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Immune response profile elicited by the model antigen ovalbumin expressed in fusion with the bacterial OprI lipoprotein

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ABSTRACT

The use of immunogenic formulations targeting pattern recognition receptors towards modulation of immune responses is a promising strategy to develop better vaccines against infectious and malignant diseases. Molecules targeting TLR2 offer interesting properties that are relevant for vaccine development, including the possibility to covalently attach the lipidic ligands to the antigens. However, the type of immune response elicited by these formulations is still controversial. In this work, we used the model antigen ovalbumin (OVA) expressed in fusion with the bacterial lipoprotein OprI in order to characterize the immunomodulatory properties of this TLR ligand. Murine bone marrow-derived dendritic cells stimulated with OprI-OVA fusion lipoprotein produced high levels of the pro-inflammatory cytokines TNF-α and IL-6 and also IL-10, IL-12(p70) and IL-27, while TGF-β and IL-23 were not detected. Using OT-II and OT-I mice, an enhancement of MHC class II and class I antigen presentation was observed for the OVA antigen in fusion with OprI. Mice immunized by intraperitoneal route with this fusion lipoprotein in prime-boost protocols developed strong specific antibody responses including IgG1, IgG2c, IgG2b, IgG3 and IgE. These results, together with data obtained by restimulation of splenocytes from the immunized mice, point to an immune response profile that does not correspond to a strict Th1 or Th2 polarization. Finally, in a challenge experiment using a melanoma syngeneic mouse model (B16-OVA), prophylactic inoculation with OprI fused with the unrelated antigen eGFP increased the tumor growth, while the fusion with the tumor-associated antigen OVA delayed the tumor growth and increased mice survival.

1. Introduction

Exploring the activation of pattern recognition receptors (PRRs) for immunomodulation is presently a promising strategy to develop novel vaccines against infectious and malignant diseases (Coffman et al., 2010; Ishii and Akira, 2007; van Duin et al., 2006; Warshakoon et al., 2009). In order to better tailor PRR-targeting adjuvants, it is of major relevance to understand the impact of different innate stimuli on the development of the specific immune response. Targeting TLR2 offers interesting properties for vaccine development, including the possibility to covalently attach lipoproteic agonists to the antigens (Basto and Leitão, 2014). This ensures the direct activation of the antigen presenting cells (APCs) that capture the antigen and enables the PRR stimulation within the same phagosomes that contain antigens being processed, enabling to explore the TLR-regulated autonomous phagosome maturation (Blander, 2008).

TLR2-targeting formulations have been shown to potentiate antibody responses (Cote-Sierra et al., 2002; Huber et al., 2002; Jackson et al., 2004; Kiura et al., 2006; Redecke et al., 2004; Zhu et al., 2004) and to be able to promote T cell specific mechanisms, including cytotoxic T lymphocyte (CTL) activity (Baz et al., 2008; Chua et al., 2008; Jackson et al., 2004; Khan et al., 2007; Lau et al., 2006; Zhang et al., 2005). Physical properties conferred by the lipid moiety of lipidated antigens have been suggested to play a role on

Abbreviations: APC, antigen presenting cells; bmDC, bone marrow-derived dendritic cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; eGFP, enhanced green fluorescent protein; EU, endotoxin unit; LPS, lipopolysaccharides; MHC, major histocompatibility complex; OM, outer membrane; OprI, outer membrane lipoprotein I; OVA, ovalbumin; PRR, pattern recognition receptor; TLR, Toll-like receptor.

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their cross-presentation by APCs (Andrieu et al., 2000; Martinon et al., 1992) but the activation through TLR2 has also been demonstrated to be determinant on the induction of *in vivo* CTL responses (Khan et al., 2009; Zhang et al., 2009). The capacity to induce mucosal immunity was also attributed to TLR2 activation either by mucosal antigen administration (Baier et al., 2000; Jackson et al., 2004; Lee et al., 2011; Nardelli et al., 1994; Zhang et al., 2009) or by imprinting lymphocyte tropism to mucosae through extramucosal dendritic cell (DC) stimulation (Wang et al., 2011). However, the type of immune response elicited by TLR2-targeting formulations is still controversial and the factors involved in the variable immunological outcomes reported in the literature are not yet clarified. In fact, a role for TLR2 in the induction of Th1 (Chua et al., 2008; Cote-Sierra et al., 2002; Ghielmetti et al., 2005; Huang et al., 2012; Huber et al., 2002; Imanishi et al., 2007; Sieling et al., 2003; Thoma-Uszynski et al., 2000; Watanabe et al., 2004; Zhu et al., 2004), Th2 (Agrawal et al., 2003; Dillon et al., 2004; Kiura et al., 2006; Pulendran et al., 2001; Redecke et al., 2004), Th17 (Aliahmadi et al., 2009) and regulatory (Dillon et al., 2006; Karumuthil-Melethil et al., 2008; Kiura et al., 2011; Manicassamy et al., 2009; Pulendran et al., 2010; Yamazaki et al., 2011) mechanisms has been reported and both inflammatory and regulatory roles have been attributed to TLR2 activation (Mele and Madrenas, 2010).

We have set-up a new *Escherichia coli* cloning system that allows the expression and purification of antigens in fusion with the OprI lipoprotein (Basto et al., 2012). OprI is the major outer membrane lipoprotein of the Gram negative bacterium *Pseudomonas aeruginosa* and we have previously shown that this lipoprotein acquires the triacylated structure typical of TLR2/1 ligands when it is expressed in *E. coli*. Upon induction of expression, the fusion products are first translated as prolipoproteins with an N-terminal signal peptide and are then translocated across the inner membrane to the periplasmic space where they are processed to their mature triacylated forms which, finally, are found anchored in the outer membrane of the expression host. Thus, to purify fusion proteins with the triacylated TLR2/1-activating forms, outer membrane fractions are first prepared in order to segregate immature forms found in the cytoplasmic/inner membrane and periplasmic fractions and, after a step of delipidation and depyrogenation of outer membranes, recombinant lipoproteins are purified by affinity chromatography (Basto et al., 2014).

Using our OprI-based *E. coli* cloning system we have previously constructed two plasmids for the production of lipidated products containing OprI lipoprotein at the N-terminus fused with sequences of the model antigens ovalbumin (OVA) and eGFP (Basto et al., 2014). Here, we produced these fusion antigens to address the immunomodulatory properties of the natural TLR ligand OprI. Our results demonstrate the capacity of OprI lipoprotein to enhance MHC class II- and class I-restricted antigen presentation and allowed us to define its *in vivo* immunomodulatory properties. These were characterized by mixed Th polarization properties and anti-tumor specific immune mechanisms as demonstrated by a delay in the development of the B16-OVA melanoma model and higher survival rates in mice immunized with OprI fused to the OVA tumor-associated antigen. Interestingly, mice immunized with the non-related OprI-eGFP antigen showed an enhancement in tumor growth.

2. Materials and methods

2.1. Production of the recombinant fusion lipoproteins OprI-OVApx and OprI-eGFP

For the expression of the model antigens OVA and eGFP fused with the OprI lipoprotein we used the previously described plasmids pOLT7-OVApx, containing the OVA partial sequence coding amino acids 203 to 386, and pOLT7-eGFP, containing the eGFP sequence coding amino acids 2 to 241 (Basto et al., 2014). Both plasmids express the antigens with OprI at the N-terminus and with a C-terminal histidine tail for purification by metal affinity chromatography with Ni^{2+}. Bacterial outer membranes were prepared by differential solubilization with N-lauroylsarcosine sodium salt solution (sarkosyl) and outer membrane depyrogenation was performed by hot phenol/water extraction as previously described (Basto et al., 2014).

For the denaturing affinity chromatography, the phenol-treated outer membrane protein precipitates from each 1 L-culture were re-suspended in 10 mL of binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 6 M guanidine hydrochloride, 2% (v/v) Triton X-100, 20 mM β-mercaptoethanol and protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche), pH 7.4), at room temperature, overnight, with mild agitation. Next day, samples were centrifuged at 20,000 × *g* for 15 min at 4 °C and, after adjusting pH to 7.4, the supernatants were incubated with Ni Sepharose 6 Fast Flow (GE Healthcare) at the ratio 1 mL protein sample/100 μL resin, for at least 2 h at room temperature. After transferring to an empty PD10 column (GE Healthcare), the chromatographic columns were washed with 20 resin-bed volumes of buffer W1-TX100 (20 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 8 M urea, 2% (v/v) Triton X-100, pH 7.4) followed by a wash at 4 °C performed with 80 resin-bed volumes of the buffer W1-TX114 (20 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 8 M urea, 0.1% Triton X-114, pH 7.4). The detergents were removed from the columns with 20 resin-bed volumes of buffer W2 (20 mM NaH_2PO_4, 0.5 M NaCl, 8 M urea, pH 7.4) and the elution was performed in acidic conditions (20 mM NaH_2PO_4, 0.5 NaCl M, 8 M urea, pH 4.5). The eluates were dialysed against PBS, aliquoted in Protein LoBind tubes (Eppendorf), flash frozen in liquid nitrogen and stored at −80 °C.

2.2. Protein analysis and endotoxin quantification assays

Purity of the protein samples was evaluated by SDS-PAGE stained with silver nitrate. Protein quantification was performed according to the microplate procedure of the BCA Protein Assay Kit (Thermo Scientific Pierce) with incubations at 60 °C for 30 min. Endotoxin levels were quantified by the Limulus Amebocyte Lysate (LAL) Kinetic-QCL (Lonza) and recombinant lipoproteins showed less than 0.02 EU/μg.

2.3. Mice and cell lines

All animal-involving procedures were conducted in compliance with the Portuguese (DL no. 129/92 and Portaria no. 1005/92) and European Union (Directive no. 86/609/EEC) legislation for the use of animals for experimental purposes. Mouse manipulation protocols were approved by the Faculty of Veterinary Medicine Ethics and Animal Welfare Committee. C57BL/6 male mice with 6 to 8 weeks of age were housed in individually ventilated cages with sawdust as bedding, in a room with controlled temperature between 22 °C and 25 °C and a 12-hours-light/12-hours-dark cycle. They were fed standard laboratory diet and water *ad libitum*. OT-I and OT-II $RAG2^{-/-}$ mice were kindly provided by Dr. Luís Graça from Instituto de Medicina Molecular, Lisboa, Portugal. The B16-OVA tumor cell line, a melanoma model expressing the ovalbumin antigen, was kindly supplied by Dr. Jon Ulf Hansen from Statens Serum Institut, Copenhagen, Denmark.

2.4. Dendritic cells differentiation

Dendritic cells were derived from bone marrow precursors obtained from femurs and tibias of naïve C57BL/6 mice, based on