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Fusion protein of TLR5-ligand and allergen potentiates activation and IL-10 secretion in murine myeloid DC

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ABSTRACT

Toll-like receptor ligands are immune-modulatory components linking innate and adaptive immune responses and are considered to be promising vaccine components. Objective of this study was to investigate the adjuvant activity of *Listeria monocytogenes*-derived TLR5-ligand flagellin A (flaA) genetically fused to ovalbumin (Ova, major chicken white egg allergen) in a murine *in vitro* system. Recombinant flaA, rOva, and a fusion protein of rflaA and rOva (rflaA:Ova) were over-expressed in *Escherichia coli* and purified by FPLC. LPS depletion was confirmed by LAL test. TLR5-binding was evaluated by human and murine TLR5-transgenic HEK 293 cells. The immune-modulatory effect of rflaA:Ova and rflaA:Ova modified by reduction and alkylation on purified BALB/c bone marrow-derived myeloid (mDC) and plasmacytoid dendritic cells (pDC) was investigated by flow cytometry and intracellular cytokine staining (ICS). Dose-dependent IL-8 secretion from transgenic HEK 293 cells confirmed binding of rflaA and rflaA:Ova molecules to human and murine TLR5. Recombinant flaA showed similar biological reactivity to TLR5-ligand fliC derived from *Salmonella typhimurium* applied as positive control. Compared to rflaA, both rflaA:Ova preparations induced higher expression of maturation markers (CD40, CD69, CD80, and CD86) on mDC, whereas only CD69 and CD40 were upregulated on pDC. Moreover, IL-6 and IL-10 production by mDC was enhanced upon stimulation with rflaA:Ova constructs in comparison to an equimolar mixture of both proteins whereas pDC did not show secretion of the investigated cytokines. Any immunological effects of LPS can be excluded by depletion of endotoxins and the lack of IL-10 production upon proteinase K digestion of rflaA:Ova. In summary, the rflaA:Ova fusion proteins showed an enhanced immune modulating capacity in comparison to rflaA or the mixture of rflaA and antigen. Since the rflaA:Ova fusion proteins induce strong IL-10 induction they are considered as potential vaccine candidates to improve allergen-specific immunotherapy.

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1. Introduction

Onset and maintenance of type I allergies are caused by exaggerated T_H2-mediated immune responses against otherwise harmless antigens. Although the mechanism of SIT is not fully

elucidated, the current concept postulates the induction of peripheral tolerance by a shift towards a T_H1-biased immune response, blocking antibodies, and/or the induction of IL-10 and TGF- β producing CD4⁺CD25⁺ regulatory Tr1 cells (Akdis et al., 2006a). Anti-inflammatory cytokines such as TGF- β and in particular IL-10 mediate tolerance by suppression of T_H2-cell expansion, mast cell activation and eosinophil function and counter-regulation of IgE and IgG4 synthesis (Akdis, 2009; Akdis and Akdis, 2009; Holgate and Polosa, 2008). Currently, conventional specific immunotherapy (SIT) is based on the application of increasing dosages of crude allergen extracts over long periods of up to 3–5 years (Bhalla and Singh, 2004). SIT frequently is not efficient, in particular for treatment of food allergies, and is accompanied by a rather high risk of adverse reactions. Moreover, due to a long treatment period, SIT is cost-intensive and inconvenient for the patients. Therefore, usage

Abbreviations: TLR, Toll-like receptor; Lm, *Listeria monocytogenes*; wt, wild-type; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; SIT, specific immunotherapy; R/A, reduced and alkylated; LAL, Limulus ameobocyte lysate; ICS, intracellular cytokine staining; BM, bone marrow.

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of adjuvants to induce production of anti-inflammatory cytokines and/or T_H1 -type cytokines is one strategy to enhance the efficacy, safety, and convenience of SIT.

Toll-like receptor 5 (TLR5)-ligand flagellin, which is the main constituent of motility conferring flagella of gram positive and negative bacteria (Hayashi et al., 2001; Lee et al., 2008), currently gained attention in clinical studies as an adjuvant for prophylactic and therapeutic immune modulation in various diseases, e.g. virus infection (Huleatt et al., 2008; McDonald et al., 2007). Early work by Ben-Yedida and coworkers suggested that flagellin might strengthen specific immune responses against exogenous antigens (Ben Yedidia and Arnon, 1998; Ben Yedidia et al., 1999). Hereby, flagellin serves both as an adjuvant and a carrier, because of its ability to activate TLR5-expressing cells which may lead to (i) an enhanced immune response to foreign epitopes, (ii) improved immune responses by local mucosal delivery, and to (iii) express multiple copies of foreign proteins or epitopes on the polymeric flagellum of transgenic bacteria (Ben Yedidia and Arnon, 2005). Flagellin has various advantages as an immuno-adjuvant such as (i) immuno-stimulatory properties at picomolar doses, (ii) enhancing effect on antigen-specific IgG and IgA production upon co-administration with the antigen, and (iii) low toxicity (Sierro et al., 2001; McDonald et al., 2007; Way et al., 2004; Lee et al., 2008; Huleatt et al., 2007, 2008).

Recently, flagellin has been considered as adjuvant in vaccines for SIT of allergic diseases. In previous preclinical studies a proof-of-concept for the beneficial effect of flagellin on the suppression of allergic immune responses has been provided (Lee et al., 2008). Lee et al. (2008) showed that intranasal co-administration of *V. vulnificus* flagellin B and Ova significantly inhibited subsequent Ova-induced airway hyperreactivity, airway inflammation, and T_H2 -cytokine production in a mouse model of allergic asthma. The administration of flagellin plus Ova did not induce detectable levels of Ova-specific T_H1 -responses, suggesting the effect of flagellin was not due to the induction of T_H1 -responses. However, detailed mechanisms of the inhibitory effect of flagellin on the T_H2 -mediated allergic responses are still unknown. A further strategy to promote the adjuvant effect of flagellin is to conjugate flagellin to the target antigen, as the proximity of the TLR-ligand to an antigen could promote the adjuvant effect by simultaneously targeting effector cells. Thus, a recombinant protein in which a deletion variant of *Salmonella typhimurium* flagellin flhC (STFΔ2) fused to four tandem copies of the conserved ectodomain of influenza matrix protein M2 (M2e), induced potent, long-lasting M2e-specific antibody responses that were sufficient to protect against otherwise lethal challenge with influenza A/Puerto Rico/8/34 (Huleatt et al., 2008). Recently, lamina propria CD11c⁺CD11b⁺ mouse mDC were identified to express high levels of TLR5 (Uematsu et al., 2006, 2008). In line with this, TLR5⁺CD11c⁺ DC were shown to be necessary for the adjuvant activity of flagellin-containing fusion proteins (Bates et al., 2009). Hence, certain DC subsets are likely to be key players in inducing flagellin-mediated immune responses. Although the effects of fusion constructs containing flagellin and antigen in adaptive immunity have been described by using different *in vivo* mouse models, the activation and maturation properties of fusion proteins on certain DC subsets have not yet been elucidated in detail.

Using Ova as model allergen, we investigated the activation, maturation, and cytokine production of murine myeloid (mDC) and plasmacytoid (pDC) by a fusion protein of *Lm.* flagellin A and rOva, compared to rflaA plus rOva and rflaA alone. The results indicate that flagellin-containing fusion constructs are powerful tools to modulate DC-controlled immune responses and are potential vaccine candidates for a variety of different disorders, including type I allergic diseases.

2. Methods

2.1. cDNA alignment of flagellin sequences

Flagellin cDNA sequences of *Listeria monocytogenes* flaA (NCBI Acc. No.: NC.003210), *S. typhimurium* flhC (NCBI Acc. No.: NC.003197), and a modified flhC, STFΔ2, (Huleatt et al., 2007, 2008) were aligned using clustalW2 (www.ebi.ac.uk). The resulting output file was formatted using Genedoc (<http://www.psc.edu/biomed/genedoc>).

2.2. cDNA cloning of flagellin flaA, Ova and flaA:Ova fusion protein

Flagellin and Ova cDNA were amplified via PCR using a synthetic gene (Genearts, Regensburg, Germany) encoding for *Lm.* flaA (Acc. No.: NC.003210, for: 5'-catatgaaagtaataactaatatcattagcttgaa-aacaagaatattc-3', rev: 5'-ctcgagttagctgttaattaattgag-3') and a plasmid encoding Ova (Acc. No.: NM205152, for: 5'-tatatatacatatgatgg-gctccatcgccgc-3', rev: 5'-tatatatactcagtcattagggaacacatctgcc-3') as templates. For the generation of the rflaA:Ova fusion protein cDNA of both proteins was elongated by PCR resulting in a 3'-elongated flaA cDNA (for: 5'-catatgaaagtaataactaatatcattagcttgaa-aacaagaatattc-3', rev: 5'-gctgcgccgatggagcccatgctgttaattagttagtt-3') and 5'-elongated Ova cDNA (for: 5'-aactcaatt-aattaacagcatgggctccatcgccgcagc-3', rev: 5'-ctcgagtcattagggaacacatctgcc-3'). Using the overlapping regions, the two elongated PCR-products were fused in an assembly PCR (for: 5'-catatgaaagtaataactaatatcattagcttgaa-aacaagaatattc-3', rev: 5'-ctcgagtcattagggaacacatctgcc-3') using the diluted and unpurified PCR-products of the initial PCR-reactions as templates. All constructs were cloned into pET15b (Novagen, Darmstadt, Germany) and transformed into BL21 Star DE3 cells (Invitrogen, Karlsruhe, Germany). cDNA sequences were verified via bidirectional Sanger sequencing.

2.3. Over-expression and purification of target proteins

For protein expression, 1.8 l of standard LB-medium supplemented with 0.5 ml Antifoam B Emulsion (Sigma-Aldrich, Munich, Germany) and 50 mg/l Carbenicillin (Roth, Karlsruhe, Germany) were inoculated with approximately 100 ml over night culture. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.75 to 1 mM, Fermentas, St. Leon-Rot, Germany) at OD₆₀₀ = 0.5. After incubation for 5–6.5 h (37 °C, 220 rpm) cells were harvested by centrifugation (7.500 × g, 10 min) and resuspended in 100 ml 50 mM phosphate buffer (pH 8) supplemented with 0.5 M NaCl, 2 mM EDTA (Titriplex III; Merck, Darmstadt, Germany), and 0.5 mg/ml Lysozyme (Boehringer, Ingelheim, Germany) (rflaA and rOva), or 50 ml 20 mM Tris (pH 8.5) plus 0.2 M NaCl and 1 tablet Protease Inhibitor (Roche, Grenzach-Wyhlen, Germany) (rflaA:Ova). After incubation for 15 min at room temperature cell suspensions were treated with 0.2–0.4% Triton X-100 (Sigma Aldrich) and 2 U benzonase/ml (Novagen) for 30 min at room temperature. MgCl₂ (4 mM) was added to rflaA and rflaA:Ova preparations. Cell lysis was performed by sonication and repeated freezing and thawing in liquid N₂. Inclusion bodies were washed twice with 150 ml phosphate buffer (50 mM, pH 8) plus 0.5 M NaCl and solubilized with 200 ml phosphate buffer (50 mM, pH 8) supplemented with 0.5 M NaCl and 6 M urea (rflaA), or washed twice with 120 ml Tris (20 mM pH 7.5) plus 10 mM EDTA and 1% Triton X-100 (rflaA:Ova). The solubilization of rflaA:Ova was performed with 125 ml CAPS (pH 11) supplemented with 0.3% (w:w) sarcosyl lauryl sulfate (SLS, Novagen) and 1 mM DTT. To deplete detergents and achieve solubility the Protein Refolding Kit (Novagen) was used according to the manufacturer's recommen-

dations. Insoluble constituents were removed via centrifugation (27.000 × g, 20 min). rOva was prepared from cell lysate under native conditions. Solubilized proteins were subjected to filtration (0.2 µm).

After addition of 20 mM imidazole the clarified cell lysate was applied either on a XK16 column (Amersham, Freiburg, Germany) loaded with 18 ml nickel nitrilotriacetic acid (Ni-NTA) agarose (Qia-gen, Hilden, Germany) (rflaA) or on a 5 ml HisTrap FF crude column (GE Healthcare, Freiburg, Germany) (rOva and rflaA:Ova). After washing with 5 CV of 50 mM phosphate buffer (0.5 M NaCl, 20 mM imidazole, pH 8) the proteins were eluted by 50 mM phosphate buffer (0.5 M NaCl, pH 8) supplemented with 100 mM imidazole (rOva) or by a linear gradient of 0–500 mM imidazole (rflaA:Ova). Recombinant flaA was refolded on the column with a linear gradient from 6 to 0 M urea (over 90 min with a flow rate of 3 ml/min) and subsequently eluted by a stepwise gradient with 150, 300, and 500 mM imidazole in 50 mM phosphate buffer (0.5 M NaCl, pH 8). Fractions containing the target protein were pooled and filtered through a 0.2 µm syringe filter (Minisart, Munich, Germany). In order to improve protein purity and remove endotoxins a size exclusion chromatography with a HiLoad™ XK16/60 Superdex™ 200 prep grade (Amersham) was performed. Running buffers were 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS, 0.5 M NaCl, pH 7.4) for rflaA and rOva and PBS (350 mM NaCl and 0.04% SLS, pH 7.1) for rflaA:Ova. The purified proteins were stored at –20 °C (rflaA and rOva) or at 4 °C (rflaA:Ova). Protein quantification was performed using the BCA Protein Assay Kit (Thermo Scientific, Rockford, USA).

2.4. Reduction and alkylation of rflaA:Ova

For reduction and alkylation (R/A) approximately 30 mg rflaA:Ova were taken up in 30 ml 200 mM Tris–HCl, 8 M urea, and 450 mM DTT and incubated at 45 °C for 1 h. Subsequently, 500 mM iodoacetamide was added and the reaction was incubated for 20 min at room temperature in the dark. Reduced and alkylated rflaA:Ova (rflaA:Ova R/A) was extensively dialyzed against PBS.

2.5. LAL test

Endotoxin concentration was determined via chromogenic *Limulus* amoebocyte lysate (LAL) test according to the manufacturers' instruction (Charles River PTS LAL, Sulzfeld, Germany).

2.6. SDS-PAGE

Recombinant proteins were analyzed by SDS-PAGE according to the method described by Laemmli (cross linker C=5%, total bis/acrylamid 15%) (Laemmli, 1970) under reducing or non-reducing conditions. Protein amounts used were 5 µg/slot.

2.7. Circular dichroism spectroscopy

Purified proteins were adjusted to a concentration of 200 µg/ml and dialyzed against 10 mM phosphate buffer. Circular dichroism (CD) spectra were recorded using a JASCO J-810 spectropolarimeter (Jasco, Gross-Umstadt, Germany) with constant N₂ flushing at 20 °C. Measurements were performed in a quartz glass cuvette (1 mm) with a step width of 1 nm and a band width of 1 nm. The spectral range was 180–260 nm at 50 nm/min. Ten scans were accumulated, spectra obtained with buffer were subtracted. The results were expressed as mean residue molar ellipticity [H]_{MRD} and served as indication for formation of secondary structure elements.

2.8. Proteolytic digestion of rflaA:Ova

To exclude immunological effects of residual LPS-contaminations contained in rflaA:Ova, rflaA:Ova was digested over night using proteinase K (7.5 mAu/mg protein, Merck, Darmstadt, Germany).

2.9. TLR5-activation assay

HEK 293 cell lines stably transfected with murine or human TLR5 (InvivoGen, Toulouse, France) were cultured in DMEM (Biochrome, Berlin, Germany) containing 10% FBS (Biochrome, Berlin, Germany), L-glutamine (0.15 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and blasticidin (10 µg/ml) (InvivoGen). Wild-type HEK 293 cells were cultured without blasticidin as indicated above. For TLR5-activation assays, 4 × 10⁴ cells/well were seeded in 48-well plates and cultured in DMEM containing 2% FBS, L-glutamine, penicillin, and streptomycin over night. Cells were stimulated for 22 h with rflaA, rOva, rflaA:Ova or rflaA:Ova R/A, and *S. typhimurium* flagellin flhC as positive control. Subsequently, human IL-8 secreted in the supernatant was quantified by ELISA.

2.10. Mice

BALB/c mice (Jackson Laboratories, Bar Harbor, Maine, USA), bred at the animal facility of the Paul-Ehrlich-Institut, were kept under specific pathogen free conditions. Mouse experimental work was carried out using 8–12-week-old mice in compliance with regulations of German animal welfare.

2.11. Generation and stimulation of bone marrow-derived dendritic cells

Bone marrow cells (BMCs) were isolated from femur and tibia of BALB/c mice. After lysis of red blood cells (Sigma Aldrich), BMCs were seeded in RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM HEPES, penicillin (100 U/ml), streptomycin (100 µg/ml), and 0.1 mM 2-mercaptoethanol. Differentiation of BMCs into mDC and pDC was achieved by culturing cells for 8 days either with 100 ng/ml GM-CSF or 100 ng/ml Flt3-L (R&D Systems, Minneapolis, USA), respectively. GM-CSF containing media was changed every 2 days, and for Flt3-L cultures once at day 4. 3.2 × 10⁵ cells/ml were stimulated (day 8) with rflaA (6.9 µg/ml), rOva (10 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml), rflaA:Ova R/A (16.9 µg/ml), and LPS (10 µg/ml, Sigma Aldrich) for 24 h or 72 h, respectively. The amounts of rflaA:Ova were adjusted to an equimolar ratio of rOva and rflaA. DC purity was confirmed by flow cytometry according to Waibler et al. (2007).

2.12. Cytokine ELISAs

Human IL-8, murine IL-1β, IL-6, IL-10, and IL-12p70 in the supernatant were quantified using the BD OptEIA™ ELISA Sets (BD Biosciences, Heidelberg, Germany). Murine IL-23 was measured using the Ready-SET-GO! ELISA kit (eBiosciences, San Diego, USA).

2.13. Flow cytometry and intracellular cytokine staining

Co-stimulatory molecules and MHC molecules on the cell surface of DC were stained with FITC-conjugated anti-mouse CD40, CD80, CD86 or MHC class II molecule mAbs (eBioscience) or phycoerythrin (PE)-conjugated mAbs raised against CD69 and MHC class I (eBioscience). Additionally, cells were stained with Pacific blue-conjugated anti-mouse CD11b (Invitrogen), allophycocyanin-conjugated anti-mouse CD11c (BD Bioscience), and PE-Cy5-

conjugated anti-mouse B220 (BD Bioscience) mAbs to gate on DC populations. FITC or PE intensity of CD11b⁺CD11c⁺B220[−] (mDC) or CD11b[−]CD11c⁺B220⁺ (pDC) cells was measured by FACS using a LSR II cytometer (BD Bioscience). Intracellular cytokine staining (ICS) was performed using the Cytofix/Cytoperm kit (BD Biosciences). ICS was performed after 24 h of stimulation using PE-conjugated antibodies directed against IL-6 and IL-10, according to the manufacturer's recommendations (eBiosciences). Isotype controls for all antibodies were performed. Data were analyzed using FlowJo V.7 (Treestar Inc., Ashland, OR).

2.14. Inhibition of endocytosis

BALB/c mDC (3.2×10^5 cells/ml) were preincubated for 1 h with cytochalasin D (5 µg/ml) and bafilomycin A1 (100 ng/ml) either provided alone or mixed (Sigma–Aldrich, Munich, Germany). Subsequently, cells were stimulated with rflaA:Ova (16.9 µg/ml). Supernatants were collected after 24 h of stimulation and the concentration of IL-6 in the supernatant was measured by ELISA.

2.15. Statistical analysis

Comparison between different treatment groups was performed by means of a mixed linear model with fixed factor *treatment group* and random factor *assay* (up to 3 assays with each two replicates). Confidence intervals for the estimated differences between treatment groups as well as *p*-values were adjusted using

the Bonferroni method in order to restrict the overall type I error α (false positive results i.e. false significant differences) to 5%.

3. Results

3.1. Expression, purification, and characterization of rflaA, rOva, and rflaA:Ova

The cDNA of natural *Lm. flaA* encodes for 287 amino acids and is characterized by a deletion of the middle hypervariable and most antigenic part (corresponding to fliC amino acids 181–407), but shows considerable sequence conservation on both N- and C-terminus with other flagellin genes, e.g. *S. typhimurium* fliC and the fliC deletion variant STFΔ2 (Huleatt et al., 2007). STFΔ2 was derived from the fliC sequence by deletion of amino acids 180–426 and inserting a flexible linker sequence (GAPVDPASPW, Fig. 1).

The amount of purified products (Fig. 2a) derived from 41 *Escherichia coli* culture were 80 mg rflaA (30.6 kDa), 10 mg rOva (44 kDa), and 12 mg rflaA:Ova (73.2 kDa). Endotoxin was depleted by chromatography to 11 pg/µg for rflaA, 0.15 pg/µg for rOva, and 2.9 pg/µg for rflaA:Ova. Protein purity was calculated at least 95% for all preparations (Fig. 2b). rflaA:Ova showed slight high molecular weight oligomerization products, which were abrogated by reduction and alkylation (R/A). Formation of secondary structure elements was analyzed by CD-spectroscopy (Fig. 2c). All proteins displayed minima at 208 and 220 nm, typical for α -helical structures, indicating the preparation of proteins with

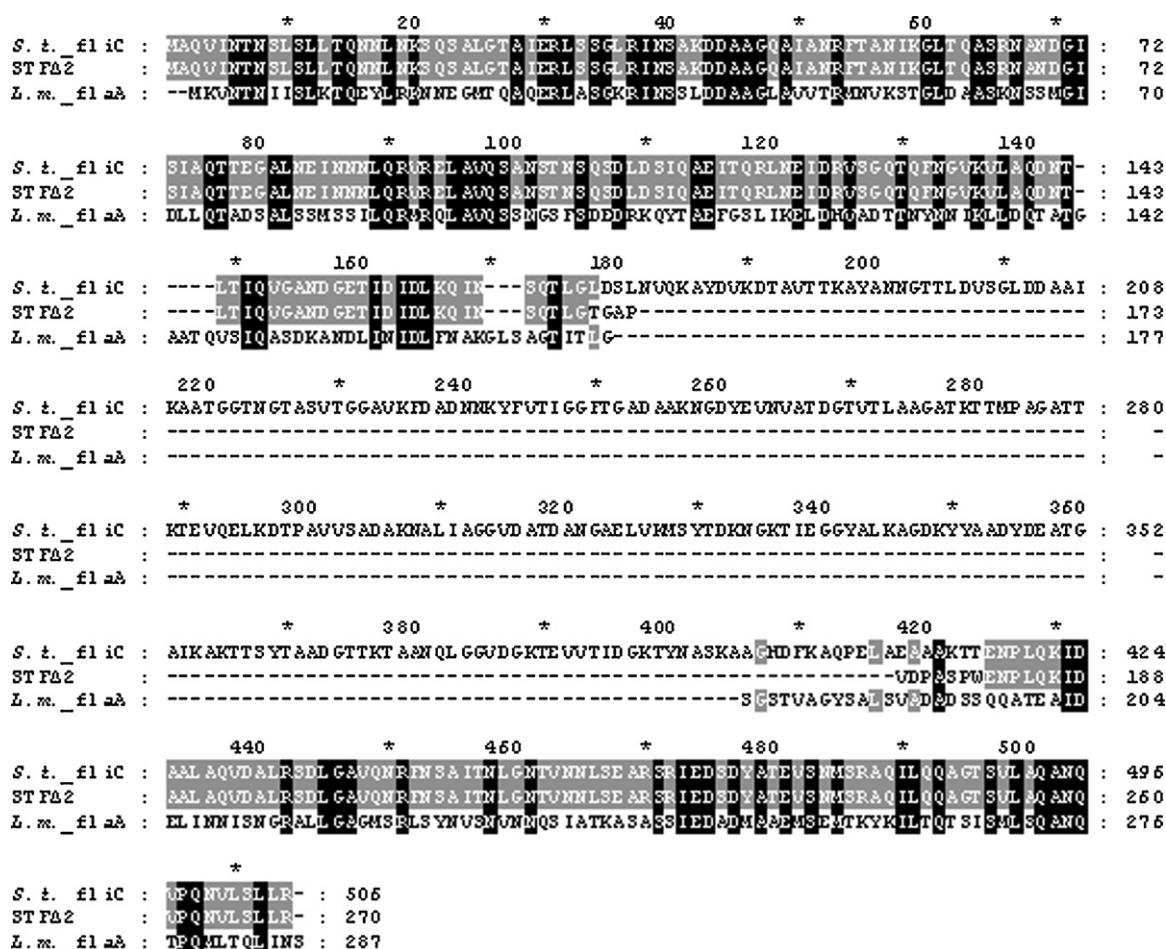


Fig. 1. *Listeria monocytogenes* flaA lacks the hypervariable region encoded by fliC. Flagellin amino acid sequences of *Listeria monocytogenes* flaA (Acc. No.: NC.003210) and *Salmonella typhimurium* fliC (Acc. No.: NC.003197) and the fliC deletion variant STFΔ2 were aligned using clustalW2 (available online on www.ebi.ac.uk). Grey color: sequence identity of 66%; black: sequence identity of 100% between the compared sequences.

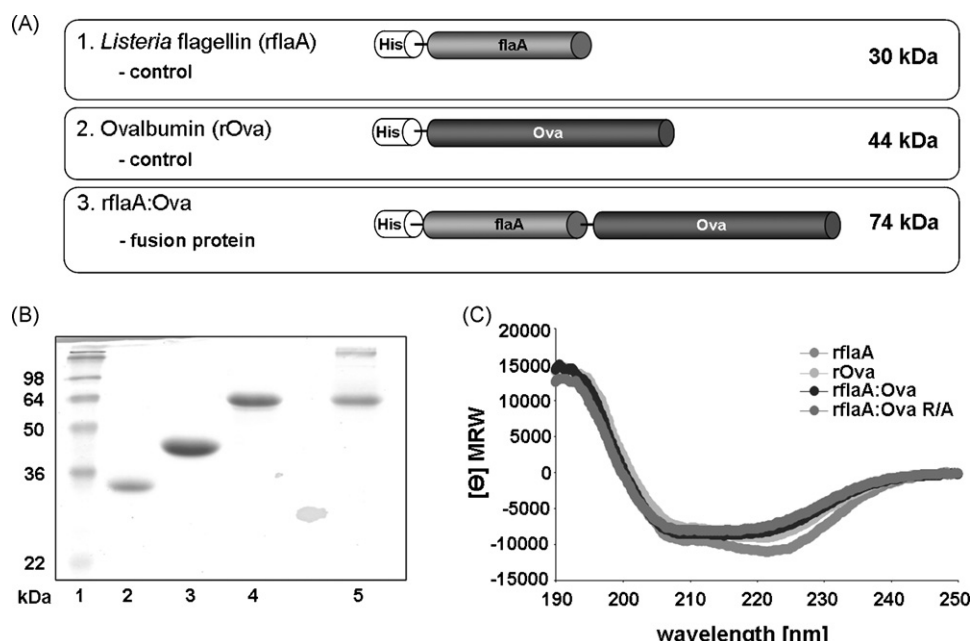


Fig. 2. Purification of recombinant Ova, flaA, and flaA-derived constructs. All proteins were cloned into pET15b, over-expressed in *E. coli*, and purified via FPLC (A). Purified and endotoxin depleted proteins were analyzed by SDS-PAGE and Coomassie staining (B); (1) molecular weight marker, (2) rflaA, (3) rOva, (4) rflaA:Ova R/A, and (5) rflaA:Ova. Circular dichroism (CD) spectra were recorded to determine folding of secondary structure elements (C). Ten scans were accumulated, spectra obtained with buffer were subtracted. Results were expressed as mean residue molar ellipticity $[H]_{MRD}$.

a considerable amount of secondary structure. CD-spectra of rflaA:Ova and rflaA:Ova (R/A) were nearly identical and showed high similarity to the CD-spectrum of rflaA and rOva. These results showed that reduction and alkylation (R/A) has no impact on the secondary structure of rflaA (comprising no cysteine residues). Collectively, we generated highly pure recombinant proteins with secondary structures closely related to the non-fused proteins.

3.2. TLR5-dependent IL-8 secretion by flaA and rflaA:Ova fusion proteins

Binding and activation of rflaA and rflaA:Ova to TLR5 was confirmed by stimulation of HEK 293 wildtype (wt) and transgenic cells expressing murine or human TLR5 (Fig. 3). Recombinant flaA and fliC dose-dependently induced comparable IL-8 secretion in TLR5 transgenic, but not in wt HEK 293 cells, indicating a similar TLR5-activation capacity for rflaA and fliC. Moreover, rflaA:Ova fusion proteins activated HEK 293 cells expressing either human or murine TLR5. However, compared with equimolar concentrations of rflaA, rflaA:Ova-triggered IL-8 responses were reduced, but using higher protein concentrations maximal secretion of IL-8 was comparable for rflaA and both rflaA:Ova preparations. Neither stimulation with Ova alone nor the stimulation of non-transfected wt HEK 293 cells induced IL-8 secretion (Ropelewski et al., 2004).

3.3. mDC are activated upon stimulation with rflaA:Ova

To assess the proteins ability to activate murine DC subsets, mDC and pDC cultures were stimulated with equimolar amounts of rflaA, rOva, rflaA plus rOva, rflaA:Ova, rflaA:Ova (R/A) and LPS and analyzed for surface expression of MHC class I/II and co-stimulatory molecules CD40, CD80, CD86, and CD69 which was previously shown to be upregulated by activated DC (Waibler et al., 2008; Young et al., 2008). Stimulation with rflaA induced slight upregulation of CD40 and CD86 on mDC, whereas CD69 and

CD80 remained unaffected (Fig. 4a). Of note, the mixture of rflaA and rOva did not alter the flaA-induced expression of maturation and activation markers. Interestingly, stimulation with rflaA:Ova and rflaA:Ova (R/A) enhanced the expression of the maturation and activation markers on mDC. Upregulation was strongest for CD69, intermediate for CD40 and CD86, and less pronounced for CD80. The expression of CD69, CD80, and CD86 upon stimulation with rflaA:Ova and LPS, a known inducer of mDC maturation, was comparable. Flow cytometric analysis revealed the activated cells (as determined by CD69 upregulation) to be neither CD49b positive NK cells, CD19 positive B cells nor CD3 positive T cells (data

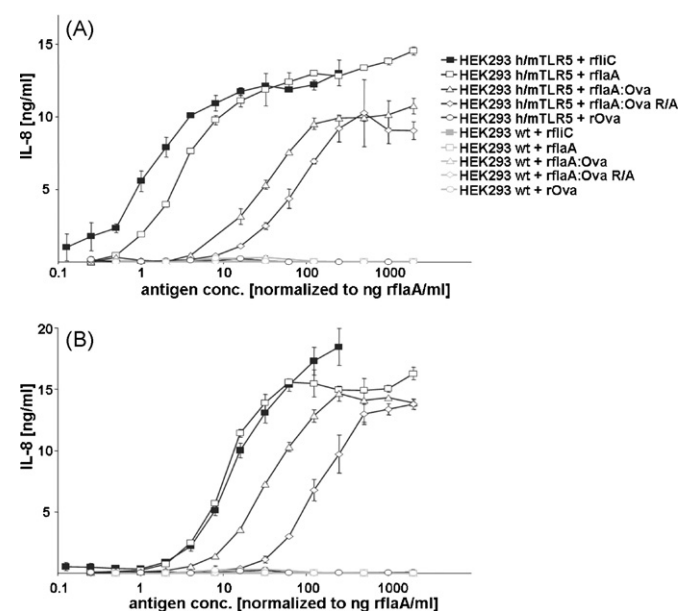


Fig. 3. Recombinant flaA and rflaA:Ova are biological active and bind to TLR5. Murine TLR5 (A) and human (B) TLR5-transgenic HEK 293 cells were stimulated with rflaA, fliC, rflaA:Ova, rflaA:Ova R/A, and rOva for 22 h. Activation was measured by IL-8 secretion. Wt HEK 293 cells were used as controls.

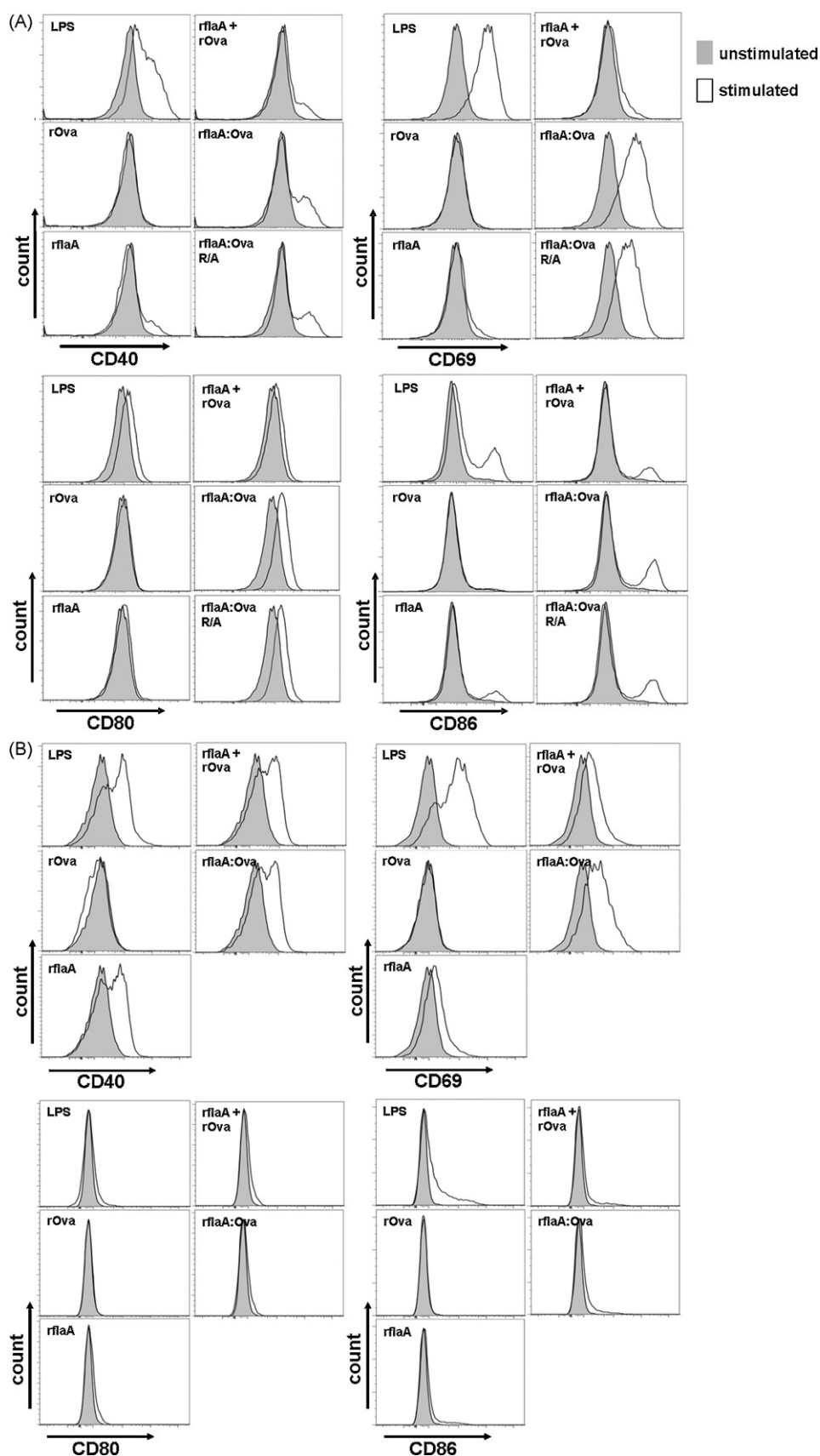


Fig. 4. rflaA:Ova fusion proteins activate *in vitro* generated DC subsets. BALB/c mDC (A) and pDC (B) were stimulated with LPS and equimolar amounts of rOva, rflaA, rflaA plus rOva, rflaA:Ova, and rflaA:Ova R/A for 24 h, gated on CD11b⁺CD11c⁺B220⁻ (mDC) or CD11b⁻CD11c⁺B220⁺ (pDC), and analyzed for CD40, CD69, CD80, and CD86 expression. Grey filled curves: unstimulated; black lines: stimulated as indicated; results represent two independent experiments.

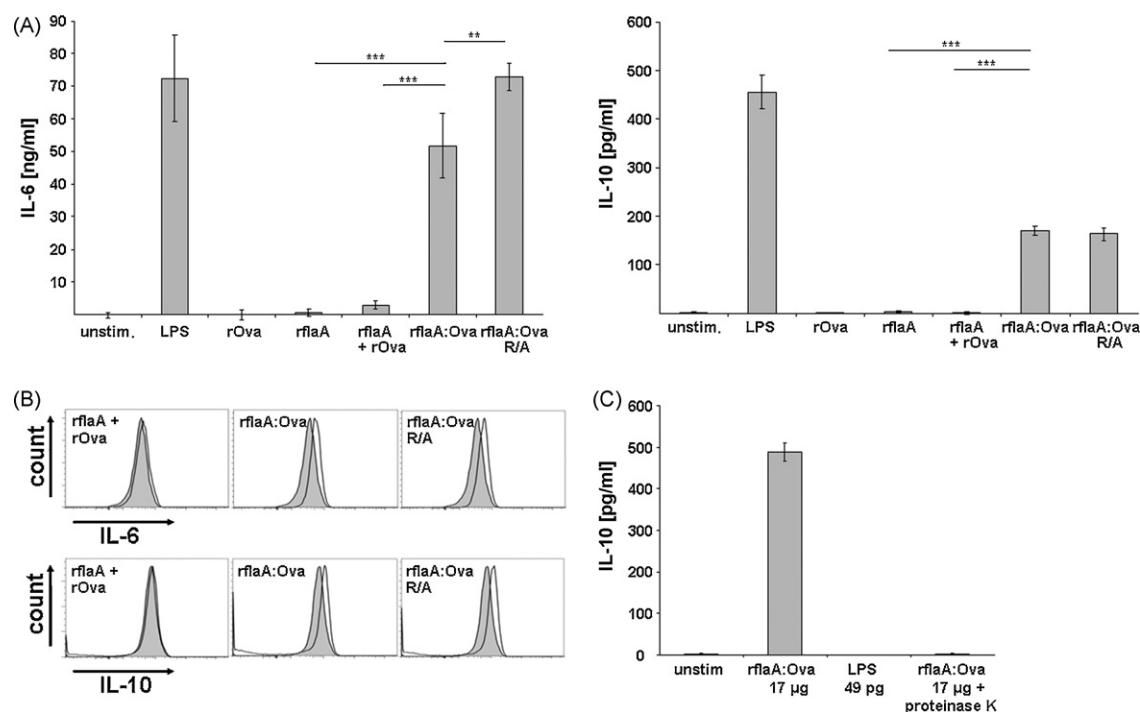


Fig. 5. rflaA:Ova induces IL-6 and IL-10 secretion from BALB/c mDC. BALB/c mDC were stimulated with LPS, rOva, rflaA, rflaA plus rOva, rflaA:Ova (untreated and R/A) and levels of IL-6 and IL-10 were detected by ELISA after 24 h and 72 h of stimulation, respectively (A). Results are mean values $\pm$ SD of three independent experiments. Induction of IL-6 and IL-10 by CD11b⁺CD11c⁺B220⁺ mDC was confirmed by intracellular cytokine staining (B). Results are representative data from two independent experiments. BALB/c mDC were stimulated with rflaA:Ova (17 μ g/ml), corresponding amounts of LPS (49 pg), and proteinase K digested rflaA:Ova (17 μ g/ml) (C). IL-10 in the supernatant was quantified via ELISA.

not shown). Expression of MHC class I and class II molecules on mDC were unaffected upon stimulation with all proteins (data not shown). rOva alone did not influence the activation of mDC. In analogous experiments using BM-derived pDC, upregulation of CD40 and slight upregulation of CD69 was observed upon stimulation with either rflaA, rflaA plus rOva or rflaA:Ova while the expression of CD80 and CD86 was unaffected (Fig. 4b). Thus, rflaA:Ova fusion proteins matured and activated mDC and to a lesser extend pDC.

3.4. Modulation of mDC-derived cytokine expression profile by rflaA:Ova

To study the cytokine production of DC subsets induced by rflaA:Ova fusion proteins, mDC and pDC preparations were stimulated with all recombinant proteins and LPS (Fig. 5). Both, untreated rflaA:Ova and rflaA:Ova (R/A) induced the production of IL-1 β (data not shown), IL-6, and IL-10 from BALB/c-derived mDC. Only minor amounts of the pro-inflammatory cytokine IL-12 could be detected (data not shown). In all experiments, rflaA:Ova induced significantly higher amounts of IL-6 (>17-fold) and IL-10 (>140-fold) from mDC than both proteins alone or provided as an equimolar mixture. LPS, applied as positive control, triggered IL-6 and IL-10 secretion from mDC (Fig. 5a). Production of IL-6 and IL-10 in rflaA:Ova stimulated CD11b⁺CD11c⁺B220⁺ mDC was confirmed by ICS (Fig. 5b). In accordance with the results obtained by ELISA, stimulation with both rflaA:Ova proteins (wt and R/A) induced IL-6 and IL-10 production in mDC, whereas rflaA and rOva applied alone or as a mixture did not induce detectable cytokine levels (Fig. 5b). When stimulating BALB/c pDC with the different constructs only minor amounts of IL-6 were produced, whereas LPS clearly induced IL-6 secretion (data not shown). Moreover, IL-10 production by pDC observed by ELISA upon stimulation with the fusion protein could not be assigned to CD11b⁺CD11c⁺B220⁺ pDC

by ICS (data not shown). Therefore, ICS confirmed that rflaA:Ova proteins induced high amounts of IL-6 and IL-10 from mDC but not from pDC. In summary, mDC were shown to be the DC-subset predominantly stimulated by rflaA:Ova.

3.5. rflaA:Ova mediated effects are independent of LPS

To exclude immunological effects of residual LPS amounts in the used protein preparations mDC were stimulated with corresponding amounts of LPS (49 pg) and analyzed for cytokine secretion (Fig. 5c). Stimulation of mDC with corresponding LPS amounts did neither induce upregulation of activation markers (data not shown), nor detectable IL-6 (data not shown) or IL-10 secretion (Fig. 5c). Furthermore, rflaA:Ova digested by proteinase K did not induce IL-10 production by BALB/c mDC (Fig. 5c). Therefore, cytokine production was shown to be mediated by the fusion protein and not by residual LPS-contamination.

3.6. Cytokine secretion is induced by high concentrations of flA

Remarkably, the applied concentration of rflaA (6.9 μ g) induced only slight DC-activation (Fig. 4) and negligible amounts of IL-6 and IL-10 (Fig. 5). Therefore, dose-dependent stimulations of mDC with equimolar amounts of rflaA and rflaA:Ova ranging from 0.25 to 64 μ g flA/ml were performed (Fig. 6). Here rflaA was able to induce IL-6 and IL-10 cytokine secretion upon stimulation with 32 μ g/ml, although in direct comparison to flA:Ova approximately 100-fold higher concentrations had to be applied. To determine the threshold for LPS-induced IL-6 and IL-10 secretion mDC were stimulated with LPS amounts ranging from 1 pg/ml to 10 μ g/ml. Hereby, IL-6 induction was observed when applying more than 0.1 ng/ml LPS, whereas IL-10 production was detectable using concentrations of at least 0.7 ng/ml LPS (Fig. 6).

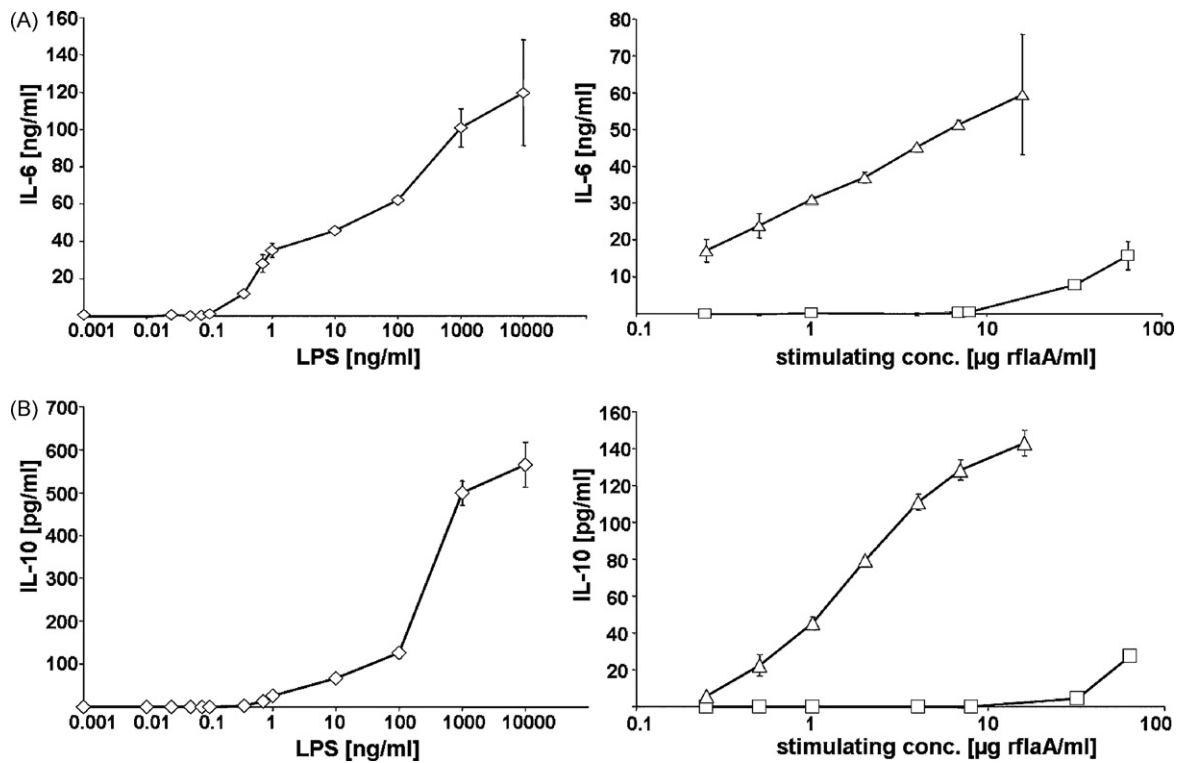


Fig. 6. Dose-dependent LPS and rflaA induced cytokine secretion. BALB/c mDC were stimulated with LPS (open diamonds), rflaA (open squares), and rflaA:Ova (open triangles). Levels of IL-6 (A) and IL-10 (B) in the culture supernatant were determined after 24 h by ELISA. Cytokine release curves were normalized to LPS free preparations. Results for IL-6 are mean values $\pm$ SD of two independent experiments, whereas results for IL-10 were obtained from one experiment $\pm$ SD.

3.7. rflaA:Ova induced IL-6 secretion depends on endocytosis

Blocking of both actin-polymerization by cytochalasin D and lysosomal fusion by bafilomycin A1 decreased IL-6 secretion by rflaA:Ova stimulated cells, suggesting endocytotic uptake of the fusion protein to contribute to the observed mDC activation. Noteworthy, simultaneous administration of both inhibitors enhanced the effect, whereas DMSO used as solvent had no impact on fusion protein induced IL-6 secretion. Since stimulation with equimolar amounts of rflaA alone did not induce cytokine secretion the influence of cytochalasin D and bafilomycin A1 pretreatment on rflaA mediated mDC activation could not be tested in this system.

4. Discussion

Bacterial TLR5-agonist flagellin is considered as an immune modulating adjuvant in various diseases. Proof-of-concept has been provided by mouse *in vivo* studies (Lee et al., 2008) mainly using flagellin fliC derived from *S. typhimurium* (Huleatt et al., 2007, 2008; McDonald et al., 2007). Since heat-killed *Listeria* have been shown to convert an allergen-specific T_H2 response into a T_H1 -biased immune response (Li et al., 2003; Frick et al., 2005; Hansen et al., 2000; Yeung et al., 1998), which likely is caused by TLR-signaling (Seki et al., 2002), but not mediated by TLR9- (Frick et al., 2005) or TLR4-activation (Li et al., 2003), we hypothesize *Lm*-derived flagellin flaA to act as one major component. In comparison to fliC flaA shows high sequence identity at both the N- and C-terminus, but lacks the middle hypervariable and major immunogenic region which was shown to be dispensable for flagellin function (Smith and Ozinsky, 2002) and thus, has been successfully removed in the fliC deletion variant STFΔ2 for vaccine development (Huleatt et al., 2007, 2008; McDonald et al., 2007). Using TLR5-transfected HEK 293 cells we could show that the capacity of rflaA to bind and activate TLR5 was comparable to fliC. Therefore, *Lm*-derived rflaA likewise can be considered as an adjuvant component for the development of immune modulating vaccines. Using rOva as a model allergen, we compared the immune modulating properties of rflaA co-administrated with rOva in a mixture and arflaA:Ova fusion protein on murine DC subsets. Of note, rflaA:Ova preserved its capability to activate TLR5. However, in direct comparison to rflaA its potency was impaired in the lower concentration ranges. This effect is likely caused by a partial sterical hindrance of rflaA receptor binding induced by the fusion to Ova. Moreover, a tendency of rflaA:Ova to aggregate could be abolished by reduction and alkylation. This modification disrupts inter- and intramolecular disulfide bonds of rOva, whereas rflaA which does not contain any cysteine residues remains unaffected. Of note, modified and

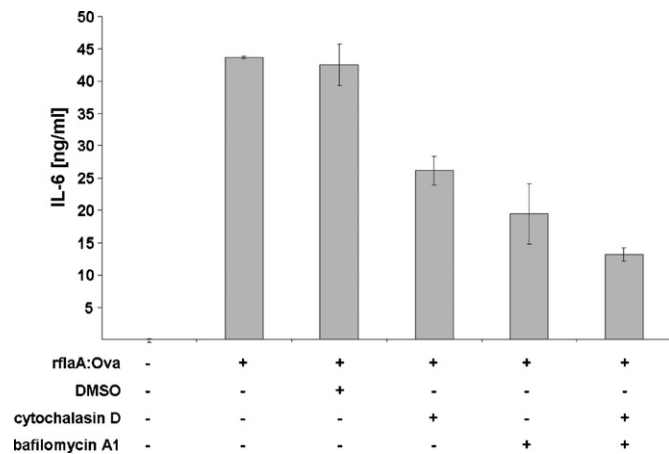


Fig. 7. rflaA:Ova induced IL-6 secretion is endocytosis-dependent. BALB/c mDC were preincubated with cytochalasin D (5.0 μg/ml) and/or bafilomycin A1 (100 ng/ml) for 1 h. Subsequently, the cells were stimulated rflaA:Ova (16.9 μg/ml). The level of IL-6 in the culture supernatant after 24 h was determined by ELISA. Results are mean values $\pm$ SD of two independent experiments.

unmodified rflaA:Ova showed similar immunological properties. Noteworthy, defolding of the antigen is one strategy to design novel allergen vaccines with reduced IgE-reactivity to minimize the risk of unwanted IgE-mediated side effects during SIT, whereas T cell epitopes are preserved (Wiedermann, 2005; de Leon et al., 2007; Cromwell et al., 2004). Hence, fusion of allergens with low IgE-binding activity to the TLR5-ligand flagellin could combine the immune modulating capacities and the increased safety of novel allergen vaccines.

However, the ability of flagellin to activate DC is controversially discussed. Means et al. (2003) reported purified bacterial flagellin to be unable to mature murine splenic DC *in vitro* due to very low TLR5 expression determined by qRT-PCR. On the other hand, Didierlaurent et al. (2004) reported monomeric *Salmonella* flagellin to induce upregulation of CD40, CD80, and CD86 on splenic DC, suggesting DC-activation. Although rflaA showed a strong induction of IL-8 in TLR5-transgenic HEK 293 cells, its potency to activate DC maturation and cytokine secretion is either weak or not detectable in the applied concentrations (6.9 µg/ml), which have been adjusted to the molar ratio used for the rflaA fusion proteins. Although, flagellin itself turned out to be only a weak stimulus in comparison to LPS, a significant IL-6 and IL-10 induction was achieved using 10-fold higher flaA concentrations. The results are in line with Smith et al. (2003) reporting flagellin-mediated IL-10 induction in mice.

In contrast to rflaA or the mixture of rflaA and rOva, we showed rflaA:Ova to strongly enhance activation of murine DC subsets and induce a distinct pattern of cytokine production. We observed direct effects of rflaA:Ova on the activation and maturation of DC subsets and much higher levels of IL-6 and IL-10 production upon stimulation of *in vitro* differentiated mDC in comparison to pDC. Although both DC subsets express TLR5 mRNA (data not shown) the immune modulating effects are predominantly attributed to mDC and less pronounced in pDC.

Results of our *in vitro* study are in accordance with an *in vivo* study showing flagellin:Ova fusion protein to have an increased adjuvant efficacy over the equimolar mixture (Bates et al., 2009). The superior immune modulating effect of rflaA:Ova likely is explained by a combination of antigen-targeting to TLR5⁺ DC and flagellin induced signaling via TLR5. Hereby, certain biophysical characteristics of the fusion protein (e.g. in respect to size or oligomerization state), and probably more important the targeting of antigen to TLR-expressing cells by conjugation to TLR-ligands likely enhances antigen uptake, processing, subsequent presentation, and DC-activation (Khan et al., 2007; Cuadros et al., 2004). In line with this, a flagellin:EGFP fusion protein was shown to be efficiently internalized, processed, and presented by mouse APC (Cuadros et al., 2004). BALB/c mice immunized with this flagellin:EGFP construct developed specific CD4⁺ and CD8⁺ T cell responses against the otherwise non-immunogenic EGFP (Cuadros et al., 2004). We observed that inhibition of endocytosis of rflaA:Ova by cytochalasin D and Bafilomycin A1 suppressed IL-6 secretion by mDC, indicating internalization into endosomal or lysosomal compartments as an important step. These observations suggest that in addition to targeting TLR5⁺ cells, fusion of flagellin to Ova changes the proteins biophysical characteristics, which in turn may influence antigen uptake and processing in DC and therefore the type of immune response induced.

In addition to IL-6, a strong production of mDC-derived IL-10 was determined upon stimulation with rflaA:Ova, whereas the different constructs did not induce considerable production of IL-12 (data not shown). IL-6 and IL-10 cytokine secretion detected in wt mice was almost abrogated in mDC-derived from MyD88^{-/-} mice, and therefore shown to be dependent on TLR-signaling (data not shown). Importantly, we can exclude any immunological effects of residual LPS in our protein preparations. Stimulation of mDC

with increasing amounts of LPS showed that threshold values for LPS-induced IL-6 and IL-10 production are higher than the residual LPS amount detected in the used protein preparations. In addition, any endotoxin-mediated effects can be excluded by proteolytic digestion of rflaA:Ova not inducing IL-10 secretion. Therefore, we conclude the observed immune modulating effects to be mediated exclusively by rflaA:Ova. IL-10 is an anti-inflammatory cytokine suppressing T_H1 and T_H2 cytokine production by CD4⁺ T cells (Jutel et al., 2003; Akdis et al., 2006b; O'Garra et al., 2008). In line with this IL-10 producing oral and pulmonary dendritic cells are suggested as potential targets for SIT (Allam et al., 2009) and have been shown to mediate tolerance (Akbari et al., 2001).

In summary, rflaA:Ova potentiated the immune modulating properties in comparison to a mixture of both proteins, probably due to targeting the antigen and adjuvant simultaneously to antigen presenting cells, resulting in enhanced antigen uptake and TLR-mediated cell activation. CD11b⁺CD11c⁺B220⁻ mDC were identified as most important target cells, which were activated and secreted cytokines IL-6 and IL-10, the latter being a desired cytokine for allergen-specific immune therapy. Therefore, flagellin fusion proteins are powerful tools to modulate DC-controlled immune responses and promising vaccine candidates for a variety of immune disorders, including type I allergic diseases.

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References

- Akbari, O., DeKruyff, R.H., Umetsu, D.T., 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2, 725–731.
- Akdis, C.A., Akdis, M., 2009. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J. Allergy Clin. Immunol.* 123, 735–746.
- Akdis, C.A., Blaser, K., Akdis, M., 2006a. Mechanisms of allergen-specific immunotherapy. *Chem. Immunol. Allergy* 91, 195–203.
- Akdis, M., 2009. Immune tolerance in allergy. *Curr. Opin. Immunol.* 21, 700–707.
- Akdis, M., Blaser, K., Akdis, C.A., 2006b. T regulatory cells in allergy. *Chem. Immunol. Allergy* 91, 159–173.
- Allam, J.P., Bieber, T., Novak, N., 2009. Dendritic cells as potential targets for mucosal immunotherapy. *Curr. Opin. Allergy Clin. Immunol.* 9, 554–557.
- Bates, J.T., Uematsu, S., Akira, S., Mizel, S.B., 2009. Direct stimulation of TLR5⁺ CD11c⁺ cells is necessary for the adjuvant activity of flagellin. *J. Immunol.* 182, 7539–7547.
- Ben Yedidia, T., Arnon, R., 1998. Effect of pre-existing carrier immunity on the efficacy of synthetic influenza vaccine. *Immunol. Lett.* 64, 9–15.
- Ben Yedidia, T., Arnon, R., 2005. Towards an epitope-based human vaccine for influenza. *Hum. Vaccin.* 1, 95–101.
- Ben Yedidia, T., Marcus, H., Reisner, Y., Arnon, R., 1999. Intranasal administration of peptide vaccine protects human/mouse radiation chimera from influenza infection. *Int. Immunol.* 11, 1043–1051.
- Bhalla, P.L., Singh, M.B., 2004. Engineered allergens for immunotherapy. *Curr. Opin. Allergy Clin. Immunol.* 4, 569–573.
- Cromwell, O., Suck, R., Kahlert, H., Nandy, A., Weber, B., Fiebig, H., 2004. Transition of recombinant allergens from bench to clinical application. *Methods* 32, 300–312.

- Cuadros, C., Lopez-Hernandez, F.J., Dominguez, A.L., McClelland, M., Lustgarten, J., 2004. Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. *Infect. Immun.* 72, 2810–2816.
- de Leon, M.P., Rolland, J.M., O'Hehir, R.E., 2007. The peanut allergy epidemic: allergen molecular characterisation and prospects for specific therapy. *Expert Rev. Mol. Med.* 9, 1–18.
- Didierlaurent, A., Ferrero, I., Otten, L.A., Dubois, B., Reinhardt, M., Carlsen, H., Blomhoff, R., Akira, S., Kraehenbuhl, J.P., Sirard, J.C., 2004. Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. *J. Immunol.* 172, 6922–6930.
- Frick, O.L., Teuber, S.S., Buchanan, B.B., Morigasaki, S., Umetsu, D.T., 2005. Allergen immunotherapy with heat-killed *Listeria monocytogenes* alleviates peanut and food-induced anaphylaxis in dogs. *Allergy* 60, 243–250.
- Hansen, G., Yeung, V.P., Berry, G., Umetsu, D.T., DeKruyff, R.H., 2000. Vaccination with heat-killed *Listeria* as adjuvant reverses established allergen-induced airway hyperreactivity and inflammation: role of CD8⁺ T cells and IL-18. *J. Immunol.* 164, 223–230.
- Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.P., Aderem, A., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099–1103.
- Holgate, S.T., Polosa, R., 2008. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol.* 8, 218–230.
- Huleatt, J.W., Jacobs, A.R., Tang, J., Desai, P., Kopp, E.B., Huang, Y., Song, L., Nakaar, V., Powell, T.J., 2007. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. *Vaccine* 25, 763–775.
- Huleatt, J.W., Nakaar, V., Desai, P., Huang, Y., Hewitt, D., Jacobs, A., Tang, J., McDonald, W., Song, L., Evans, R.K., Umlauf, S., Tussey, L., Powell, T.J., 2008. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine* 26, 201–214.
- Jutel, M., Akdis, M., Budak, F., Aebischer-Casaulta, C., Wrzyszc, M., Blaser, K., Akdis, C.A., 2003. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur. J. Immunol.* 33, 1205–1214.
- Khan, S., Bijker, M.S., Weterings, J.J., Tanke, H.J., Adema, G.J., van Hall, T., Drijfhout, J.W., Melief, C.J., Overkleef, H.S., van der Marel, G.A., Filippov, D.V., van der Burg, S.H., Ossendorp, F., 2007. Distinct uptake mechanisms but similar intracellular processing of two different Toll-like receptor ligand-peptide conjugates in dendritic cells. *J. Biol. Chem.* 282, 21145–21159.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lee, S.E., Koh, Y.I., Kim, M.K., Kim, Y.R., Kim, S.Y., Nam, J.H., Choi, Y.D., Bae, S.J., Ko, Y.J., Ryu, H.J., Koh, J.T., Choy, H.E., Rhee, J.H., 2008. Inhibition of airway allergic disease by co-administration of flagellin with allergen. *J. Clin. Immunol.* 28, 157–165.
- Li, X.M., Srivastava, K., Huleatt, J.W., Bottomly, K., Burks, A.W., Sampson, H.A., 2003. Engineered recombinant peanut protein and heat-killed *Listeria monocytogenes* coadministration protects against peanut-induced anaphylaxis in a murine model. *J. Immunol.* 170, 3289–3295.
- McDonald, W.F., Huleatt, J.W., Foellmer, H.G., Hewitt, D., Tang, J., Desai, P., Price, A., Jacobs, A., Takahashi, V.N., Huang, Y., Nakaar, V., Alexopoulou, L., Fikrig, E., Powell, T.J., 2007. A West Nile virus recombinant protein vaccine that coactivates innate and adaptive immunity. *J. Infect. Dis.* 195, 1607–1617.
- Means, T.K., Hayashi, F., Smith, K.D., Aderem, A., Luster, A.D., 2003. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *J. Immunol.* 170, 5165–5175.
- O'Garra, A., Barrat, F.J., Castro, A.G., Vicari, A., Hawrylowicz, C., 2008. Strategies for use of IL-10 or its antagonists in human disease. *Immunol. Rev.* 223, 114–131.
- Ropelewski, A.J., Nicholas Jr., H.B., Deerfield, D.W., 2004. Mathematically complete nucleotide and protein sequence searching using Ssearch. *Curr. Protoc. Bioinform.*, Unit 3; Chapter 3.
- Seki, E., Tsutsui, H., Tsuji, N.M., Hayashi, N., Adachi, K., Nakano, H., Futatsugi-Yumikura, S., Takeuchi, O., Hoshino, K., Akira, S., Fujimoto, J., Nakanishi, K., 2002. Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of *Listeria monocytogenes* in mice. *J. Immunol.* 169, 3863–3868.
- Sierro, F., Dubois, B., Coste, A., Kaiserlian, D., Kraehenbuhl, J.P., Sirard, J.C., 2001. Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13722–13727.
- Smith, K.D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M.A., Barrett, S.L., Cookson, B.T., Aderem, A., 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat. Immunol.* 4, 1247–1253.
- Smith, K.D., Ozinsky, A., 2002. Toll-like receptor-5 and the innate immune response to bacterial flagellin. *Curr. Top. Microbiol. Immunol.* 270, 93–108.
- Umetsu, S., Fujimoto, K., Jang, M.H., Yang, B.G., Jung, Y.J., Nishiyama, M., Sato, S., Tsujimura, T., Yamamoto, M., Yokota, Y., Kiyono, H., Miyasaka, M., Ishii, K.J., Akira, S., 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat. Immunol.* 9, 769–776.
- Umetsu, S., Jang, M.H., Chevrier, N., Guo, Z., Kumagai, Y., Yamamoto, M., Kato, H., Sougawa, N., Matsui, H., Kuwata, H., Hemmi, H., Coban, C., Kawai, T., Ishii, K.J., Takeuchi, O., Miyasaka, M., Takeda, K., Akira, S., 2006. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c⁺ lamina propria cells. *Nat. Immunol.* 7, 868–874.
- Waibler, Z., Anzaghe, M., Konur, A., Akira, S., Muller, W., Kalinke, U., 2008. Excessive CpG 1668 stimulation triggers IL-10 production by cDC that inhibits IFN-alpha responses by pDC. *Eur. J. Immunol.* 38, 3127–3137.
- Waibler, Z., Detje, C.N., Bell, J.C., Kalinke, U., 2007. Matrix protein mediated shutdown of host cell metabolism limits vesicular stomatitis virus-induced interferon-alpha responses to plasmacytoid dendritic cells. *Immunobiology* 212, 887–894.
- Way, S.S., Thompson, L.J., Lopes, J.E., Hajjar, A.M., Kollmann, T.R., Freitag, N.E., Wilson, C.B., 2004. Characterization of flagellin expression and its role in *Listeria monocytogenes* infection and immunity. *Cell Microbiol.* 6, 235–242.
- Wiedermann, U., 2005. Prophylaxis and therapy of allergy by mucosal tolerance induction with recombinant allergens or allergen constructs. *Curr. Drug Targets Inflamm. Allergy* 4, 577–583.
- Yeung, V.P., Gieni, R.S., Umetsu, D.T., DeKruyff, R.H., 1998. Heat-killed *Listeria monocytogenes* as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. *J. Immunol.* 161, 4146–4152.
- Young, L.J., Wilson, N.S., Schnorrer, P., Proietto, A., ten Broeke, T., Matsuki, Y., Mount, A.M., Belz, G.T., O'Keefe, M., Ohmura-Hoshino, M., Ishido, S., Stoorvogel, W., Heath, W.R., Shortman, K., Villadamos, J.A., 2008. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nat. Immunol.* 9, 1244–1252.