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Galectin-8 activates dendritic cells and stimulates antigen-specific immune response elicitation

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ABSTRACT

Galectin-8 (Gal-8) is a mammalian β-galactoside-binding lectin, endowed with proinflammatory properties. Given its capacity to enhance antigen-specific immune responses in vivo, we investigated whether Gal-8 was also able to promote APC activation to sustain T cell activation after priming. Both endogenous [dendritic cells (DCs)] and bone marrow-derived DCs (BMDCs) treated with exogenous Gal-8 exhibited a mature phenotype characterized by increased MHC class II (MHCII), CD80, and CD86 surface expression. Moreover, Gal-8-treated BMDCs (Gal-8–BMDCs) stimulated antigen-specific T cells more efficiently than immature BMDCs (iBMDCs). Proinflammatory cytokines IL-3, IL-2, IL-6, TNF, MCP-1, and MCP-5, as well as growth factor G-CSF, were augmented in Gal-8–BMDC conditioned media, with IL-6 as the most prominent. Remarkably, BMDCs from Gal-8-deficient mice (*Lgals8*$^{-/-}$ BMDC) displayed reduced CD86 and IL-6 expression and an impaired ability to promote antigen-specific CD4 T cell activation. To test if Gal-8-induced activation correlates with the elicitation of an effective immune response, soluble Gal-8 was coadministrated with antigen during immunization of BALB/cJ mice in the experimental foot-and-mouth disease virus (FMDV) model. When a single dose of Gal-8 was added to the antigen formulation, an increased specific and neutralizing humoral response was developed, sufficient to enhance animal protection upon viral challenge. IL-6 and IFN-γ, as well as lymphoproliferative responses, were also incremented in Gal-8/antigen-immunized animals only at 48 h after immunization, suggesting that Gal-8 induces the elicitation of an inflammatory response at an early stage. Taking together, these findings argue in favor of the use of Gal-8 as an immune-stimulator molecule to enhance the adaptive immune response.

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Abbreviations: BHK-21 = baby hamster kidney 21, BMDC = bone marrow-derived dendritic cell, CRD = carbohydrate-recognition domain, DC = dendritic cell, dpi = days postimmunization, FMDV = foot-and-mouth disease virus, Gal-8 = Galectin-8, Gal-8_{A488} = $Alexa_{488}$-labeled Galectin-8, Gal-8–BMDC = Galectin-8-treated bone marrow-derived dendritic cell, iBMDC = immature bone marrow-derived dendritic cell, iDC = immature dendritic cell,

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The online version of this paper, found at www.jleukbio.org, contains supplemental information.

Introduction

Gals constitute a family of soluble mammalian lectins characterized by the presence of conserved CRDs that bind to *N*-acetyl-lactosamine-containing glycans on target cells to exert multiple biologic effects [1]. Of note, even though Gals recognize similar glycosidic structures, different Gals can induce distinct or even opposed activities. At present, enough evidence has been accumulated as to position Gals among the major mediators of innate and adaptive immune responses [2]. In the particular case of Gal-8, from the tandem-repeat group, with 2 different CRDs covalently fused, there are a growing number of reports that endorse its participation in the immune response. Regarding the innate immunity, it has been described that Gal-8 activates neutrophil function by inducing superoxide production, an essential event in the bactericidal role of these cells [3]. On the other hand, Stowell et al. [4] observed that Gal-8 is able to kill directly *Escherichia coli* bacteria, expressing human blood group antigens by altering membrane integrity, hence, protection against pathogens that bears autoantigen-like epitopes. Finally, Gal-8 was also described as a "danger signal" by labeling bacteria-invaded vacuoles for their destruction by autophagy [5]. In this case, authors postulate that Gal-8 detects invading bacteria by binding host glycans exposed on damaged vacuoles,

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suggesting an ability to sense a wide range of pathogen infections.

Regarding the adaptive immune response, we have previously demonstrated that Gal-8 costimulates the antigen-specific CD4 T cell response in the DO11.10TCR$_{OVA}$ mouse model [6]. Later, we found that Gal-1 shared the costimulatory capacity with Gal-8, whereas Gal-3 was not only unable to induce costimulation but also inhibited both Gal-1 and Gal-8 effects, thus evidencing redundant and antagonistic effects among these Gals in the T cell response. Furthermore, the coadministration of a single dose of Gal-1 or Gal-8 together with a limited amount of OVA antigen in DO11.10 mice was sufficient to enhance the antigen-specific CD4 T cell response in vivo [7]. Even though the costimulatory process previously observed in vitro could mediate the in vivo effect, it should be considered that Gals could exert different activities through many types of cells when inoculated in the organism. Thus, Gals may activate APCs to support the T cell activation after priming with the antigen.

APCs are responsible for the link between the innate and adaptive immune responses. DCs are the main APC that participate in the elicitation of T cell responses, thus playing an essential role in the beginning and control of the magnitude and type of the adaptive immune response. iDCs act as immunologic sensors that alert the presence of microbes, both by direct recognition of microbial compounds or by detecting signals from the innate immune system. iDCs decode and integrate these signals and transport this information to the cells from the adaptive immune system; therefore, the type of the elicited response depends on the stimulus received by the DC. To promote the antimicrobial response, DCs undergo a complex process of maturation from cells specialized in antigen capture to APCs [8, 9].

Many authors have described that Gal-1 regulates DC differentiation, signaling, and migration. In particular, Fulcher et al. [10] observed that the addition of Gal-1 induced a mature phenotype in GM-CSF/IL-4 monocyte-derived human DCs, characterized by an increased expression of costimulatory molecules; secretion of proinflammatory molecules, such as IL-6 and TNF; an enhanced ability to stimulate T cell proliferation; and reduced endocytic capacity. These same authors also described that Gal-1-treated DCs displayed an increased migration through cellular matrix, suggesting its participation in the elicitation of the immune response [10, 11]. In agreement, Perone et al. [12] reported that Gal-1 overexpression in transgenic GM-CSF/IL-4 murine BMDCs resulted in cell activation, evidenced by a mature phenotype, an increased expression of proinflammatory messengers (IL-6 and IL-12, among others), and a better ability to stimulate an antigen-specific T cell response.

At difference with Gal-1, no information about the potential effect of Gal-8 on DC functionality is available. In fact, the only report regarding Gal-8 is that it is constitutively expressed in GM-CSF/IL-4 monocyte-derived human DCs; however, no function has been described so far [13]. To gain a deeper insight in the participation of Gal-8 in the elicitation of the immune response, the aim of the present work was to investigate whether Gal-8 is able to induce DC activation and to promote in vivo protection against pathogen challenge.

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iFMDV = inactivated foot-and-mouth disease virus, INTA = Instituto Nacional de Tecnología Agropecuario, KO = knockout, *Lgals8*$^{-/-}$ = Galectin-8-deficient (knockout) mice, mBMDC = LPS-matured bone marrow-derived dendritic cell, MHCII = MHC class II, sTNFRI = soluble TNFRI, TCID$_{50}$ = median tissue culture-infective dose, *wt* = wild-type

MATERIALS AND METHODS

Mice

BALB/cJ, C57BL6/J, DO11.10 [C.Cg-Tg(DO11.10)10Dlo/J], and OTII [B6.Cg-Tg(TcraTcrb)425Cbn/J] breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in our facilities. Mice KO for the Gal-8 coding sequence gene [B6;129S5-*Lgals8*$^{Gt(OST314218)Lex}$/Mmucd] was obtained from Mutant Mouse Resource & Research Centers (MMRRC; University of California, Davis, CA, USA) as heterozygous. After in-house, 12 backcrosses to C57BL/6J, a homozygous KO colony with 95% of a C57BL/6J genetic background was established, as assessed at The Jackson Laboratory Genotyping Resources. The Ethical Committee Boards of the Universidad Nacional de San Martín and INTA approved all procedures involving animals.

Gals

Recombinant Gal-8 was obtained, as described previously [14]. In brief, the Gal-8L mouse isoform (GenBankEF524570) was expressed in *E. coli* BL-21 and purified by lactosyl-Sepharose (Sigma-Aldrich, St. Louis, MO, USA), followed by immobilized metal affinity chromatography. Lectin activity was tested by hemagglutination, as described [14]. Endotoxin levels were <0.5 IU/mg recombinant protein, as determined by the *Limulus* amebocyte lysate gel-clot assay (Pyrotell; Associates of Cape Cod, East Falmouth, MA, USA).

DCs

DCs were generated as described earlier [15], with minor modifications. Bone marrow was obtained from femurs and tibias of 6- to 8-wk-old BALB/cJ males, disrupted in cold PBS, and filtered (30 μm) to remove bone and muscle debris. Then, RBCs were lysed, and cells were resuspended in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with gentamicin and 10% of FBS (complete medium). After 4–5 h incubation at 37°C and 5% CO_2, nonadherent cells were recovered and cultured in the presence of 20 ng/ml GM-CSF and 10 ng/ml IL-4 (both from BioLegend, San Diego, CA, USA). At day 6, nonadherent cells were transferred to a new dish, and cytokine-containing media were replaced every 48–72 h. At day 12, cells were harvested, and percentage of BMDC was determined by labeling of the CD11c surface molecule, followed by flow cytometry analysis. CD11c$^+$ cells represented ~60%.

Splenocytes and CD4$^+$ T cell purification

Mouse splenocytes were obtained as described previously [7]. MiniMACS columns and anti-CD4-coupled paramagnetic particles (Miltenyi Biotec, Auburn, CA, USA) were used for CD4 T cell purification, following the manufacturer's instructions. Cell purity (>90%) was checked by flow cytometry.

Analysis of endogenous CD11c$^+$ cells

BALB/cJ spleens were cut into small pieces and disaggregated in the presence of 2.5 mg/ml Collagenase D (Roche, Basel, Switzerland) and 10 μg/ml DNAase (Sigma-Aldrich) in PBS during 30 min with mild agitation at 37°C. Then, RBCs were lysed, and CD4 T cells were depleted using anti-CD4-coupled paramagnetic particles and MiniMACS columns (Miltenyi Biotec). Cells in the flowthrough were collected (splenocytes^{-CD4}), washed, and resuspended in complete medium. CD4 T cell depletion was confirmed by flow cytometry. For costimulatory molecule expression, 2×10^6 splenocytes^{-CD4} were incubated overnight (ON) at 37°C, 5% CO_2, with 2 μM Gal-8 or PBS as control; when indicated, 50 mM lactose was added, 30 min before Gal-8 stimulus.

CD80, CD86, and MHCII surface expression

BMDCs (1×10^6) or splenocytes^{-CD4} (2×10^6) were incubated in 100 μl cold PBS–azide plus anti-FcγR mAb (CD16/32; Clone 93) for 30 min on ice. Then, anti-CD80 (Clone 16-10AI), anti-CD86 (Clone PO3), anti-MHCII (Clone M5/114.15.2), anti-CD11c (Clone N418), or anti-CD11b (Clone M1/70) mAb, conjugated to distinct fluorochromes, were added in the recommended concentrations. After 1 h, cells were washed, fixed with 1% *p*-formaldehyde in PBS, and analyzed by flow cytometry. All mAb and their isotype controls were from BioLegend.

Gal-8 binding

Splenocytes^{-CD4} (1×10^5) were resuspended in 100 μl cold PBS–azide and treated with 0.2 μM Gal-8_{A488} or left untreated. Then, cells were incubated for 30 min on ice and washed with ice-cold PBS or PBS plus 100 mM lactose. Finally, the CD11c molecule was labeled, as described before, and analyzed by flow cytometry.

BMDC microscopy

BMDCs (1×10^4) were cultured ON in 8-well plates suitable for microscopy observation (Nunc Lab-Tek; Thermo Fisher Scientific) in the presence of 2 μM Gal-8 in a final volume of 0.2 ml complete medium. Polymyxin B (10 μg/ml, USB; United States Biological, Life Sciences, Cleveland, OH, USA) was added previously to Gal-8 treatment. Then, cells were permeabilized with 0.1% Triton X-100–PBS and incubated for 1 h with 1/1000 $Alexa_{488}$–phalloidin (ThermoFisher Scientific). Nuclei were labeled with DAPI (Sigma-Aldrich). Images were acquired with a Nikon Eclipse 80i microscope (Plan APO 60× Oil, 1.4 NA, 0.13 mm working distance objective) with the CoolLED pE excitation system.

Antigen-specific T cell activation

BMDCs were seeded at 1×10^4/well in a 96-well plate and incubated ON at 37°C and 5% CO_2 in the presence of Gal-8 (0.5 or 1 μM) or LPS (0.5 μg/ml) in a final volume of 0.2 ml complete medium (preactivation). Then, after mild washing with PBS, 1×10^5 CD4 T cells purified from $DO11.10TCR_{OVA}$ or OTII mice were added to BMDC cultures in the presence of 0.1 μg/ml $OVA_{323–339}$ peptide (GenScript, Piscataway, NJ, USA). For cell proliferation assessment, methyl[^{3}H]-thymidine (1 μCi; PerkinElmer, Wellesley, MA, USA) was added to each well, 18 h before harvesting. Proliferation assays were performed in quadruplicate.

Cytokine array and quantification

Commercial arrays (RayBiotech, Norcross, GA, USA), displaying immobilized capture antibodies specific for G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40p70, IL-12p70, IL-13, IL-17, IFN-γ, MCP-1, MCP-5, RANTES, stem cell factor, sTNFRI, TNF, thrombopoietin, and vascular endothelial growth factor, were used to evaluate the cytokine secretion profile of activated BMDCs, following the manufacturer's protocol. In brief, membranes were incubated ON with supernatants from BMDC cultures pretreated with 0.5 μM Gal-8 or untreated BMDC (control). After washing steps, arrays were incubated for 2 h with a biotinylated-detection antibody cocktail, washed, and further incubated for 1 h with HRP–streptavidin. Signal was revealed by chemiluminescence and quantified by densitometry using ImageJ software (NIH, Bethesda, MD, USA). For the analysis, each array was first normalized to its internal loading sample control, and then the ratio of Gal-8–BMDC/control–BMDC signals was calculated for each cytokine and expressed as fold increase. Cytokine [IL-12(p70), IL-6, and IFN-γ] concentrations were determined in BMDC culture supernatants by ELISA (BioLegend), using calibration curves of cytokine standards.

Endogenous Gal-8 expression

For Western blot analysis, BMDCs (1×10^6) were stimulated ON with LPS (0.5 μg/ml) or PBS (vehicle). Then, cells were lysed in TBS buffer, 0.5× Triton, PMSF 1×, and protease inhibitor cocktail 1× (Sigma-Aldrich). Cell extracts were run in 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (GE Healthcare Limited, Amersham Place, Little Chalfont, Buckinghamshire, UK). Blots were probed with an in-house-made, affinity-purified rabbit IgG anti-mouse Gal-8, followed by HRP-labeled goat secondary antibodies 1/5000 (Sigma-Aldrich) or anti-tubulin-HRP antibody (GenScript) and developed by chemiluminescence (Thermo Fisher Scientific). For flow cytometry analysis, BMDCs, stimulated as for Western blot, were incubated in 100 μl cold PBS–azide plus anti-FcγR (CD16/32; Clone 93) mAb for 30 min on ice. CD11c molecule was labeled as described previously, before permeabilization in the presence of 0.1× Triton–PBS. Then, cells were labeled with anti-mouse Gal-8, followed by anti-rabbit IgG $Alexa_{488}$ (Thermo Fisher Scientific). Finally, cells were fixed with 1% *p*-formaldehyde in PBS and analyzed by flow cytometry.

iFMDV formulations and animal immunization

Binary ethylenimine-iFMDV serotype O1 Campos was used to formulate antigens and for ELISA assays. Infectious virus (same serotype) was used for viral challenge and was provided by the National Agri-Food Quality and Health Service (Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), Buenos Aires, Argentina). All experiments involving infectious virus were performed at INTA Biosafety Level 4 World Organisation for Animal Health (OIE) facilities. Female BALB/cJ mice (8 wk old) were i.p. vaccinated with the iFMDV antigen formulated with PBS (iFMDV) in a total volume of 0.2 ml. iFMDV dose was 0.1 μg/mouse and was always preselected from a dose-response curve to obtain a mild protection (30–40%) upon homologous viral challenge. A dose of 100 μg Gal-8 was used in combination with the iFMDV antigen in PBS (iFMDV/Gal-8) or alone (Gal-8). The negative control group was inoculated with PBS (vehicle group). To evaluate the humoral response, animals were bled at 7, 14, 21, and 30 dpi. A booster was administered at 21 dpi and consisted of the same doses of antigen and Gal-8.

Determination of anti-FMDV antibodies

Total antibodies against FMDV were assessed by trapping ELISA. In brief, microtitre ELISA plates (Nunc MaxiSorp; Thermo Fisher Scientific) were coated with anti-FMDV rabbit serum in 0.05 M carbonate–bicarbonate buffer, pH 9.6, ON at 4°C. Then, purified iFMDV was added. After washing, diluted serum samples were added, followed by incubation with HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Enzymatic activity was developed by the addition of the substrate/chromophore mixture H_2O_2/2,2-azino-bis-3-ethyl-benzothiazoline-6 sulfonic acid diammonium salt (Sigma-Aldrich). The reaction was inhibited after 30 min incubation by the addition of 0.2% NaFl. The OD readings were measured using an automatic microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 405 nm. Fifty microliter volumes were used throughout the assay. Between each step, plates were washed 5 times with PBS. Except for the capture antibody, all reagents were diluted with PBS containing 2% NaCl, 1% Tween, pH 7.6 (ELISA dilution buffer). To eliminate unspecific signal from each serum sample, control wells, sensitized with capture antibodies but in the absence of antigen, were included in the plates.

Sera-neutralizing antibodies were measured as described [16]. In brief, serial dilutions of sera were incubated with 100 $TCID_{50}$/well FMDV. The FMDV–serum mixtures were transferred onto BHK-21 cell monolayers and incubated for 40 min; then, cells were washed, and fresh media were added. The appearance of cytopathic effects was recorded after 48 h of incubation at 37°C.

Lymphoproliferation

Splenocytes (5×10^5) from immunized (iFMDV/Gal-8 and iFMDV) or control (Gal-8 and vehicle) animals, obtained at 48 h postinfection, were cultured in a 96-well plate in a final volume of 0.2 ml complete medium containing 1 μCi methyl[^{3}H]-thymidine. Cells were harvested after 24 h. Splenocytes, obtained from immunized animals at 5 and 21 dpi, were restimulated with 1 μg/ml purified iFMDV for 72 h, previous to the addition of [^{3}H]methyl-thymidine. Proliferation assays were performed in quadruplicate.

Viral challenge

Protection against FMDV was assessed, as described previously [17]. In brief, mice were inoculated i.p. at 21 dpi with 10^4 $TCID_{50}$ infectious FMDV. After 24 h, collected, heparinized blood was spread onto BHK-21 cell monolayers and incubated for 40 min at 37°C in a 5% CO_2 atmosphere. Then, cell monolayers were washed 3 times with sterile PBS. Fresh DMEM, supplemented with 2% FCS, was added, and cells were incubated for 72 h at 37°C, 5% CO_2. Animals were considered protected if the cell monolayer did not present a cytopathic effect after a blind passage. Percentages of protection were calculated as: 100 × (protected/challenged mice). Viremia titers were calculated by the method of Reed and Muench [18].

Flow cytometry analysis

FlowMax cytometer Particle Analysing System PAS-III (Sysmex Partec GmbH, Görlitz, Germany) and FlowJo software (FlowJo, Ashland, OR, USA) were used throughout this work.

Statistical analyses

Student's *t*-test was used, except for the analysis (see Fig. 5B and C and Supplemental Fig. 1A) where ANOVA test was used. $P < 0.05$ was considered significant.

RESULTS

Gal-8 induces DC activation

We have previously shown that Gal-8 is able to enhance the T cell-specific immune response in the DO11.10 TCR_{OVA} transgenic mouse model [7]. With the possibility that Gal-8 may stimulate APC to sustain T cell activation after priming, we investigated whether this lectin displayed a direct effect on DC function. BMDCs, differentiated in vitro from GM-CSF/IL-4 conditioned mice bone marrow cultures, constitute a DC model that has been largely used to study many aspects of APCs [19–23]. Recently, it has been demonstrated that $CD11c^+$ BMDC cultures comprise a heterogeneous population of DCs (GM-DC) and macrophages (GM-M) [24]. Therefore, we phenotypically characterized our $CD11c^+$ BMDC cultures from BALB/cJ mice into $MHCII^{high}/CD11b^{int}$ (GM-DC) and $MHCII^{int}/CD11b^{high}$ (GM-M) subsets (**Fig. 1A**). Percentages for each subset (29.2 vs. 61.6, respectively) were in agreement with previous reports [23, 24]. Incubation with 2 μM Gal-8 for 18 h induced the expression of CD86 in both $CD11c^+$ BMDC subsets, whereas expression of CD80 was induced only in $MHCII^{high}/CD11b^{int}$ cells (Fig. 1B). The increment of costimulatory molecules in $CD11c^+$ BMDCs was evident from 0.2 μM Gal-8 and after 2 h of incubation, as shown for CD86 in Supplemental Fig. 1A. To rule out the presence of unspecific effects of endotoxin contamination in recombinant protein preparations, BMDC cultures were preincubated with Polymyxin B. As controls, mBMDCs, in the presence or absence of Polymyxin B, were also included (Supplemental Fig. 1B).

Having demonstrated that $CD11c^+$ BMDCs are specifically activated by Gal-8, we analyze this activity further in endogenous DCs from BALB/cJ mice spleen. First, Gal-8 binding to $CD11c^+$ splenocytes was analyzed by flow cytometry using $Gal\text{-}8_{A488}$. As shown in Fig. 1C, Gal-8 bound to all $CD11c^+$ populations in a lectin-dependant manner, as lactose addition displaced Gal-8 from the cell surface. Before test Gal-8 activity, splenocytes were depleted of CD4 T cells ($splenocytes^{-CD4}$) to avoid background, given that these cells are a target of Gal-8-induced proliferation in the absence of antigen [6]. Then, $splenocytes^{-CD4}$ were incubated ON in the presence of 2 μM Gal-8, and expression of costimulatory molecules was analyzed by flow cytometry. As shown in Fig. 1D, Gal-8 induced the expression of CD86, CD80, and MHCII in endogenous $CD11c^+$ splenocytes. Moreover, preincubation with lactose (50 mM) prevented the Gal-8 stimulatory effect, indicating that the lectin–glycan interaction is actually involved. Of note, CD80 induction was not affected in the presence of this sugar, suggesting that higher affinity ligands, different from lactose, could be involved in this particular process, as we have previously reported for Gal-8 costimulatory activity [25]. In line with this evidence, only CD86 induction was affected in the presence of lactose in $CD11c^+$ BMDCs, whereas CD80 and MHCII expression was slightly reduced (data not shown).

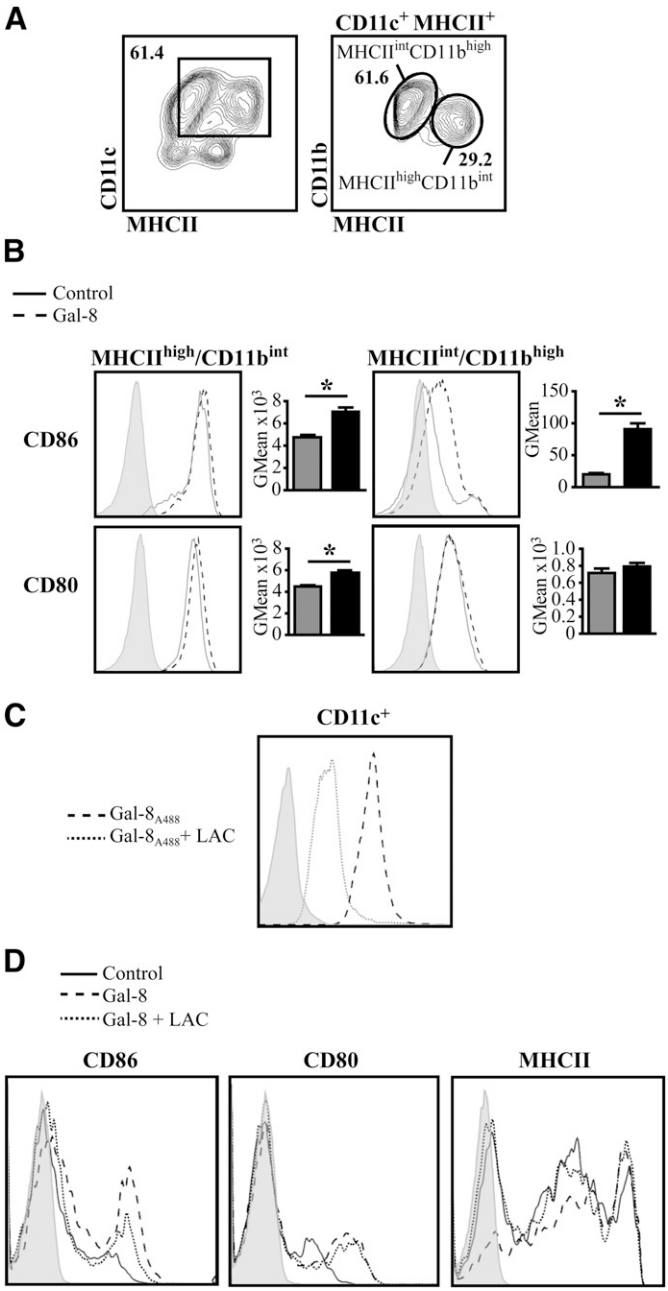
Given that the increased expression of CD80, CD86, and MHCII is characteristic of the mature DC phenotype, results depicted until here strongly suggest that exogenous Gal-8 induces DC activation.

Gal-8–BMDCs stimulate a specific T cell response and secrete high levels of IL-6

Gal-8–BMDCs displayed a morphology characteristic of a mature phenotype with prolonged dendrites, as observed in **Fig. 2A**. To determine whether Gal-8–BMDCs were capable of activating an antigen-specific T cell response, CD4 T cells, purified from transgenic $DO11.10TCR_{OVA}$ mice, were coincubated in the presence of a low dose of OVA peptide ($OVA_{323–339}$) with Gal-8–BMDC, mBMDC, or untreated DC (iBMDC). A significant increment in T cell proliferation was observed in the presence of Gal-8–BMDC compared with iBMDC (Fig. 2B). This increment was similar to that observed with mBMDC, indicating that Gal-8 actually activates BMDC to present efficiently antigen to T cells. For all conditions, controls of BMDC plus T cells in the absence of antigen did not proliferate beyond background levels, thus discarding any unspecific effect of BMDC on T cell activation.

IL-12 is one of the major cytokines produced by mature DCs that participates in the Th1 polarization response. To determine if this cytokine was also induced in the presence of Gal-8, IL-12p70 level was tested in Gal-8–BMDC supernatants. Surprisingly and at difference with mBMDC, IL-12p70 was undetected in Gal-8–BMDC supernatants (data not shown). To search for other cytokines induced in the presence of Gal-8, commercial arrays displaying immobilized anti-cytokine antibodies were incubated with BMDC conditioned media. Secretion level was quantified by densitometry for each target molecule, based on its signal in the array (**Fig. 3A**). Cytokine fold increase was calculated as the ratio of the signal obtained in the array incubated with supernatant from Gal-8–BMDC vs. iBMDC. As shown in Fig. 3B, Gal-8 induced the secretion of several proinflammatory cytokines, such as IL-3, IL-2, IL-6, TNF-α, MCP-1, and MCP-5, as well as growth factors, such as G-CSF. No increase in IL-12p70 signal was found in the array assayed with the Gal-8–BMDC supernatant, thus confirming our previous ELISA result. It is noteworthy to highlight the detection of IL-3, G-CSF, and to a lesser degree, GM-CSF, as they are cytokines that regulates the survival, proliferation,

and differentiation of hematopoietic cells [26]. This could explain, at least in part, the increased percentage of $CD11c^+$ cells observed repeatedly after 18 h of incubation with Gal-8 (Supplemental Fig. 2). This interesting finding suggests that Gal-8 could favor BMDC differentiation. IL-6 was selected further to confirm array results, given its well-defined proinflammatory properties and its striking increment. The pronounced augmentation of IL-6 was confirmed by ELISA in conditioned media from Gal-8–BMDC cultures, as well as its dependence on the lectin–glycan interaction, as preincubation with lactose almost inhibited cytokine secretion (Fig. 3C). For this assay, in particular, BMDCs were differentiated from bone marrow of C3H/HeJ mice (an LPS-resistant mouse strain) to assign the activating effect exclusively to Gal-8.



Gal-8 deficiency impairs the BMDC ability to stimulate a T cell response

GM-CSF/IL-4 human monocyte-derived DCs are known to express Gal-8 [13]; thus, we tested Gal-8 expression in BMDC differentiated from BALB/cJ mice bone marrow. As observed in **Fig. 4A** (upper), not only the expression of Gal-8 was confirmed in mouse BMDC by Western blot but also a marked increment of this Gal was observed after LPS activation. This increment was also demonstrated by flow cytometry in permeabilized $CD11c^+$ BMDC (Fig. 4A, lower). The fact that Gal-8 is increased in mBMDC, together with its activating role on this cell population, led us to investigate whether the absence of Gal-8 could somehow affect BMDC functionality. To this purpose, BMDCs were differentiated from *Lgals8*$^{-/-}$ mice or its counterpart, C57BL/6J *wt* mice. CD80, CD86, and MHCII expression was then analyzed in iBMDC or mBMDC, matured in the presence of a low dose of LPS (0.05 μg/ml). As shown in Fig. 4B, CD86 expression was decreased significantly in *Lgals8*$^{-/-}$ BMDC, either immature or mature, compared with its *wt* counterpart. The reduction in CD86 expression was still evident in *Lgals8*$^{-/-}$ BMDC treated with higher LPS doses, such as 0.5 μg/ml (data not shown). Regarding CD80 and MHCII expression, no differences were observed between *Lgals8*$^{-/-}$ and *wt* BMDC in any condition (data not shown). Next, we evaluated whether IL-6 secretion was impaired in *Lgals8*$^{-/-}$ BMDC. Conditioned media from immature *Lgals8*$^{-/-}$ BMDC contained significant lower levels of IL-6 than *wt* cells; however, no differences were recorded after LPS treatment (Fig. 4C). Finally, we asked whether the absence of Gal-8 affects BMDC ability to stimulate an antigen-specific T cell response. To this purpose, iBMDC or mBMDC from *Lgals8*$^{-/-}$ and *wt* mice were cocultured with purified CD4 T cells from

Figure 1. Gal-8 increases the expression of costimulatory molecules. (A) Phenotype analysis of GM-CSF/IL-4 bone marrow-derived cultures. Bone marrow-derived cells displaying higher expression of CD11c and MHCII (box inset, left) were gated to analyze CD11b and MHCII expression further. (Inset, right) Circles show 2 defined cell subsets, $MHCII^{high}CD11b^{int}$ and $MHCII^{int}CD11b^{high}$. Numbers indicate the percentage of cells in each subpopulation. (B) Gal-8 activation of BMDCs. BMDCs were treated ON in the presence of 2 μM Gal-8 (dashed lines) or left untreated (solid lines). Then, cells were labeled with specific mAb or the isotype control (filled histograms) and analyzed by flow cytometry. Histograms depict fluorescence intensity of CD86 or CD80 molecules on gated $MHCII^{high}/CD11b^{int}$ and $MHCII^{int}/CD11b^{high}$ subsets. Bars indicate the geometric mean of fluorescence intensity (Gmean). (C) Gal-8 binding to endogenous DC. Splenocytes from BALB/cJ depleted in CD4 T cells ($splenocytes^{-CD4}$) were incubated in the presence (dashed line) or absence (filled histogram) of 0.2 μM $Gal\text{-}8_{A488}$ and washed with PBS or 100 mM lactose (LAC)–PBS (dotted line). Then, cells were labeled with anti-CD11c. Histograms indicate fluorescence intensity of $Gal\text{-}8_{A488}$ on gated $CD11c^+$ cells. (D) Gal-8 activation of endogenous DC. $Splenocytes^{-CD4}$ were activated ON in the presence of 2 μM Gal-8 (dashed lines), Gal-8 plus 50 mM lactose (dotted line) or left untreated (solid lines). Lactose was added 30 min before activation with Gal-8. Then, cells were labeled with specific mAb or the isotype control (filled histograms). Histograms indicate fluorescence intensity of CD80, CD86, and MHCII molecules on gated $CD11c^+$ cells. Depicted assays are representative of 2 (A–C) or 3 (D) independent experiments and were carried out, each time, with different recombinant protein preparations. $^*P < 0.05$.

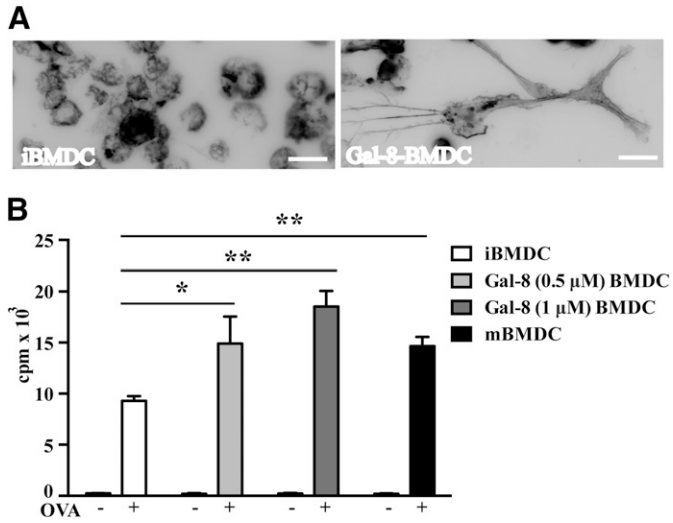


Figure 2. Gal-8–BMDCs display an activated morphology and promote antigen-specific T cell proliferation. (A) BMDC morphologic analysis. BMDCs from BALB/cJ mice were preincubated with 10 μg/ml Polymyxin B before ON treatment with vehicle (iBMDC; left) or 2 μM Gal-8 (Gal-8–BMDC; right). Then, cells were labeled with $Alexa_{488}$–phalloidin and analyzed by epifluorescence microscopy. Original scale bars, 10 μm. (B) Antigen-specific T cell activation. BMDCs were treated ON in the presence of 0.5–1 μM Gal-8 (Gal-8–BMDC), 0.5 μg/ml LPS (mBMDC), or vehicle (iBMDC). Then, preactivated BMDCs were cocultured with CD4 T cells purified from $DO11.10TCR_{OVA}$ mice in the presence of 0.1 μg/ml $OVA_{323–339}$ peptide. After 48 h, proliferation was assessed by incorporation of methyl[^{3}H]-thymidine. Depicted assays are representative of 2 (A) and 3 (B) independent experiments and were carried out, each time, with different recombinant protein preparations. *$P < 0.05$; **$P < 0.01$.

$OTII\text{-}TCR_{OVA}$ transgenic mice in the presence of $OVA_{323–339}$ peptide. As shown in Fig. 4D, no differences in an antigen-specific T cell response were observed in the presence of iBMDC from either *Lgals8*$^{-/-}$ or *wt* mice. However, a reduction in T cell stimulation capacity was readily observed in *Lgals8*$^{-/-}$ BMDC compared with their *wt* counterpart after activation in the presence of 0.05 μg/ml LPS. In an attempt to counterbalance Gal deficiency, *Lgals8*$^{-/-}$ BMDCs were stimulated with LPS in the presence of 0.2 μM Gal-8. However, as shown in Supplemental Fig. 3, no differences in CD86 expression or T cell priming capacity were registered between LPS and LPS/Gal-8-stimulated *Lgals8*$^{-/-}$ BMDC, thus suggesting that exogenous Gal-8 was not able to rescue the KO phenotype. Overall, these results show that BMDC from *Lgals8*$^{-/-}$ mice displayed a more immature phenotype characterized by a reduced expression of CD86 and IL-6 and an impaired ability to induce an antigen-specific T cell response after maturation. With the consideration that these differences became increasingly evident after BMDC activation with LPS (except for IL-6), together with the fact that this same activation induced Gal-8 expression, we postulate a role for the endogenous lectin in the development of mature and functional DC.

Gal-8 enhances the elicitation of the immune response against the FMDV antigen

It is reported that Gal-8 promotes plasma cell formation and Ig secretion [27]. On the other hand, IL-6 is a pleiotropic cytokine that plays a prominent role in the antigen-specific response [28], also involved in Ig production by promoting B cell differentiation into plasma cell [29]. With the consideration that Gal-8 activates BMDC to secrete high levels of IL-6 (Fig. 3C) and also improves the elicitation of the immune response in the transgenic TCR_{OVA} mouse model [7], we decided to test critically the Gal-8 ability to stimulate a specific humoral response by using a complex antigen. FMDV infects cloven-hoofed animals, causing a highly contagious illness [30], which is currently prevented by a strong virus-neutralizing antibody response elicited through vaccination. The experimental BALB/cJ murine model of FMDV correlates with the humoral immune and protective response in cattle [31, 32]. Adult BALB/cJ mice can be experimentally infected with FMDV O1 Campos by i.p. inoculation, the virus replicates in pancreatic cells, and viremia lasts 72 h without clinical signs. In the following assays, we used Gal-8 as a stimulant of the immune

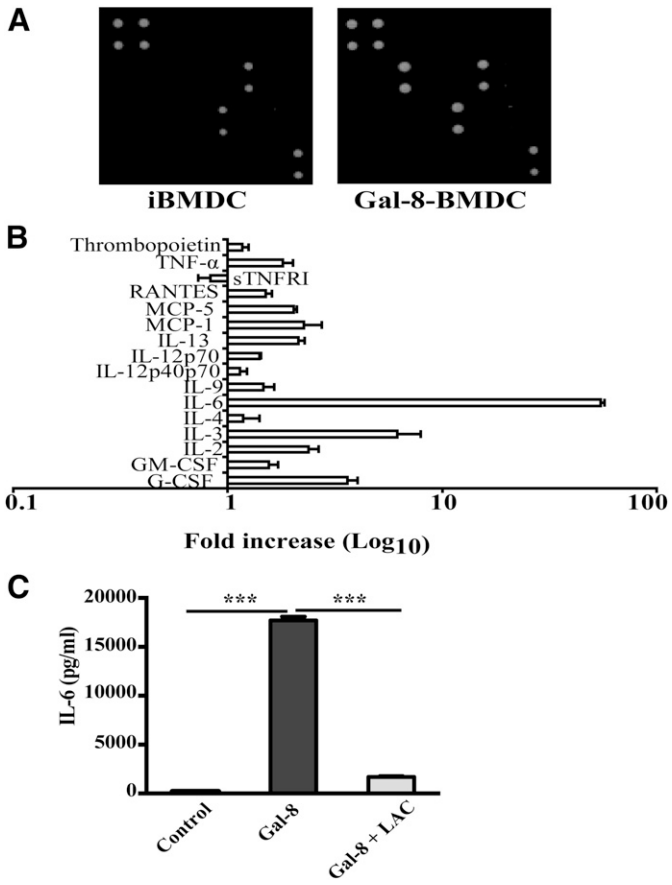


Figure 3. Gal-8–BMDC cytokine secretion profile. (A) Cytokine membrane arrays were incubated with conditioned media from BMDC stimulated ON in the presence of 0.5 μM Gal-8 (Gal-8–BMDC; right) or left untreated (iBMDC; left). (B) Fold increase was calculated for each cytokine as the ratio between Gal-8–BMDC and iBMDC signals. (C) IL-6 in supernatants from iBMDC (Control), BMDC treated ON with 0.5 μM Gal-8 (Gal-8), or treated with 0.5 μM Gal-8 plus 50 mM lactose (Gal-8 + LAC) was quantified by ELISA. Lactose was added 30 min before activation with Gal-8. ELISA assay is representative of at least 3 independent experiments, carried out, each time, with different recombinant protein preparations. ***$P < 0.001$.

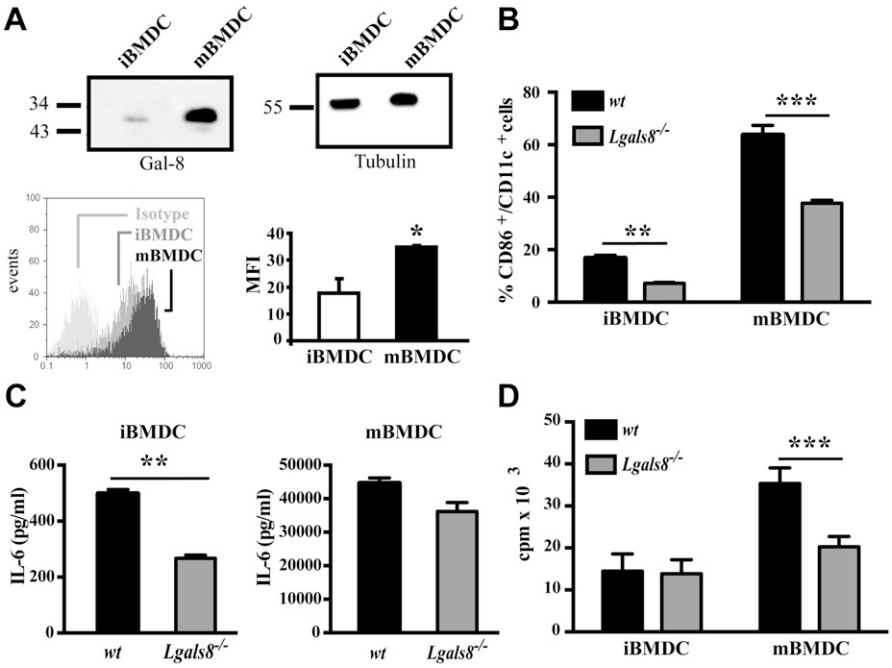


Figure 4. Gal-8 deficiency impairs BMDC functionality. (A) Analysis of endogenous Gal-8 expression on iBMDC and mBMDC by Western blot (upper) and flow cytometry (lower). In both assays, affinity-purified rabbit polyclonal anti-mouse Gal-8 antibody was used. Anti-tubulin antibody was included as sample load control. Isotype, Normal rabbit IgG; MFI, mean fluorescence intensity. (B) Flow cytometry analysis of CD86 expression in $CD11c^+$ BMDC, differentiated from *Lgals8*$^{-/-}$ and *wt* mice. (C) IL-6 in conditioned media from *Lgals8*$^{-/-}$ and *wt* mice-derived iBMDC or mBMDC was quantified by ELISA. (D) Antigen-specific T cell activation. iBMDC and mBMDC from *Lgals8*$^{-/-}$ or *wt* mice were cocultured with CD4 T cells purified from OTII mice in the presence of 0.1 μg/ml $OVA_{323–339}$ peptide. After 48 h, proliferation was assessed by incorporation of [^{3}H]methyl-thymidine. mBMDCs were obtained by ON treatment with 0.5 μg/ml (A) or 0.05 μg/ml (B–D) LPS. Depicted assays are representative of 3 (A, C, and D) and 4 (B) independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

response during immunization of BALB/cJ mice with the iFMDV antigen. Remarkably, animals immunized with iFMDV plus Gal-8 (iFMDV/Gal-8) displayed a significant increment of the specific humoral response elicited compared with animals vaccinated with iFMDV alone (**Fig. 5A**). This increment was observable from 14 dpi and was even more evident at 30 dpi after the administration of a booster at 21 dpi. These findings demonstrate that a defined dose of soluble recombinant Gal-8 was enough to enhance significantly a specific humoral response against the iFMDV antigen.

It has been demonstrated that a proinflammatory response characterized by IL-6 and IFN-γ secretion is associated with the early protection observed in iFMDV-vaccinated mice [17]. To investigate if Gal-8 was inducing this inflammatory response, the presence of IL-6 and IFN-γ was analyzed in the supernatants of splenocyte cultures from mice at 2, 5, and 21 dpi. As shown in Fig. 5B, a significant increase of IL-6 and IFN-γ secretion was evident in the iFMDV/Gal-8-immunized group at 2 dpi compared with the iFMDV-alone group. At 5 dpi, IL-6 and IFN-γ from iFMDV/Gal-8 immunized animals returned to control levels, and no further increments were recorded until 21 dpi, even after restimulation with iFMDV (data not shown). In agreement, an increased lymphoproliferative response was only evident at 2 dpi in the iFMDV/Gal-8 immunized group (Fig. 5C). These results suggest that Gal-8 activates the elicitation of an adaptive immune response against FMDV by inducing secretion of proinflammatory cytokines at an early stage.

Coadministration of Gal-8 with the iFMDV antigen enhanced animal protection

Given that FMDV infection is controlled by elicitation of neutralizing antibodies upon vaccination, we next investigated whether the elicited humoral response of those animals immunized in the presence of Gal-8 was also able to neutralize the virus. As shown in **Fig. 6A**, serum samples obtained at 21 dpi from the iFMDV/Gal-8-immunized group displayed higher neutralizing antibody titers compared with those from iFMDV alone group. Having demonstrated that Gal-8-containing formulation increased the anti-FMDV-neutralizing humoral response, we next investigated whether this response was able to protect effectively in vivo upon homologous viral challenge. For this purpose, mice were immunized as before and challenged at 21 dpi. As shown in Fig. 6B (left), those animals receiving iFMDV, formulated with Gal-8, displayed a significant reduction in viremia titers, with respect to those receiving only antigen. Furthermore, the number of protected animals (with undetectable viremia) from the iFMDV/Gal-8-vaccinated group doubled those from the iFMDV control group (75 vs. 29% protection, respectively; Fig. 6B, right). Control mice receiving only Gal-8 displayed similar viremia titers as the vehicle control group, indicating that the Gal-8-induced response was antigen specific. Taken together, our findings demonstrate that the use of a single dose of Gal-8, in combination with the iFMDV antigen, is sufficient for the elicitation of an effective immune response that significantly enhanced the protection against homologous viral challenge.

DISCUSSION

Here, we described for the first time an activating role for Gal-8 on the DC population, evidenced by an increased expression of costimulatory molecules, an augmented capacity to activate an antigen-specific T cell response, the development of a typical mature morphology, and a strong production of IL-6, among other proinflammatory cytokines. Gal-8-induced IL-6 and CD86 expression was partially prevented in the presence of lactose,

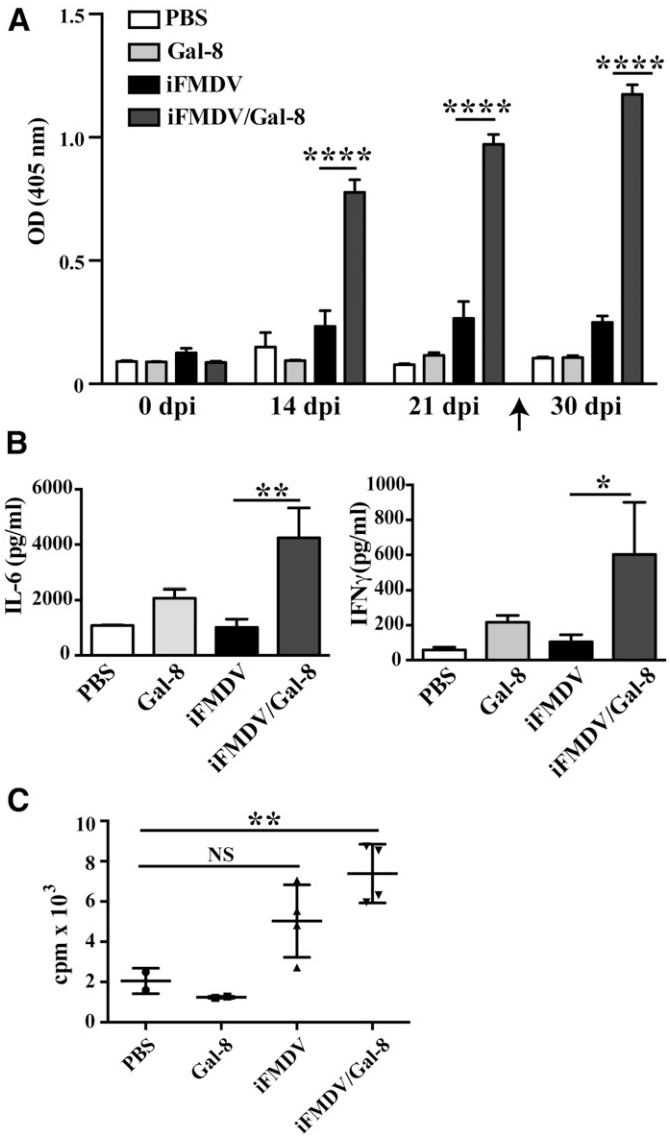


Figure 5. Gal-8 enhances the elicitation of the immune response against FMDV antigen. (A) Anti-FMDV total IgG was determined by ELISA in serum samples from immunized mice. Arrow indicates booster inoculation at 21 dpi. (B) IL-6 and IFN-γ were determined by ELISA in supernatants of splenocyte cultures from animals killed at 2 dpi. Cells were cultivated for 96 h. (C) Lymphoproliferation assays. Splenocytes from animals killed at 2 dpi were cultured for 24 h in the presence of [^{3}H]methyl-thymidine. Mice per group: (A) PBS ($n = 3$), Gal-8 ($n = 3$), iFMDV ($n = 5$), iFMDV/Gal-8 ($n = 5$); (B and C) PBS ($n = 2$), Gal-8 ($n = 2$), iFMDV ($n = 4$), iFMDV/Gal-8 ($n = 4$). Depicted assays are representative of at least 3 (A) and 2 (B and C) independent experiments and were carried out, each time, with different recombinant protein preparations. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

indicating that lectin activity was actually implicated. However, CD80 induction was not affected in the presence of this sugar, thus suggesting that other types of glycans might be also involved. In fact, we have recently demonstrated by using a 2-CRD mutated recombinant Gal-8, in which substitution of canonical arginine residues on each CRD precluded low- and middle- but not high-affinity glycan interaction, that blood group antigens and some polylactosamines are actually mediating the Gal-8 costimulatory effect on the CD4 T cell response [25]. Despite several cytokines, such as IL-6, IL-3, TNF-α, and IL-13, produced in response to Gal-8, the absence of IL-12 induction was an unexpected result given that its secretion is a hallmark of DC activation. A priori, this finding suggests that Gal-8 promotes a specific activation mechanism, different from other stimulus, such as LPS. As mentioned above, the most abundant cytokine induced in response to Gal-8 was IL-6. Rochman et al. [28] demonstrated that IL-6 plays an important physiologic role in the regulation of an antigen-specific T cell response. These results are in agreement with our observations, where the costimulatory effect exerted by Gal-8 on antigen-specific CD4 T cell response is mediated by IL-6 [unpublished results]. On the other hand, it is worthy to highlight the detection of IL-3, G-CSF, and to a lesser extent, GM-CSF, as these molecules regulate the survival, proliferation, differentiation, and activation of hematopoietic cells [26]. IL-3 is a myeloid hematopoietic growth factor, and it has been described as potent as GM-CSF in generating human myeloid DC [33–35]. Moreover, the presence of IL-3 in mouse-derived bone marrow cultures increases myeloid DC production [36], thus probably explaining the increased frequency of CD11c$^+$ cells observed in the presence of Gal-8 (Supplemental Fig. 2). IL-13, MCP-1, and MCP-5 secretion was also induced by Gal-8, to a lesser extent. IL-13 is responsible for a great variety of effects on different cell types, such as the increment of MHCII in monocytes or the production of IgG and IgM by B cells [37]. MCP-1 and MCP-5 are chemokines involved in the migration and infiltration of monocytes, T lymphocytes, and NK, among others cells [38, 39]. In the same line of evidence, we have previously reported that Gal-8 also induces the endothelium to secrete GM-CSF, as well as several cytokines and chemokines, such as MCP-1, RANTES, and IL-6, among others, which favors the recruitment of the cells involved in the inflammatory process [40]. Taken together, our results demonstrate an integral activating role of Gal-8 on DC functionality.

Over the last few years, our group has been proposing Gal-8 participation in the inflammatory process. Several findings support this hypothesis, principally, the activating role on platelets, where Gal-8 addition induced the spreading and release of granule content [41], the strong activation of mouse and human naïve CD4 T cells [6, 42], and more recently, the activation of endothelial cells, characterized by von Willebrand factor exposure, secretion of proinflammatory molecules, and NF-κB phosphorylation [40]. Finally, the activating effects on DC described in the present work are in line with our previous results. All of these findings were evidenced by the exogenous addition of recombinant Gal-8 in a wide range from 0.1 to 5 μM. However, we have also detected an increment of endogenous Gal-8 upon activation; for example, human platelets treated with thrombin exposed higher levels of Gal-8 on their surface, and the LPS-activated endothelium secreted higher amounts of Gal-8, which in turn, spread its activity all over the organism [40, 41]. Regarding BMDCs, we observed that they also express higher amounts of Gal-8 after activation with LPS (Fig. 4A). The existence of incremented Gal-8 levels in those inflammatory environments further supports the participation of the endogenous lectin. In fact, mature *Lgals8*$^{-/-}$ BMDCs displayed lower

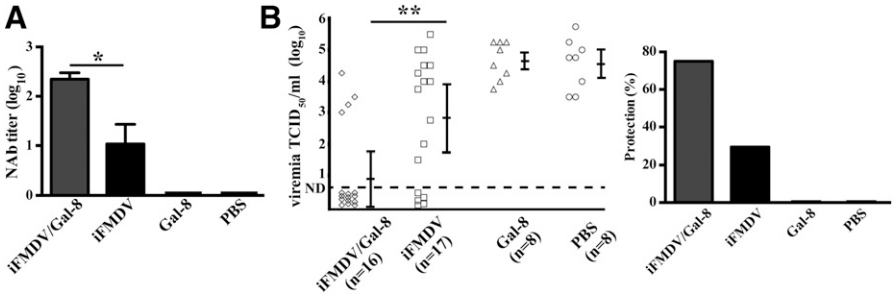


Figure 6. Immunization with iFMDV/Gal-8 increases the neutralizing humoral response and enhances protection against viral challenge. (A) Neutralizing antibody (NAb) titers were determined in serum samples obtained from animals at 21 dpi. (B) Mice were immunized, as described before, and challenged at 21 dpi. After 24 h, blood samples were collected, and viremia was calculated as described in Materials and Methods. Viremia titers from 3 independent challenge assays were pooled, and the total animal number per group is indicated (left). ND, Viremia not detected. Protection percentages (right) were calculated as 100 × (protected mice/challenged mice). $*P < 0.05$; $**P < 0.01$.

levels of CD86 after activation and stimulate T cell proliferation less efficiently than their *wt* counterpart (Fig. 4B and D, respectively). However, addition of recombinant Gal was unable to rescue the *Lgals8*$^{-/-}$ BMDC phenotype. It might be possible that endogenous Gal-8 could exert additional effects, not totally recapitulated with the exogenous protein. In support, it was previously reported a key role for intracellular Gal-8 in the selective autophagy [5], a mechanism that has been associated with DC capability of T cell priming [43]. Notably, IL-6 secretion was also impaired in *Lgals8*$^{-/-}$ iBMDC; however, no significant differences were registered between *Lgals8*$^{-/-}$ and *wt* DC after activation with LPS, probably as a result of a compensation effect (Fig. 4C). Even though CD86 expression and IL-6 production were both impaired in *Lgals8*$^{-/-}$ iBMDC, no differences were recorded in T cell stimulation between *Lgals8*$^{-/-}$ and *wt* iBMDC. Probably the lower magnitude in costimulatory molecule expression and cytokine levels from iBMDC compared with mBMDC may result insufficiently to generate a visible difference in antigen-specific T cell proliferation. Moreover, given that LPS treatment leads to Gal-8 up-regulation in mBMDC, it is more likely that an impaired T cell stimulation ability became evident in *Lgals8*$^{-/-}$ cells, only after maturation. Nevertheless, it is worthy to point out that other costimulatory molecules or cytokines, distinct from CD86 and IL-6, could also be responsible for the differences observed in the T cell stimulation ability between *Lgals8*$^{-/-}$ and *wt* BMDCs. Taken together, the increment in Gal-8 expression may represent an endogenous mechanism to fuel the elicitation of the immune response in the inflammation site.

The immunostimulatory capacity of Gal-8 was critically tested using a complex antigen, such as iFMDV. In this model, animals immunized with antigen alone elicited per se a humoral response that confers 30–40% of protection (depending on the antigen dose) upon infective viral challenge. Remarkably, the administration of Gal-8 together with iFMDV increased total specific and neutralizing antibody titers, which resulted in an increased protection rate. Conversely, when Gal-1 was coadministered with iFMDV, no differences in specific antibodies titers were observed, even after a booster administration at 21 dpi (data not shown). Maybe a higher amount of Gal-1 would be necessary, mainly considering that 100-fold more Gal-1 was required to equal Gal-8 costimulatory activity [7]. A feasible explanation is that different than Gal-8, Gal-1 has to dimerize to achieve its active form, which only occurs at elevated concentrations [44–46]. However, the fact that the same dose of 25 μg of both Gal-8 and Gal-1 was shown to stimulate in vivo the antigen-specific T cell response in the DO11.10TCR$_{OVA}$ model [7] led us to postulate alternative explanations; for example, the differences in fine specificity among CRDs could be responsible for the different activities of these Gals. Another possibility could be a dependence on the antigen used, as it is well known that different antigens can elicit different responses. The Gal-8-induced anti-FMDV response was preceded by an increment of IL-6 and IFN-γ production, observed at 48 h postimmunization, which was also accompanied by an enhanced splenocyte proliferation rate. Interestingly, both cytokines and proliferation increment were transient and returned to basal levels by day 5 after immunization, suggesting that Gal-8 circumscribed its effects to the very beginning of the immune response induction. IL-6 is a pleiotropic cytokine, which has been shown to be involved in Ig production by promoting B cell differentiation to plasma cells [29]. Notably, Tsai et al. [27] observed that both Gal-8 and Gal-1 play an important role in the generation of plasma cells; however, only Gal-8 induced IL-6 secretion on B cells. This difference could also account for the absence of the anti-FMDV response when immunizing with Gal-1, especially if it is considered that DCs pulsed in vitro with the inactivated virus secrete IL-6, which is crucial for the anti-FMDV response [47, 48]. All of these data further link Gal-8-induced DC activation and IL-6 secretion with its capacity to stimulate the humoral response in the FMDV experimental model. It is necessary to emphasize at this point that endogenous macrophages could also participate in a Gal-8-induced immune response in this model. In fact, Helft et al. [24] reported that the MHCII^{int}CD11b^{high} BMDC subpopulation, which became activated in the presence of Gal-8 (Fig. 1B, left), actually resembles monocyte-derived macrophages rather than conventional DCs. Further studies are needed to address specifically whether Gal-8 could activate endogenous macrophages.

Taken together, our findings demonstrate that a single dose of iFMDV antigen formulated with soluble Gal-8 triggers an effective immune response that enhanced protection against homologous viral challenge, thus allowing us to postulate this Gal as a potential adjuvant in some vaccine preparations. This is the first time that an adjuvant activity is demonstrated for a member of the Gal family, offering potentially new applications for these molecules in vaccine design. Among the possible advantages of Gal-8 application, it should be stressed that soluble Gal-8 does not

persists in the organism, overcomes the need of an antigen depot, and avoids granuloma formation associated with oil-based vaccination. Furthermore, recombinant Gal-8 is easy to produce, and unlike oily adjuvants, it does not induce denaturalization of the antigenic proteins, thus preserving conformational epitopes that might be crucial to induce effective humoral responses. As mentioned above, we have developed a mutated form of Gal-8 that preserved its ability to promote an antigen-specific T cell response but has lost the antigen-independent proliferative induction [25]. We consider that the use of this modified Gal-8 may constitute an alternative tool to assay in the immunization model in the future, given the lack of potentially unspecific proinflammatory effects. Therefore, all of these characteristics of Gal-8 would add to the design of improved vaccine formulations.

Our data demonstrate that Gal-8 induces a full DC activation, which is probably one of the mechanisms involved in the elicitation of the adaptive immune response.

AUTHORSHIP

J.C. and M.V.T. designed and performed experimental work, analysed data and wrote the manuscript. V.Q. and P.Z. performed FMDV NAb response and viral challenge assays. A.D. performed anti-FMDV IgG ELISA. M.V.T. and O.C. conceived the project and supervised work.

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DISCLOSURES

The authors declare no conflicts of interest.

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