A Polymeric Bacterial Protein Activates Dendritic Cells via TLR4[1]

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The enzyme lumazine synthase from *Brucella* spp. (BLS) is a highly immunogenic protein that folds as a stable dimer of pentamers. It is possible to insert foreign peptides and proteins at the 10 N terminus of BLS without disrupting its general folding, and these chimeras are very efficient to elicit systemic and oral immunity without adjuvants. In this study, we show that BLS stimulates bone marrow dendritic cells from mice in vitro to up-regulate the levels of costimulatory molecules (CD40, CD80, and CD86) and major histocompatibility class II Ag. Furthermore, the mRNA levels of several chemokines are increased, and proinflammatory cytokine secretion is induced upon exposure to BLS. In vivo, BLS increases the number of dendritic cells and their expression of CD62L in the draining lymph node. All of the observed effects are dependent on TLR4, and clearly independent of LPS contamination. The described characteristics of BLS make this protein an excellent candidate for vaccine development. ***The Journal of Immunology,* 2006, 176: 2366–2372.**

Over recent years, a clear relationship between protein quaternary and higher order organization and immunogenicity has emerged (1–6). There is a close correlation between the degree of repetitiveness and organization of an Ag and the efficiency of induction of the B cell immune response (1, 2). Many viruses exhibit a quasicrystalline, highly organized surface that displays a regular array of epitopes, which efficiently cross-link epitope-specific membrane Igs on B cells (7). A very successful example of the use of virus-like particles as vaccines is the surface Ag of hepatitis B virus. Expression of the S domain in yeast results in the production of 22-nm virus-like particles with ~100 polypeptides per particle. This Ag elicits high levels of Abs to hepatitis B surface Ag and is now widely used as a vaccine against hepatitis B (8).

Molecular order could affect immunogenicity in at least three related ways. One is the capacity to efficiently cross-link Ag-specific surface Igs on B cells (BCRs). A repetitive and spatially ordered presence of the same epitope would produce a strong signal transduction mediated by BCRs, as has been described using haptens as model Ags (9). The second effect is that a homopolymeric quaternary structural order implies an increase on the overall stability of the polymer. The higher stability increases the $t_{1/2}$, and thus the probability of effectively stimulating Ag-specific B lymphocytes.

As a third factor, the ability of protein particles to induce potent immune responses suggests that their highly ordered structure could impact APCs, and especially dendritic cells (DCs),[4] through pattern recognition receptors, including the Toll family. Importantly, the stimulation of DC innate responses could be responsible for shaping strong adaptive immune responses to these pathogen or vaccine Ags.

The enzyme lumazine synthase from *Brucella* spp. (BLS) is a highly immunogenic protein (10–12), and behaves as a potent oral or systemic immunogen when injected as a protein or as a DNA vaccine (G. Rosas, G. Fragoso, N. Ainciart, F. Esquivel, A. Santana, R. Bobes, P. Berguer, A. Toledo, C. Cruz-Revilla, G. Meneses, et al., submitted for publication) (13, 14). BLS has been used as a protein carrier of foreign peptides and proteins (15–17). Crystallographic and spectroscopic solution studies allowed us to determine the mechanisms of dissociation and unfolding of the decameric structure, serving as a platform for protein engineering purposes (18, 19). BLS folds as a highly stable dimer of pentamers, displaying an intertwined structure in which each monomer has ~45% of its exposed surface area buried on monomer-monomer and pentamer-pentamer contacts (18, 19). In this regard, the high immunogenicity and stability of the decameric BLS resemble the cases of the cholera toxin B subunit and the heat-labile toxin of *Escherichia coli*, two pentameric and highly stable bacterial proteins (20, 21). Noteworthily, BLS is resistant to urea denaturation and is more stable to thermal denaturation than the cholera toxin.

In the present work, we studied the capacity of BLS to activate and mature DCs. The results presented in this study clearly show that BLS is a potent activator of bone marrow DCs (BMDCs) via the TLR4, explaining at least in part its immunogenic properties.

Materials and Methods

Mice

BALB/cJ and congenic C.C3H-Tlr4^{lps-d} mice, C57BL/10J, C57BL/10ScNJ, and C57BL/6J mice obtained from The Jackson Laboratory, and TLR2 knockout (KO) mice (22) were bred in the animal facility of the División Medicina Experimental, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina. TLR2 KO mice were provided by S. Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). All mice were bred under specific

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[4] Abbreviations used in this paper: DC, dendritic cell; BLS, *Brucella* spp. lumazine synthase; BMDC, bone marrow DC; KO, knockout; mGM-CSF, mouse GM-CSF; PGN, peptidoglycan; PMB, polymyxin B; RPA, RNase protection assay.

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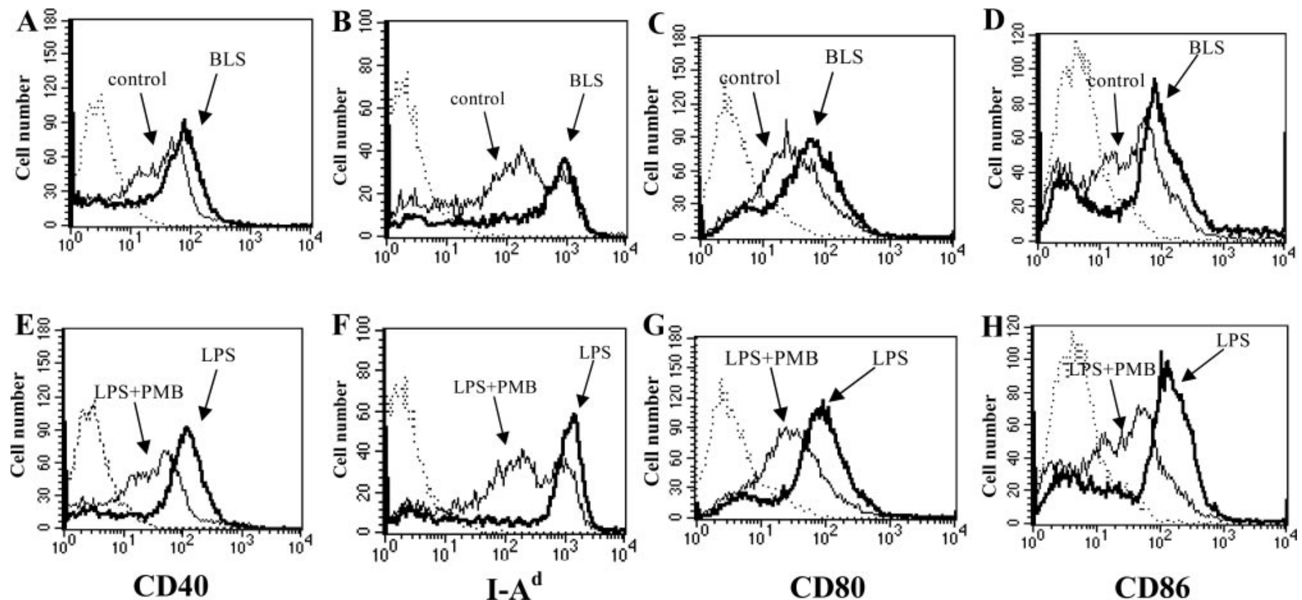

FIGURE 1. Expression of costimulatory molecules on BMDCs after BLS stimulation. BMDCs from BALB/c mice were incubated for 18 h with PBS preincubated with PMB-agarose (control) or 90 μg of BLS preincubated with PMB-agarose (*A–D*) or with 100 ng of LPS or 100 ng of LPS preincubated with PMB-agarose (*E–H*). Expression of CD40 (*A* and *E*), I-A^d (*B* and *F*), CD80 (*C* and *G*), or CD86 (*D* and *H*) on CD11c$^+$ cells was analyzed by FACS. The dashed lines represent isotype Ab control. The histograms depict representative results.

pathogen-free conditions and were used at 8–10 wk of age. Mice were housed and treated according to the policies of the Academia Nacional de Medicina (National Institutes of Health Guide for the Care and Use of Laboratory
Animals) (23).

Generation of mouse BMDCs

BMDCs were generated according to the method of Lutz et al. (24). Briefly, femurs and tibiae of mice were removed and freed of muscles and tendons. The bones were placed in 70% ethanol for 120 s and subsequently washed in PBS. Both bone ends were cut off, and the marrow was flushed out with RPMI 1640 medium. The cells were centrifuged for 8 min at 360 × *g*. The cells were seeded in bacterial petri dishes at a density of 2 × 10^5/ml in 10 ml of RPMI 1640 with 2 nM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin, 50 μM 2-ME, and 10% FBS (R10 medium) supplemented with 30% mouse GM-CSF (mGM-CSF)-containing supernatant from a J558 cell line stably transfected with mGM-CSF. On day 3 of culture, another 10 ml of R10 medium with mGM-CSF was added. On days 6 and 8, the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 20 ml of R10 medium with mGM-CSF. On day 9, nonadherent cells were collected by gentle pipetting, centrifuged at 300 × *g* for 10 min, and resuspended in R10 medium; 85% of these cells were CD11c positive (data not shown).

Protein expression and purification

The cloning, recombinant expression, and purification of BLS protein were described previously (15, 25). Briefly, the BLS gene cloned in pET11a vector (Novagen) was transformed and expressed as inclusion bodies on strain BL21(DE3) *E. coli*. The inclusion bodies were solubilized in 50 mM Tris, 5 mM EDTA, and 8 M urea (pH 8.0) at room temperature overnight with agitation. The solubilized material was refolded by dialysis during 72 h against PBS containing 1 mM DTT. This preparation was purified in a Mono-Q column in a fast performance liquid chromatography apparatus (Amersham Biosciences) using a linear gradient of NaCl between 0 and 1 M in buffer 50 mM Tris (pH 8.5). The peak enriched with BLS was further purified on a Superdex-200 column with PBS and 1 mM DTT. The purity of the BLS preparation was determined on SDS-PAGE 15% (w/v) polyacrylamide gels. BLS was concentrated (2 mg/ml), frozen in liquid N_2, and stored at −20°C. Purified BLS was detoxified by two incubations of 1 mg of BLS with 500 μl of polymyxin B-agarose (PMB-agarose) overnight at 4°C. The supernatant was assayed for LPS content using the *Limulus* amebocyte lysate kit, giving a value of 0.10–0.15 ng/ml.

Stimulation of DCs

BMDCs were cultured for 18 h in R10 medium with LPS from *E. coli* 0111:B4 (100 ng/ml; Sigma-Aldrich) or BLS (18, 90, or 180 μg) preincubated with PMB or PMB-agarose. For blocking experiments, BLS was preincubated with rabbit Fab anti-BLS polyclonal serum at 37°C for 1 h. C.C3H-Tlr4^{lps-d} and C57BL/10ScNJ BMDCs were incubated with 20 μg/ml peptidoglycan (PGN) of *Staphylococcus aureus* (Fluka BioChemica) as a positive control.

Flow cytometry

Cells were stained with the following mAbs (BD Pharmingen) and subjected to FACS analysis: PE-conjugated anti-CD11c (HL3), FITC-conjugated anti-CD40 (HM40-3), FITC-conjugated anti-CD80 (16-10A1), FITC-conjugated anti-CD86 (GL-1), FITC-conjugated anti-I-A^d (AMS-32.1), and FITC-conjugated anti-CD62L (Mel-14). Cells were acquired on

Table I. *Expression of CD40, I-A^d, CD80, or CD86 on BMDCs after BLS stimulation*[a]

	CD40	I-Ad	CD86	CD80
BLS	62 ± 5** (88 ± 12*)	61 ± 8* (1200 ± 142*)	65 ± 6** (149 ± 14*)	68 ± 8** (95 ± 11*)
Control	32 ± 4 (62 ± 3)	35 ± 3 (512 ± 65)	18 ± 2 (81 ± 4)	50 ± 6 (70 ± 3)
LPS	79 ± 8 (187 ± 17)	76 ± 10 (1400 ± 112)	62 ± 7 (170 ± 7)	84 ± 16 (120 ± 12)
LPS + PMB	36 ± 5 (65 ± 5)	32 ± 4 (521 ± 80)	21 ± 3 (75 ± 10)	54 ± 5 (71 ± 7)

[a] Costimulatory molecule expression by BMDCs from BALB/c mice was determined by two-color fluorescence cytometry as in Fig. 1. Values represent the mean percentages of CD86^{high}, CD80^{high}, CD40^{high}, or I-A$^{d\ high}$ gated on CD11c$^+$ cells ± SD ($n = 8$) or the mean fluorescence intensity (between parentheses) of CD86$^+$, CD80$^+$, CD40$^+$, or I-A^{d+} gated on CD11c$^+$ cells ± SD ($n = 8$). *, $p < 0.02$, **, $p < 0.001$ compared with the control group. Pooled data of three independent experiments are shown.

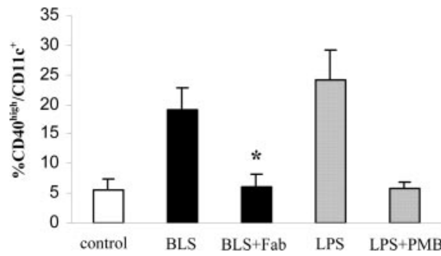

FIGURE 2. Effect of the preincubation of BLS with an anti-BLS Fab on BMDC stimulation. Percentage of BALB/c mice BMDCs expressing high levels of CD40 ($CD40^{high}/CD11c^{+}$) after 18 h of incubation with 90 μg of detoxified BLS (BLS), detoxified PBS (control), detoxified BLS preincubated with rabbit anti-BLS Fab (BLS+Fab), 100 ng of LPS, or 100 ng of LPS preincubated with PMB-agarose (LPS+PMB). Results are expressed as the mean percentage + SD ($n = 6$). *, $p < 0.002$ compared with BLS. Data of two independent experiments have been pooled.

a FACScan cytometer (BD Biosciences). Data were analyzed by using CellQuest software (BD Immunocytometry Systems).

Determination of cytokines

Cytokine contents in the DC supernatants after 18 h of stimulation were determined using ELISA for TNF-α, IL-6, IL-12 p70, and IL-4 (all OptEIA sets; BD Pharmingen), following the manufacturer's instructions. The reaction was developed by adding 50 μl of a solution containing 2 μg/μl ortho-phenylenediamine and 0.03% H_2O_2 in 0.1 M citrate-phosphate buffer and was stopped with 50 μl of 4 N H_2SO_4. The final color was read at 492 nm in an ELISA reader (SLT Labinstruments). The detection limit was 15.6 pg/ml for TNF-α and IL-6, 31.3 pg/ml for IL-12 p70, and 7.6 pg/ml for IL-4. Data are presented as means ± SDs of triplicate observations of three independent experiments.

RNase protection assays (RPAs)

Total RNA (4–10 μg) was analyzed by RPAs using the BD Pharmingen Riboquant kit, according to the manufacturer's recommendations. The mCK-5c multiprobe template set was used. The bands corresponding to the various chemokines were quantified using a Scion Image software. For quantitation, chemokine levels were expressed as percentages of the mean levels of the L32 and GAPHD housekeeping genes for each RNA sample. Data are presented as the increase (*n*-fold) in mRNA expression in stimulated vs unstimulated cells.

Recruitment assay

Ten-week-old BALB/c mice and C.C3H-$Tlr4^{lps-d}$ mice were given a 50-μl s.c. injection of 10 or 50 μg of BLS in the right hind footpad and a 50-μl injection of PBS in the left hind footpad. Both BLS and PBS were previously incubated with PMB-agarose. At 48 h, the popliteal lymph nodes were removed and processed for cytofluorometric analysis. Blocking of DC migration to the lymph nodes was achieved by a single dose of i.v. injection of purified anti-CD62L (L-selectin) Abs (clone MEL-14; BD Pharmingen) 1 day before BLS inoculation (26). Control mice were i.v. injected with rat IgG2a. A group of C.C3H-$Tlr4^{lps-d}$ mice was injected in the right hind footpad with 20 μg of PGN as a positive control.

Table II. *Cytokines production by BLS-stimulated BMDCs*[a]

	IL-6	IL-12 p70	TNF-α	IL-4
BLS	22,571 ± 4,850	1,255 ± 512	320 ± 110	27.4 ± 20.2
Control	ND	ND	ND	ND
LPS	12,121 ± 5,433	9,560 ± 856	2,317 ± 1,102	ND
LPS + PMB	ND	ND	ND	ND

[a] BMDCs from BALB/c mice were incubated with 90 μg of detoxified BLS, detoxified PBS (control), 100 ng/ml LPS, or 100 ng/ml LPS preincubated with PMB-agarose. After 18 h, the concentrations of IL-6, IL-12p70, TNF-α, or IL-4 in the supernatant were measured by ELISA. The results are presented as means ± SD ($n = 8$) in pg/ml. Pooled data of three independent experiments are shown.

PMB treatment

A total of 1 mg of BLS or 100 ng of LPS in PBS or PBS only was preincubated before use with 0.5 ml of PMB-agarose (Sigma-Aldrich) overnight at 4°C with agitation. The PMB-agarose was centrifuged, and the supernatant was collected. BLS or PBS was subjected to an additional PMB-agarose overnight incubation. They were then added to DCs. After 18 h, activation of the DCs was assessed, as noted above. For the in vivo experiments, BLS and PBS were also detoxified, as described.

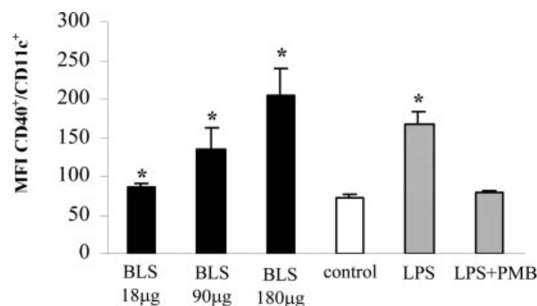

FIGURE 3. Dose-dependent effect of BLS in BMDC activation. BMDCs from BALB/c mice were incubated for 18 h with 18, 90, or 180 μg of detoxified BLS, detoxified PBS (control), 100 ng of LPS, or 100 ng of LPS preincubated with PMB-agarose. Values represent mean fluorescence intensity (MFI) of CD40-positive BMDCs + SD ($n = 9$). *, $p < 0.01$ compared with the control group. Data of three independent experiments have been pooled.

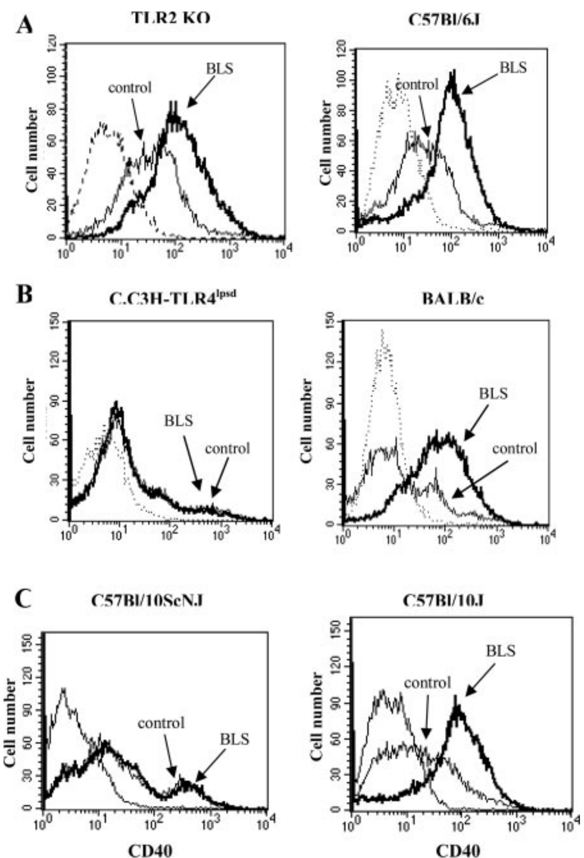

FIGURE 4. Expression of CD40 on BMDCs from TLR2 KO mice, C.C3H-$Tlr4^{lps-d}$ mice, and C57BL/10ScNJ mice after BLS stimulation. BMDCs from each mice strain and from the respective control strains were incubated for 18 h with detoxified PBS (control) or 90 μg of detoxified BLS. Expression of CD40 on $CD11c^{+}$ cells was analyzed by FACS. *A*, TLR2 KO (*left*), C57BL/6J (*right*); *B*, C.C3H-$Tlr4^{lps-d}$ (*left*), BALB/c (*right*); and *C*, C57BL/10ScNJ (*left*), C57BL/10J (*right*). The dashed lines represent isotype Ab control. The histograms depict representative results.

Table III. *Expression of CD40, CD80, or CD86 on TLR2 KO BMDCs after BLS stimulation*[a]

	CD86	CD80	CD40
TLR2 KO + BLS	8.5 ± 1.2* (79 ± 6*)	38.4 ± 3.6* (70 ± 5*)	33.0 ± 5.1* (191 ± 29*)
TLR2 KO + PBS	5.1 ± 0.5 (72 ± 2)	23.9 ± 1.8 (55 ± 5)	9.5 ± 2.0 (96 ± 13)
C57BL/6J + BLS	11.2 ± 1.9 (97 ± 1)	52.1 ± 1.0 (89 ± 3)	29.9 ± 2.8 (160 ± 12)
C57BL/6J + PBS	4.2 ± 0.5 (64 ± 1)	32.0 ± 3.1 (62 ± 1)	8.1 ± 1.1 (87 ± 15)

[a] Costimulatory molecule expression by TLR2 KO or C57BL/6J mice BMDCs was determined by FACS analysis. Values represent the mean percentages of $CD86^{high}$, $CD80^{high}$, or $CD40^{high}$ gated on $CD11c^+$ cells ± SD (n = 6) or the mean fluorescence intensity (between parentheses) of $CD86^+$, $CD80^+$, or $CD40^+$ gated on $CD11c^+$ cells ± SD (n = 6). Results were significantly different from BMDCs stimulated with PBS (control): *, $p < 0.01$. Pooled data of two independent experiments are shown.

Measurement of endotoxin activity

The *Limulus* amebocyte lysate test was performed using the Gel-clot method for the detection and quantification of Gram-negative bacterial endotoxins (LPS) (Pyrotell), following the manufacturer's instructions. The detection limit of the assay was 0.03 EU/ml (0.003 ng/ml).

Statistical analysis

Results were expressed as the mean ± SD. Levels of significance were determined using two-tailed Student's t test, and a confidence level of greater than 95% ($p < 0.05$) was used to establish statistical significance.

Results

BLS increases the expression of BMDC costimulatory molecules

To test whether the high immunogenicity of BLS is related to its ability to activate DCs, we first investigated the in vitro effect of BLS on the expression of BMDC costimulatory molecules. Taking into account that this protein is produced by genetically engineered *E. coli* cells, BLS preparations were preincubated twice with PMB-agarose to remove the endotoxin activity. The final BLS preparation contained 0.10–0.15 ng/ml LPS (<0.38 pg LPS/μg BLS). As a control, 100 ng/ml LPS was preincubated with the same concentrations of PMB-agarose used to detoxify BLS preparations, and the final amount of remnant LPS was measured. These procedures were conducted in each of the individual experiments reported in this work. BALB/c BMDCs were first incubated with 90 μg of BLS for 18 h. The maturation status of BMDCs was determined by the expression of cell surface markers, such as CD40, CD80 (B7.1), CD86 (B7.2), and $I\text{-}A^d$ on CD11c-positive cells. Following BLS exposure, BMDCs increased the expression of cell surface costimulatory molecules, including those essential for optimal activation of T cells: CD40, CD80, and CD86, as assessed by cytofluorometric analysis (Fig. 1 and Table I). A cell surface molecule important for Ag presentation, such as major histocompatibility class II Ag $I\text{-}A^d$, was also up-regulated on BLS-exposed BMDCs (Fig. 1 and Table I). The increments of the expression levels of all these markers induced with 100 ng/ml LPS were reverted to the control level by treating LPS with PMB-agarose (Fig. 1 and Table I), 60–70 pg being the amount of LPS in the culture. This amount of remnant LPS is larger than the LPS content of the added BLS (33 pg), thus assuring that the observed activation is not due to LPS contamination. BLS was also able to induce significant increases in the expression of costimulatory molecules when cocultured with BMDCs from C3H/HeN and C57BL/6J mice (data not shown). Preincubation of BLS with an anti-BLS polyclonal Fab blocked maturation of BMDCs (Fig. 2). BMDCs from BALB/c mice were then incubated with 18, 90, or 180 μg of BLS, and FACS analysis was conducted to determine the level of expression of maturation markers. Results obtained showed a dose-dependent effect of BLS in BMDC activation (Fig. 3, CD40 as an example).

BLS is able to induce the production of proinflammatory cytokines

In addition to phenotypic maturation, BMDCs should also undergo functional maturation to stimulate optimal innate and adaptive immune responses. To assess functional maturation, BLS-treated BMDCs from BALB/c mice were analyzed for cytokine production. We tested IL-6, IL-12p70, TNF-α, and IL-4 levels in the supernatants of BMDCs stimulated with 90 μg of BLS for 18 h, using sandwich ELISAs. As shown in Table II, incubation of BMDCs with BLS greatly increased the production of cytokines IL-6, IL-12p70, and TNF-α. BLS induced low levels of IL-4 (Table II). As a control, LPS treated with PMB-agarose did not induce detectable cytokine production (Table II). These results show that BLS is able to induce in BMDCs the production of proinflammatory cytokines essential for potentiation of effective immune responses. Thus, results reported in this study show that BLS is able to induce murine BMDC phenotypic and functional maturation.

BLS induction of BMDC maturation is TLR4 dependent

To test whether TLRs have a role in the BLS-dependent maturation of BMDCs, we analyzed the expression of costimulatory molecules in BMDCs from TLR2- and TLR4-deficient mice and their respective control strains. In BMDCs from TLR2 KO mice, the activation markers were significantly increased to the same extent

Table IV. *Expression of CD40, CD80, or CD86 on C.C3H-Tlr4$^{lps\text{-}d}$ BMDCs after BLS stimulation*[a]

	CD86	CD80	CD40
C.C3H-Tlr4$^{lps\text{-}d}$ + BLS	3.8 ± 4.6 (22 ± 2)	12.9 ± 2.0 (18 ± 1)	5.1 ± 1.8 (141 ± 16)
C.C3H-Tlr4$^{lps\text{-}d}$ + PBS	4.0 ± 1.0 (23 ± 2)	14.1 ± 1.6 (20 ± 1)	6.7 ± 1.0 (149 ± 4)
C.C3H-Tlr4$^{lps\text{-}d}$ + PGN	23.9 ± 3.8 (59 ± 9)	24.1 ± 5.1 (40 ± 5)	30.0 ± 6.9 (191 ± 15)
BALB/c + BLS	15 ± 1.2 (41 ± 1)	36.1 ± 0.7 (49 ± 5)	18.1 ± 3.3 (170 ± 9)
BALB/c + PBS	3.0 ± 0.8 (21 ± 2)	18.1 ± 1.1 (29 ± 3)	4.5 ± 0.9 (120 ± 5)

[a] Costimulatory molecule expression by BMDCs was determined by FACS analysis. Values represent the mean percentages of $CD86^{high}$, $CD80^{high}$, or $CD40^{high}$ gated on $CD11c^+$ cells ± SD (n = 9) or the mean fluorescence intensity (between parentheses) of $CD86^+$, $CD80^+$, or $CD40^+$ gated on $CD11c^+$ cells ± SD (n = 9). Pooled data of three independent experiments are shown.

Table V. *Proinflammatory cytokines production by TLR4-deficient BMDCs*[a]

	IL-6	IL-12 p70	TNF-α
C.C3H-Tlr4^{lps-d} + BLS	264 ± 562	ND	ND
C.C3H-Tlr4^{lps-d} + PBS	152 ± 116	ND	ND
BALB/c + BLS	26,500 ± 9,275	3,104 ± 980	587 ± 177
BALB/c + PBS	ND	ND	ND

[a] BMDCs from C.C3H-Tlr4^{lps-d} or BALB/c mice were incubated with 180 μg of detoxified BLS or detoxified PBS (control). After 18 h, the concentrations of IL-6, IL-12p70, or TNF-α in the culture supernatant was measured by ELISA. The results are presented as means ± SD (n = 6) in pg/ml. Pooled data of two independent experiments are shown.

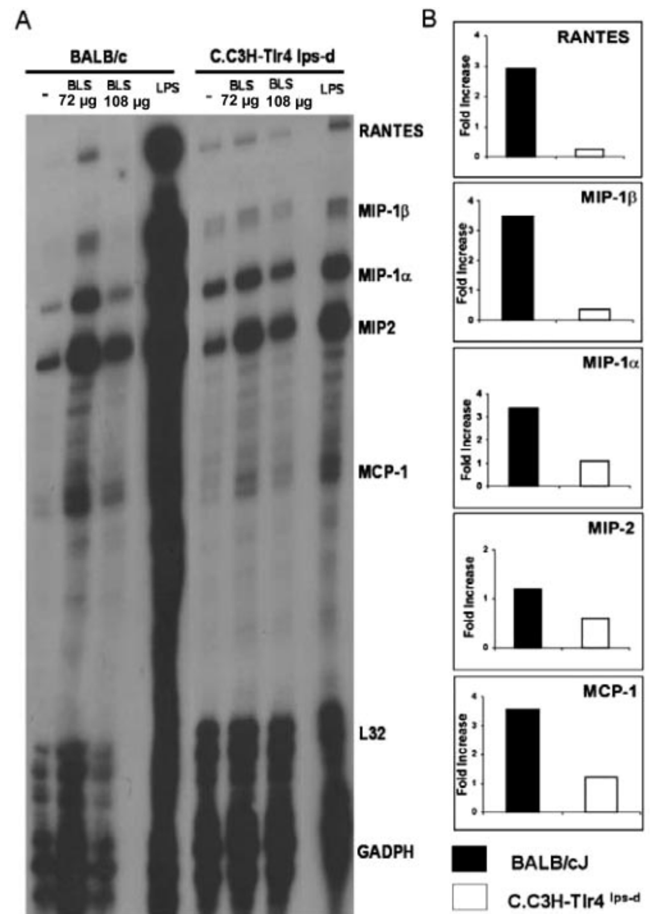

FIGURE 5. Chemokine mRNA levels in BLS-induced BMDCs. BMDCs from C.C3H-Tlr4^{lps-d} or BALB/c mice were incubated with 72 or 108 μg of detoxified BLS or detoxified PBS (−). After 18 h, chemokine mRNA levels were measured by RPA, using multiprobe sets (*A*). Quantitation of relative expression of chemokine mRNAs (*B*), as described in *Materials and Methods*. One experiment of two is shown.

as in control mice (Fig. 4*A* and Table III). On the contrary, BLS was not able to increase the expression of costimulatory molecules on BMDCs from C.C3H-Tlr4^{lps-d} mice, which have a point mutation in their *TLR4* gene that makes the molecule unable to transduce signals (Fig. 4*B* and Table IV). Similar results (Fig. 4*C*) were obtained in C57BL/10ScNJ mice, which have a null mutation in their *tlr4* gene. The lack of response to BLS stimulation in TLR4-negative BMDCs rules out possible contamination with lipoproteins.

BLS did not induce TNF-α, IL-6, or IL-12p70 significant secretion by C.C3H-Tlr4^{lps-d} BMDCs (Table V). We also analyzed chemokine mRNA levels in BLS-stimulated BMDCs obtained from BALB/c and C.C3H-Tlr4^{lps-d} mice by RPAs. After 18 h of BLS stimulation, BALB/c BMDCs showed significant increases in the levels of MIP-1α, MIP1β, MIP-2, MCP-1, and RANTES mRNAs. In contrast, none of these mRNAs was induced by BLS in C.C3H-Tlr4^{lps-d} BMDCs (Fig. 5). Taken together, these results show that BLS is able to induce BMDC maturation through interaction with TLR4 molecules.

BLS inoculation induces a TLR4-dependent recruitment of CD11c$^+$ cells to lymph nodes

A total of 10 or 50 μg of BLS was s.c. injected into the footpads of BALB/c mice. Two days later, popliteal lymph nodes were excised, and the absolute number of CD11c$^+$ cells was assessed using FACS analysis. As can be observed in Fig. 6*A*, BLS inoculation in BALB/c mice was able to induce a significant increase in the absolute number of CD11c$^+$ cells in the draining lymph node. A marked increase in the percentage and absolute number of DCs expressing high levels of CD62L could be recorded (Fig. 6*B*). BALB/c mice were i.v. inoculated with anti-CD62L mAb 1 day before BLS footpad inoculation. As can be observed in Fig. 6*C*, the absolute number of CD11c$^+$ cells in the draining lymph node decreased to control levels, thus demonstrating that BLS induces the occurrence of a CD62L-dependent recruitment of blood DC precursors. BLS inoculation in C.C3H-Tlr4^{lps-d} mice did not lead to increases either in the absolute number of CD11c$^+$ cells or in the absolute number of DCs expressing high levels of CD62L (Fig. 6, *A* and *B*). These results show that BLS inoculation is able to induce a TLR4-dependent recruitment of DCs to the draining lymph node.

Discussion

DCs are specialized APCs, critical for initiating and regulating immune responses. Conserved structural motifs on pathogens trigger pattern recognition receptors present on DCs. An important class of such receptors is constituted by the TLRs. TLRs are a key component of the innate immune system that detect microbial infection and trigger antimicrobial host defense responses. They activate multiple steps in the inflammatory reactions that help to eliminate the invading pathogens and coordinate systemic defenses (27). In addition, TLRs control multiple DC functions and activate signals that are critically involved in the initiation of adaptive immune responses. TLR signaling in DCs causes an increase in the display of MHC peptide ligands for T cell recognition, up-regulation of costimulatory molecules important for T cell clonal expansion, and secretion of immunomodulatory cytokines, which direct T cell differentiation into effectors (28).

Recent findings on the function of TLRs and their adaptors facilitated the elucidation of the molecular basis of adjuvant activity (29–31). TLR signaling was found to induce IFNs, chemokines, proinflammatory cytokines, and mature DCs for enhanced efficiency in Ag presentation. Although an accepted hallmark of DC maturation as a consequence of TLR signaling is an eventual down-regulation of DC endocytic capacity, it has been shown recently that TLR ligands first acutely stimulate Ag macropinocytosis, leading to enhanced presentation on class I and class II MHC molecules (32).

We had demonstrated previously that systemic and oral immunization with the recombinant enzyme lumazine synthase from *Brucella* spp. in the absence of adjuvants induced high titers of Abs in mice (12). To test whether the high immunogenicity of BLS is related to its ability to activate DCs through TLRs, we investigated the in vitro and in vivo effects of BLS on DCs. As BLS is produced by genetically engineered *E. coli* cells, the final preparations may be contaminated with bacterial cell-wall products such

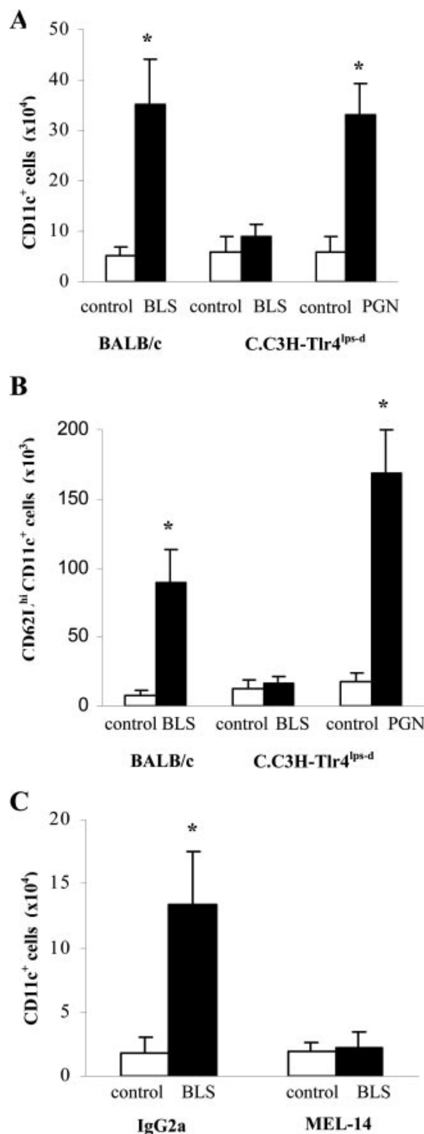

FIGURE 6. DC recruitment to lymph nodes. BALB/c or C.C3H-Tlr4^{lps-d} mice were inoculated in the right hind footpad with 10 μg (*A* and *B*) or 50 μg (*C*) of detoxified BLS and in the left hind footpad with detoxified PBS (control). A group of C.C3H-Tlr4^{lps-d} mice was inoculated with 20 μg of PGN (*A* and *B*). *C*, BALB/c mice were i.v. inoculated with anti-CD62L (MEL-14) or with control Ab (IgG2a) 1 day before BLS inoculation. After 48 h, the popliteal lymph nodes were excised and analyzed by FACS. Data represent: *A* and *C*, absolute number of CD11c$^+$ cells $\times$ 10^4; *B*, absolute number of DCs expressing high levels of CD62L $\times$ 10^3 (mean + SD, n = 6). Data of two independent experiments have been pooled. $*$, $p < 0.01$ compared with the respective control group.

as LPS and lipoproteins (33, 34), ligands for TLR4 and TLR2, respectively (35, 36). As an example, recent evidence suggests that the reported cytokine effects of heat shock proteins may be a result of the contaminating LPS and LPS-associated molecules (37–39). It has been reported recently that LPS at a concentration as low as 0.1 ng/ml induced a marked release of TNF-α from murine macrophages (37). Taking this into account, several procedures were conducted to rule out endotoxin and lipoprotein contamination. The BLS preparations were always preincubated with PMB-agarose to remove the remaining endotoxin activity. In each experiment, a control group was assessed in parallel with 100 ng/ml LPS preincubated with the same concentrations of PMB-agarose used to treat BLS preparations. PMB-agarose treatment of 100 ng/ml LPS was able to revert all endotoxin effects tested to control levels. Most of the experiments presented in this work have been performed with 90 μg of BLS; thus, the mass of added LPS was 33.8 pg. In our hands, even 180 pg of LPS (6-fold of the amount present in our BLS experiments) was not able to activate BMDCs. It has been reported recently that traces of LPS can act synergistically with hemoglobin in the induction of cytokine production (40). In the experimental conditions used in our work, we could not find synergism. However, the putative existence of synergism between LPS and BLS in other experimental conditions cannot be discarded.

In this work, we were able to show that BMDCs from BALB/c mice were able to mature upon exposure to BLS. The expression of CD40, CD80, CD86, and I-A^d was up-regulated; the mRNA levels of several chemokines were increased; and proinflammatory cytokine IL-6, IL-12p70, and TNF-α secretion was induced. Up-regulation of costimulatory molecules was shown to be BLS dose dependent, and was also observed in BMDCs from C3H/HeN and C57BL/6J mice. Besides, pretreatment of BLS with an anti-BLS Fab was able to revert the stimulatory effect of BLS on BMDCs. BLS was also able to induce similar increases of activation molecules in BMDCs from TLR2 KO and control mice, showing that TLR2 was not involved in BLS effect on BMDCs. On the contrary, BLS was not able to induce an increase in the expression of costimulatory molecules on BMDCs from both C57BL/10ScNJ and C.C3H-Tlr4^{lps-d} mice. None of the proinflammatory cytokines tested increased when C.C3H-Tlr4^{lps-d} BMDCs were incubated with BLS. BLS stimulation of BALB/c BMDCs induced increased levels of MIP-1α, MIP1β, MIP-2, MCP-1, and RANTES mRNAs. In contrast, none of these mRNAs was induced by BLS in C.C3H-Tlr4^{lps-d} BMDCs. Taken together, these results clearly show that BLS is able to induce the maturation of BMDCs through interaction with TLR4 molecules.

In a previous study, we had reported that the mouse mammary tumor virus was able to activate DCs through interaction with TLR4 (41). In vivo, this pathogen was able to induce the recruitment of DCs to neonatal Peyer's patches, being this effect was also dependent on TLR4. We report in this study that BLS inoculation was able to induce a TLR4-dependent recruitment of DCs to the draining lymph node. This effect is probably mediated by the increase in the expression of chemokines, such as MIP-1α, MIP-1β, and RANTES, which are known to attract immature DCs into the vicinity of pathogens (42, 43).

BLS was able to elicit a partially protective immunity in a murine model of brucellosis, used as a protein or a DNA vaccine (14). BLS is also a promising carrier for the polymeric delivery of Ags. Structural analysis has shown that it is possible to insert foreign peptides and proteins at the 10 N terminus of BLS without disrupting its general folding. These chimeras are very efficient to elicit systemic and oral immunity without the need of adjuvants. Moreover, BLS used as a carrier is capable of eliciting strong immune responses against an autologous protein, overcoming tolerance mechanisms (17). As we have demonstrated previously, BLS has adjuvant properties when a foreign Ag is covalently linked to it, whereas no adjuvant effect is observed by mixing or coinjecting BLS with peptide or protein Ags. In the case of the autologous domain RBD3, only mice boosted with the covalently linked domain produced a significant response, whereas coinjection of both proteins did not produce specific Abs (17). BLS was not able to induce anti-lysozyme Abs in BALB/c mice coinjected s.c. with 50 μg of BLS and 50 μg of hen egg lysozyme (results not shown). Thus, BLS has adjuvant properties only when the foreign Ag is covalently attached to it. The strong link between adjuvancy and carrier effect observed on BLS suggests that this protein produces the targeting of the attached Ag to activated DCs. The fact

that BLS is able to activate DCs through TLR4 would explain its high immunogenicity and carrier capacity. Insertion of a 14-residues-long peptide does not impair BLS ability to activate DCs through TLR4 (data not shown). Therefore, results reported in this work explain the ability of BLS to act as a carrier for eliciting potent immune responses against covalently attached proteins and peptides.

Although previous works have shown recognition by the TLRs and consequent stimulation of DCs by virus-like particles (44, 45), we show in this work that a polymeric bacterial protein that forms a particle of medium size (180 kDa) is a potent activator of DCs via a TLR. The described characteristics of BLS make this polymeric bacterial protein an excellent candidate for linking innate and adaptive immunity for vaccine development.

Disclosures

The authors have no financial conflict of interest.

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