFEKL. C, Spleen cells from C57BL/6 WT mice were cocultured with B3Z hybridoma cells and EDA-SIINFEKL (20 nM) in the presence or absence of anti-mouse TLR4 Abs. B3Z cells incubated in the absence of Ag (PBS) is included as a negative control. The amount of IL-2 released to the culture supernatant was measured by a CTLL-based bioassay. D, Effect of different drugs on the presentation of the EDA-SIINFEKL protein to specific T cells. BMDC were incubated for 1 h in the absence or presence of 30 μM chloroquine (Chloroq), monensin (Monen), brefeldin (Brefeld), or 4 μg/ml cycloheximide (Cyclohex) before the addition of EDA-SIINFEKL (filled bars) or the OVA synthetic peptide (open bars). After 10 h of culture, treated BMDCs were fixed with glutaraldehyde and used as APCs ($10^6$ cells/well) in coculture with nonadherent cells from OT-1 mice ($10^6$ cells/well). Twenty-four hours after, the amount of IFN-γ released was measured by ELISA. Results are representative of three independent experiments. No inhib, No inhibitor.

**FIGURE 4.** Immunization of mice with EDA-SIINFEKL induces IFN-γ production and activation of CTL specific for the OVA T cell epitope. A, C57BL/6 mice (three mice per group) were immunized i.v. with 1.5 nmol of EDA-SIINFEKL or the OVA peptide in PBS on days 0 and 10. On day 20, spleen cells were incubated for 48 h in the presence or absence of SIINFEKL, and the IFN-γ released into the culture supernatants was measured by ELISA. B, Analysis of OVA-specific CTL. The data represent the mean percentages of the net specific lysis values from triplicate samples. Results are representative of two independent experiments.
BMDCs cultured in the presence of EDA-OVA stimulated a stronger production of IFN-γ by OT-1 T cells than after culture with equimolecular quantities of OVA protein. When BMDCs were cocultured with OVA plus EDA, the production of IFN-γ was also enhanced as compared with the OVA protein alone (Fig. 6B). This result suggests that the maturation of BMDCs induced by EDA may improve T cell activation. As shown above, EDA-SIINFEKL Ag uptake was dependent on TLR4 (Fig. 3C) and was not mediated by macropinocytosis (Fig. 3D), suggesting that EDA-SIINFEKL is processed via the MHC class I cytosolic pathway. It has been reported that certain proteins containing CTL epitopes can be delivered into the cytoplasm of APCs by linking these molecules with membrane-translocating proteins (30). Once in the cytoplasm, these proteins are processed via the MHC class I pathway, which involves some proteolysis followed by transport into the endoplasmatic reticulum by the peptide transporter TAP. To test whether

FIGURE 5. EDA-SIINFEKL protects mice from challenge with EG7-OVA tumor cells. A, To study the capacity of the EDA-SIINFEKL fusion protein to protect mice against the graft of EG7-OVA tumor cells, C57BL/6 mice (seven per group) were immunized s.c. on days 0 and 10 with 3 nmol of EDA-SIINFEKL (●), the OVA peptide (○), or PBS (△). Twenty days after the second immunization, mice were challenged s.c. with 10⁵ EG7-OVA cells. Tumor growth was monitored using a caliper and average tumor size was expressed in cubic millimeters. Mice were sacrificed when tumor size reached a volume greater than 8 cm³. B, Kaplan-Meier plot of mice survival. Results are representative of two independent experiments.

FIGURE 6. EDA as a delivery system for large Ags. A, Analysis by SDS-PAGE of the recombinant EDA-OVA protein. B, In vitro assays of Ag presentation. BMDCs were cultured with 10⁵ OT-1 nonadherent cells in the presence of medium alone or different concentrations (Conc) of OVA (○), EDA-OVA (●), EDA alone (△), or EDA plus OVA (▲). IFN-γ production was measured by ELISA. C, EDA-OVA Ag presentation is TAP independent. EL-4 or T2-Kb cells were incubated overnight with EDA-OVA or with OVA and used as APCs in coculture with B3Z cells. After 24 h of coculture, B3Z stimulation was determined by measuring LacZ activity. D, Mice (three per group) were immunized with 1 nmol of EDA-OVA or OVA. Seven days after immunization, the capacity to induce specific CTL against EL-4 target cells pulsed with SIINFEKL was measured by a conventional chromium release assay. The data represent the mean percentage of the net specific lysis values from triplicate samples. E, Treatment of EG7-OVA established tumors. Mice were injected with 10⁵ EG7-OVA cells and, once the tumors were palpable (≥125 mm³), received an intratumoral injection of PBS or 1 nmol of EDA-OVA. Tumor size was monitored twice a week with a caliper. Average tumor size is expressed in cubic millimeters using the formula \( V = \frac{\text{length} \times \text{width}^2}{2} \). Mice were sacrificed when tumor size reached a volume >8 cm³.
the presentation of the OVA epitope by APCs incubated with the EDA-OVA fusion protein was TAP dependent, we conducted an assay of Ag presentation using TAP-competent EL-4 cells or TAP-deficient T2 cells transfected with H-2-K\(^\text{b}\) (T2-K\(^\text{b}\)) as APCs. These cells were incubated with OVA or EDA-OVA protein, and after 16 h of culture they were used as APCs for the stimulation of B3Z hybridoma cells. EDA-OVA, in the presence of either EL-4 or T2-K\(^\text{b}\) cells, induced a strong and comparable stimulation of B3Z cells, suggesting that its presentation was TAP independent (Fig. 6C). In contrast, the OVA protein in the presence of EL-4 or T2-K\(^\text{b}\) APCs was unable to stimulate B3Z, indicating that the EDA delivery strongly improves its capture and/or processing.

We then tested the in vivo capacity of EDA-OVA to activate T cells and reject established EG7-OVA tumors. Immunization of mice with 1 nmol EDA-OVA, but not 1 nmol of the OVA protein, induced a strong OVA-specific CTL response (Fig. 6D). Moreover, a therapeutic effect was observed after a single intratumoral immunization of EDA-OVA to mice grafted with EG7-OVA tumor cells and carrying 125-mm\(^3\) tumors. A clear retardation on tumor growth was found in three of seven mice (with one complete remission) injected with EDA-OVA (Fig. 6E).

**Discussion**

TLR triggering by pathogen-associated molecular patterns has been demonstrated to be one of the most potent in vivo inducers of DC maturation, leading to the production of proinflammatory cytokines and the up-regulation of costimulatory molecules (reviewed in Ref. 17). Thus, oligodeoxynucleotides containing CpG motifs that bind to TLR9 exhibit immunostimulatory activities and have been used as powerful vehicles and adjuncts for the induction of cellular and humoral immune responses (31). In mice, TLR9 is expressed on all major DC subtypes (32). However, in humans TLR9 is selectively expressed on plasmacytoid DCs and B cells only. Thus, the induction of adaptive immune responses in humans using CpG-DNA-conjugated proteins will depend on the efficiency of direct and/or indirect Ag presentation by plasmacytoid DCs and B cells. One of the best characterized pathogen-associated molecular patterns is LPS, which is recognized by TLR4. TLR4 can also recognize other molecules not related to TLR4. TLR4 can also recognize other molecules not related to TLR4-dependent NF\(\kappa\)B activation (20). In the present study we developed a new strategy for Ag delivery to DCs based on EDA, an endogenous TLR ligand capable of both binding to a receptor expressed on DC and inducing their maturation.

Our results clearly show that the recombinant fusion protein EDA-SIINFEKL can bind directly to HEK-293 cells expressing human TLR4/MD2/CD14 and can activate TLR4 signaling pathway, leading to the production of high amounts of IL-12 (p70) and TNF-\(\alpha\) by BMDCs. Proteasine K completely abrogated TLR4 signaling and cytokine production, strongly suggesting that these activities are indeed due to EDA-SIINFEKL, and not to residual LPS contamination. The EDA-SIINFEKL fusion protein induced DC maturation in vitro as well as in vivo by a process dependent upon the TLR4 molecule. However, although DC activated by either LPS or EDA-SIINFEKL produced comparable amounts of IL-12, their surface costimulatory molecules such as CD40 and CD86 were more strongly up-regulated by LPS. To exclude the possibility that this difference was due to some toxicity of EDA-SIINFEKL on DCs, we analyzed the viability of dendritic cells after 24 and 48 h of culture with LPS or the EDA-SIINFEKL fusion protein by measuring apoptosis and necrosis with flow cytometry using annexin V-PE and 7-aminoactcinomycin D, respectively. This experiment did not show any significant difference between either of the stimuli. It thus could be speculated that other cytokines such as TNF-\(\alpha\) (which is more efficiently produced by LPS-treated DC than by EDA-SIINFEKL-treated DC), IL-6, IL-1, type I IFNs, IL-15 or other proinflammatory cytokines might be implicated in the higher up-regulation of CD40 and CD86 by LPS.

We also showed that APCs incubated with the EDA-SIINFEKL protein take up, process, and present the OVA CTL epitope to specific T cells. Although low concentrations of the OVA peptide were more efficiently presented to specific T cells than EDA-SIINFEKL, high concentrations of EDA-SIINFEKL stimulated higher levels of IFN-\(\gamma\) by specific T cells than did the OVA peptide alone at equivalent concentrations. Whereas the OVA peptide can bind directly to the H-2K\(^\text{b}\) molecule without the need of an intracellular processing, EDA-SIINFEKL needs to be captured by the APC and processed inside the cell to generate SIINFEKL-H-2K\(^\text{b}\) complexes presented at the surface of the APC. Our results suggest that the maturation of DCs induced by EDA-SIINFEKL increases specific T cell activation. Interestingly, when we compared the presentation efficiency of the EDA-OVA and OVA proteins, which both require Ag processing, EDA-OVA was always more efficient than OVA alone. Therefore, linking EDA with an antigenic polypeptide not only facilitates binding of the immunogen to DC but also activates the immunostimulatory functions of these cells, thus leading to a more potent T cell response.

The finding that chloroquine did not affect the formation of K\(^\text{b}\)-SIINFEKL complexes when BMDCs were incubated with EDA-SIINFEKL indicates that EDA internalization is independent on vacular acidification or of endolysosomal degradation. However, sensitivity to Golgi disruption by brefeldin or monensin and sensitivity to protein synthesis inhibition by cycloheximide suggest that the OVA epitope derived from EDA-SIINFEKL protein binds to nascent MHC class I molecules in the lumen of the endoplasmic reticulum and that the complexes formed are exported to the cell surface via the Golgi apparatus by the conventional secretory pathway. These results suggest that EDA-SIINFEKL is processed like a cytosolic Ag and thus implies that EDA-SIINFEKL is delivered to the cytosol of an APC. However, in vitro assays of Ag presentation using a TAP-deficient T2-K\(^\text{b}\) cell line incubated with the EDA-OVA protein indicated that Ag presentation is TAP independent. EDA-SIINFEKL presentation by APCs is greatly dependent on the presence of TLR4, suggesting that binding of EDA-SIINFEKL to TLR4 may favor its delivery into the MHC class I presentation pathway. LPS recognition is a complex biological cascade that involves the formation of activation clusters containing TLR4/MD2/CD14 and other molecules including Hsp70, Hsp90, CXCR4, and growth differentiator factor 5 (reviewed in Ref. 33). Although it is not clear how all these molecules fit together in the “LPS-sensing apparatus” to trigger TLR4-mediated signaling, it has been described that LPS added to cells is rapidly internalized by a lipid raft-dependent pathway (35, 36). It is tempting to speculate that this internalization process is used by the EDA-SIINFEKL protein to access the trans-Golgi compartment and be processed by endopeptidases such as furin (37, 38) and by carboxypeptidases and to access the MHC class I presentation pathway in a TAP-independent and brefeldin A-sensitive process. However, this issue requires further investigation.

Finally, and more importantly, immunization of mice with EDA-SIINFEKL or EDA-OVA in PBS induces a strong OVA-specific CTL response, whereas the OVA peptide or the OVA protein in PBS failed to stimulate such response. Moreover, immunization with these EDA recombinant proteins also protects mice from challenge with EG7-OVA tumor cells. Altogether, these results suggest that the EDA from fibronectin may serve as a suitable carrier to develop adjuvants or vaccines to induce and boost.
immune responses. These results might be of great interest for the development of antitumoral or antiviral vaccines.

Disclosures

The authors have no financial conflict of interest.

References