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β 2-Adrenoreceptor Agonist Inhibits Antigen Cross-Presentation by Dendritic Cells

Julie Hervé,^{*,†} Laurence Dubreil,^{†,‡} Virginie Tardif,[§] Mickael Terme,^{*,†} Sylvie Pogu,^{*,†} Ignacio Anegón,[§] Bertrand Rozec,[¶] Chantal Gauthier,[¶] Jean-Marie Bach,^{*,†} and Philippe Blancou^{*,†}

Despite widespread usage of β -adrenergic receptor (AR) agonists and antagonists in current clinical practice, our understanding of their interactions with the immune system is surprisingly sparse. Among the AR expressed by dendritic cells (DC), β 2-AR can modify *in vitro* cytokine release upon stimulation. Because DC play a pivotal role in CD8⁺ T cell immune responses, we examined the effects of β 2-AR stimulation on MHC class I exogenous peptide presentation and cross-presentation capacities. We demonstrate that β 2-AR agonist-exposed mature DC display a reduced ability to cross-present protein Ags while retaining their exogenous peptide presentation capability. This effect is mediated through the nonclassical inhibitory G ($G_{\alpha i0}$) protein. Moreover, inhibition of cross-presentation is neither due to reduced costimulatory molecule expression nor Ag uptake, but rather to impaired phagosomal Ag degradation. We observed a crosstalk between the TLR4 and β 2-AR transduction pathways at the NF- κ B level. *In vivo*, β 2-AR agonist treatment of mice inhibits Ag protein cross-presentation to CD8⁺ T cells but preserves their exogenous MHC class I peptide presentation capability. These findings may explain some side effects on the immune system associated with stress or β -agonist treatment and pave the way for the development of new immunomodulatory strategies. *The Journal of Immunology*, 2013, 190: 3163–3171.

Large amounts of catecholamines are released during stress and physical activity from postganglionic adrenergic fibers and adrenal glands following activation of the sympathetic nervous system (SNS). The SNS principal neurotransmitters are norepinephrine (NE) and epinephrine (E) that bind both α - (subtypes α 1 and α 2) and β -adrenergic receptors (AR) (subtypes β 1, β 2, and β 3). Modulation of the adrenergic system has been successful in many diseases, making β -adrenergic modulators among the best selling drugs in the world. However, their effects on the immune system have been questioned for different pathologies (1, 2).

AR are expressed by many cell types throughout the body, including immune cells. Many authors have documented that β -AR agonists (referred to as β -agonists) display a deep impact

on T and B cell functions both *in vitro* and *in vivo* (reviewed in Ref. 3). In particular, catecholamines induce elevation in the number of circulating lymphocytes both in humans (4) and mice (5). The global effect of β -agonists on immunity was mainly studied by chemically depleting natural catecholamine stocks using 6-hydroxydopamine, which results in increased innate immune responses against bacteria (6), increased adaptive immune responses against virus (7), and decreased immune responses against Gram-positive bacteria (7–9). Additionally, surgical removal of the ocular sympathetic nerve affects the generation and maintenance of immune privilege in the eye (10). However, although these reports strongly suggest that the SNS regulates the magnitude and quality of immune responses via AR agonists, the molecular and cellular basis of this phenomenon remain unclear.

Conflicting results were obtained regarding AR expression on immune cells (11). Concerning APC, the effect of AR agonists is being revealed. AR (α 1, α 2, β 1, β 2) stimulation of macrophages decreases their phagocytic activity (12) and TNF- α secretion (13) and increases apoptosis (14). As APC, dendritic cells (DC) appear to be uniquely specialized for cross-presentation with the capacity to acquire exogenous proteins, process them into peptide, load and display MHC class I (MHC-I)/peptide complexes on their surface, and prime naive CD8⁺ T cells. Interestingly, bone marrow-derived DC (bmDC) and splenic DC express β 2-AR receptor mRNA (7, 15). Similar to macrophages, DC are sensitive to AR stimulation, which modifies their cytokine secretion (16), migration capabilities (17), and Ag uptake (18) via the β 2, α 1b subtype, and α 2-AR, respectively. Recently, it was demonstrated that β 2-AR blocking enhances antiviral CD8⁺ T cell responses, supporting a chronic effect of the β 2-AR on immune responses (7). Moreover, deletion of the adrenergic system by chemical sympathectomy was associated to an enhanced cross-presentation capacity of CD11c⁺CD8 α ⁺ DC (7). However, we do not know whether β 2-AR agonists can directly modify cross-presentation capabilities of DC.

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Abbreviations used in this article: AC, adenylate cyclase; AR, adrenergic receptor; bmDC, bone marrow-derived dendritic cell; DC, dendritic cell; HA, hemagglutinin; MHC-I, MHC class I; PTX, pertussis toxin; SNS, sympathetic nervous system; wt, wild-type.

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In this study, we sought to determine the effects of *in vitro* and *in vivo* β 2-AR stimulation on cross-presentation of Ag by DC and on ensuing Ag-specific CD8⁺ T cell responses using both pharmacological and genetic approaches. We also investigated the effects of β 2-AR stimulation on the steps involved in cross-presentation, including Ag uptake and processing. Collectively, our findings provide new insights into molecular and cellular mechanisms by which β -agonist impairs Ag-specific immune responses by interfering with cross-presentation by DC, a process with broad implications in many diseases.

Materials and Methods

Chemicals

LPS *Escherichia coli* 0111:B4 was purchased from InvivoGen. Salbutamol hemisulfate and pertussis toxin (PTX) originated from Sigma-Aldrich. ICI-118,551, Rp-cAMP, NF-449, forskolin, and KT 5720 came from Tocris Biosciences. PTX, Rp-AMPC, NF-449, forskolin, and KT 5720 were, respectively, used at 2 μ g/ml (19), 300 μ M (20), 30 μ M (21), 10 μ M (22), and 10 μ M (23).

Mice and cells

C57BL/6J, OT-I, and BALB/c mice were originally purchased at The Jackson Laboratory (Bar Harbor, ME). β 2-adrenoreceptor knockout mice (24) were provided by M. Barrot (Strasbourg University, Strasbourg, France) and backcrossed for seven generations on the C57BL/6J background. CL4 mice were provided by R. Liblau. Splenic OT-I and CL4 CD8⁺ T cells were purified using CD8⁺ magnetic beads (Miltenyi Biotec). The B3Z cell line was a gift from A. Savina (INSERM Unité 932, Paris, France). bmDC were derived from C57BL/6J or BALB/c mice as previously described (25).

DC treatment

After CD11c⁺ magnetic cell sorting, bmDC were classically treated for 3 h with LPS (1 μ g/ml) with or without salbutamol (1 μ M) and with or without ICI-118,551 (10 μ M). For each experiment, supernatants were collected 24 h afterward and secretion of IL-10 and IL-12 was determined by ELISA (BD Pharmingen). In parallel, bmDC were analyzed for expression of CD40, CD80, CD86, and MHC-I and viability (7-aminoactinomycin D staining) was determined by flow cytometry. We did not observe any effect of treatment on cell viability (as assessed by 7-aminoactinomycin D staining; viability was >95% in all experiments presented).

In vitro CD8⁺ T cell activation assays

In vitro CD8⁺ T cell activation was performed either using OT-I (anti-OVA) or CL4 (anti-hemagglutinin [HA]) CD8⁺ T cells.

For OT-I CD8⁺ T cells, bmDC were incubated either with soluble OVA (endotoxin-free OVA; Hyglos) (50 μ g/ml), OVA-coated beads, or with the H-2K^b-restricted OVA peptide SIINFEKL (OVA₂₅₇₋₂₆₄) (1 nM) (Neosystem/PolyPeptide Laboratories). For CL4 CD8⁺ T cells, bmDC were incubated either with an inactivated influenza A PR8 virus (produced on embryonated chicken eggs provided by Nicolas Escirou, Institut Pasteur, Paris, France) or with the H-2K^d-restricted HA peptide IYSTVASSL (HA₅₁₂₋₅₂₀) (1 nM) (Neosystem/PolyPeptide Laboratories). For lentiviral infection, DC were infected in a minimal volume of medium for 1 h with a lentivirus encoding HA Ag (pRRLSIN.cPPT.PGK/HA.WPRE; Lentiviral Vector Production Unit, Swiss Institute of Technology, Lausanne, Switzerland) 2 d before coculture. In all cases, after incubation, DC were thoroughly washed and cocultured with CD8⁺ T from either OT-I or CL4 transgenic mice or B3Z cells. IL-2 release was measured by ELISA and proliferation was assessed either using [³H]thymidine incorporation or CFSE staining.

Assessment of Ag internalization and processing by DC

OVA endocytosis and phagocytosis were determined using Alexa 488-OVA (Invitrogen) and PE beads (BD Pharmingen), respectively. DC were pulsed with fluoroprobes for 30 min and chased for 0, 15, 30, and 90 min at 37°C. After chase, cells were analyzed by flow cytometry. A control at 4°C was included to evaluate the percentage of nonspecific endocytosis or phagocytosis.

Intraphagosomal degradation was determined using OVA-coated beads as previously described (26). DC were pulsed-chased at the indicated times

(0, 30, 60, and 120 min) with OVA-coated beads, washed, immediately disrupted in lysis buffer, and centrifuged (150 × *g*, 4 min, 4°C). Supernatants containing beads were collected and stained with a rabbit polyclonal anti-OVA (MP Biomedicals) and FITC-coupled anti-rabbit Abs (Jackson ImmunoResearch). Percentage of degraded OVA was determined for each condition. A control tube containing a mixture of protease inhibitors (cOmplete tablets; Roche) was included.

Detection of specific peptide/MHC-I complexes on DC

By immunofluorescence, DC were incubated with soluble OVA for 3 h. Then, immunofluorescence was performed according to a protocol previously described by Kurts and colleagues (27). Briefly, cells were cocultured with mouse 25-D1.16 mAb (gift from Dr. R.N. Germain), then revealed using Alexa 568-conjugated donkey anti-murine IgG and counterstained with TO-PRO3 (1 μ g/ml; Molecular Probes/Invitrogen). Slides were observed with a confocal microscope. Mean fluorescence intensity was measured for each condition in each channel on a total of at least 1500 analyzed cells by conditions.

By flow cytometry, as previously described (27), DC were incubated with soluble OVA (5 mg/ml) for 16 h, thoroughly washed twice, and incubated with PE-coupled 25-D1.16 mAb (BioLegend) for 30 min on ice. Cells were then washed and fluorescence intensity was analyzed using a BD FACSAria. Controls included DC incubated with the OVA peptide SIINFEKL.

NF- κ B immunofluorescence experiments

DC were incubated with anti-p65 NF- κ B Ab (SC 372; Santa Cruz Biotechnology), revealed using Alexa 488-conjugated donkey anti-rabbit IgG, and cell nuclei were counterstained with TO-PRO3. To evaluate the ratio of NF- κ B protein in the cytoplasm versus nucleus, we developed a macro using the Nikon instrument software image analysis. The sum intensity of total NF- κ B was measured for image 1, and then the nuclear image (referred to as image 2) was subtracted from the total NF- κ B image 1 and the result of this subtraction was identified as image 3, which was quantitated as the “cytosolic NF- κ B fluorescence” sum intensity. The “nuclear NF- κ B fluorescence” sum intensity was determined by subtracting total NF- κ B sum intensity (referred to as image 1) to cytosol NF- κ B intensity (referred to as image 3). Finally, mean nuclear intensity was obtained by dividing nuclear sum intensity by nuclear area determined from image 2. The total fluorescence mean intensity was determined by dividing total NF- κ B sum intensity by total NF- κ B area. Analyses were performed at least on 5000 cells by condition. Pearson’s correlation coefficient calculation as in Ewins et al. (28) gave comparable results (data not shown).

In vivo cross-presentation assay

Cross-presentation *in vivo* was assessed in C57BL/6J wild-type (wt) and β 2-AR knockout mice previously injected *i.v.* with 5×10^6 OT-I CD8⁺ T cells that were CFSE stained. On day 0, mice received *i.p.* either OVA protein (250 μ g/mouse) or SIINFEKL peptide (100 μ g/mouse) along with LPS (80 μ g/mouse) with or without salbutamol (200 μ g/mouse). On day 5, mice were sacrificed and CD8⁺ proliferation was assessed by cytometry.

Ex vivo cross-presentation experiments

Cross-presentation by splenic DC was assessed on splenic CD11c⁺ cells originating from C57BL/6J mice previously injected *i.v.* either with OVA protein (500 μ g) or SIINFEKL peptide (100 μ g) along with LPS (80 μ g) with or without salbutamol (200 μ g). Three to 5 h later, splenic DC were collected in the presence of brefeldin A as previously described (29). Splenic DC were cocultured with CFSE-stained OT-I CD8⁺ T cells and proliferation was assessed after 40 h.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software). For multiexperimental group analysis, data were subjected to one-way and two-way ANOVA followed by a post hoc test (Bonferroni or Tukey multiple comparison test). When experiments were paired, a Wilcoxon matched paired *t* test was applied.

Results

β 2-AR agonist modifies DC IL-12/IL-10 secretions through *G α i0* signaling

Using salbutamol (β 2-AR agonist) alone or in combination with ICI-118,551 (β 2-AR antagonist), we demonstrated that inhibition

of IL-12 secretion ($86 \pm 14\%$, $n = 13$) and increase of IL-10 secretion ($215 \pm 46\%$, $n = 8$) by bmDC were specifically mediated by $\beta 2$ -AR receptor (Fig. 1A). This observation was confirmed with bmDC originated from $\beta 2$ -AR-deficient mice, which were insensitive to salbutamol effects on IL-12/IL-10 secretion following TLR4 stimulation (Fig. 1A). Along with this effect, salbutamol slightly diminished costimulatory molecule expression (CD86, CD40) in a $\beta 2$ -AR-dependent fashion (Fig. 1B, Supplemental Fig. 1A) but did not change MHC-I molecule expression (data not shown).

To investigate the $\beta 2$ -AR transduction pathway in DC, we screened modulatory molecules of the G protein-coupled receptor transduction pathway for their ability to restore IL-12 secretion following $\beta 2$ -AR stimulation. Inhibition of either G_s protein by NF 449 or protein kinase A by KT 5720 and Rp-cAMP did not restore IL-12 secretion following treatment of DC with salbutamol (Fig. 1C). Similarly, protein kinase C (Go6983) and phospholipase A_2 (AACOCF3) inhibitors were also tested without success for IL-12 restoration (Supplemental Fig. 1B). In contrast, PTX restored both IL-12 and IL-10 secretions (Fig. 1D), supporting a critical role for the $G_{\alpha i/0}$ pathway in the effects displayed by $\beta 2$ -AR agonists on DC. Furthermore, the activation of adenylate cyclase (AC) using forskolin did not reproduce the effects of salbutamol on IL-12 secretion, which strengthens the fact that $\beta 2$ -AR-mediated effects on DC are not mediated by an increase in intracellular cAMP through AC activation (Fig. 1E).

$\beta 2$ -AR-mediated inhibition of cross-presentation is dependent on $G_{\alpha i/0}$

We then investigated whether cross-presentation by DC was directly affected by salbutamol using the widely used in vitro OVA cross-presentation assay. Depending on their size, Ag are processed either through the early endosomal compartment for soluble Ag or

through the phagocytic pathway for particulate Ag ($>0.5 \mu m$) (30). LPS-matured bmDC were incubated with soluble OVA, particulate OVA-coated beads, or OVA peptide and treated using salbutamol with or without ICI-118,551. Cells were then extensively washed and cocultured with naive anti-OVA $CD8^+$ T cells (OVA₂₅₇₋₂₆₄ SIINFEKL, H2-K^b-restricted) isolated from OT-1 TCR transgenic mice. DC were impaired in their capacity to cross-present soluble or particulate Ag when treated with the highly specific $\beta 2$ -AR agonist salbutamol while retaining their exogenous MHC-I/peptide presentation capabilities toward OVA-specific $CD8^+$ T cells (Fig. 2A). Besides proliferation, IL-2 production was dramatically reduced in cross-presentation assays whereas it was conserved for exogenous MHC-I presentation upon salbutamol treatment of DC (data not shown). One can argue that the use of high peptide concentration may hide the potential effect of salbutamol on MHC-I/peptide presentation capabilities. However, lower peptide concentration did not reveal different priming capacities of untreated versus salbutamol-treated DCs (Fig. 2D). PTX completely restored cross-presentation (Fig. 2B), once again supporting the coupling of $\beta 2$ -AR with $G_{\alpha i/0}$ protein in DC functions.

The massive effect of $\beta 2$ -AR treatment on cross-presentation by DC cannot be explained by its marginal effect on costimulatory molecules expression (Fig. 1B, Supplemental Fig. 1A). To test whether $\beta 2$ -AR inhibition of cross-presentation results from the decrease in IL-12 secretion (Fig. 1A) or from a decrease in peptide/MHC-I complex expression at the DC surface, we used the anti-OVA B3Z hybridoma T cells in an OVA cross-presentation assay. This cell line is known to be activated exclusively upon OVA₂₅₇₋₂₆₄ SIINFEKL peptide/MHC-I complex expression (31). When cocultured with salbutamol-treated DC, B3Z T cells exhibited inhibition of activation, which is in favor of a decrease in OVA₂₅₇₋₂₆₄ SIINFEKL peptide/MHC-I complex expression at the DC surface induced by salbutamol treatment (Fig. 2C, 2D). This phenomenon

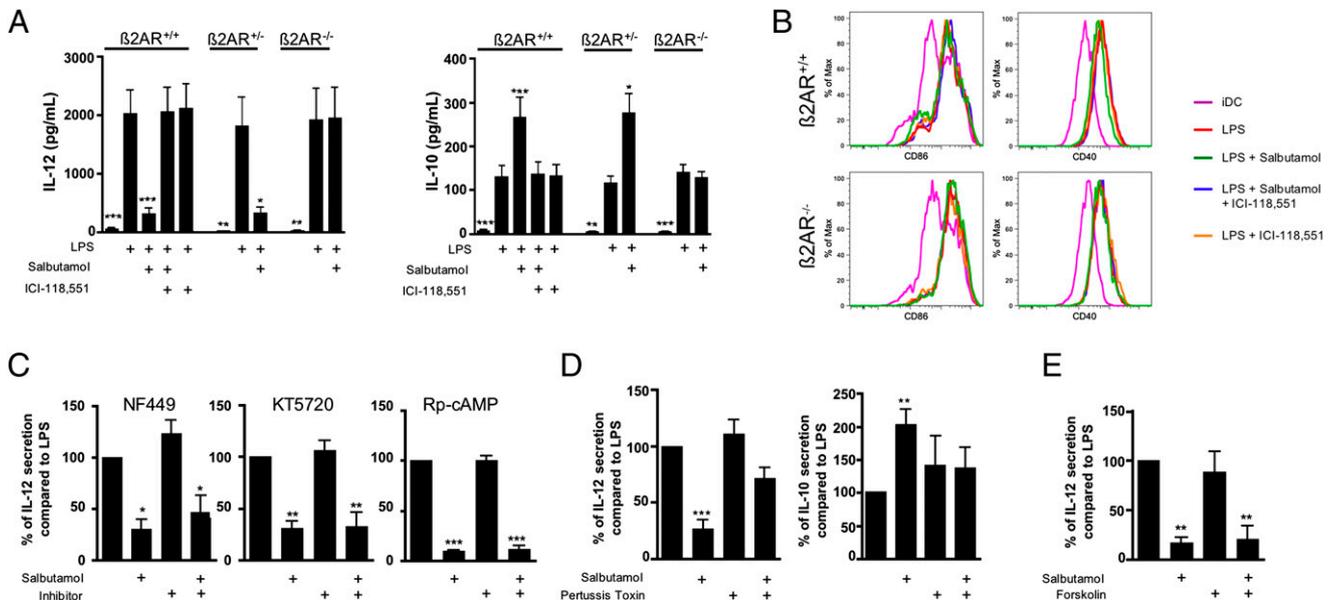


FIGURE 1. $\beta 2$ -AR signaling impairs IL-12 secretion and costimulation marker expression but increases IL-10 production by DC in a $G_{\alpha i/0}$ -dependent fashion. (**A** and **B**) bmDC from C57BL/6J wt or $\beta 2$ -AR-deficient mice were either left untreated (iDC), LPS ($1 \mu g/ml$) matured, or treated with salbutamol ($1 \mu M$). $\beta 2$ -AR specificity was tested by addition of the $\beta 2$ -AR antagonist ICI-118,551 ($10 \mu M$). (**C–E**) $CD11c^+$ cells were LPS matured and pretreated with inhibitors for 1 h before being incubated with LPS and salbutamol in at least three independent experiments. IL-12, IL-10 secretion (**A**, **C–E**), and costimulation marker (CD86, CD40) expression (**B**) were evaluated by ELISA and flow cytometry, respectively, after 24 h culture. Results are either presented as amount of cytokine released in the supernatant (**A**) or percentage of secretion as compared with the LPS group (**C–E**). Each experiment was performed independently at least three times \pm SEM except in (**B**) where a representative histogram from cytometric analysis of costimulation marker (CD86, CD40) expression on DC is presented. For statistical analysis, a one-way ANOVA was performed. Only significant p values of the different groups compared with the LPS group are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

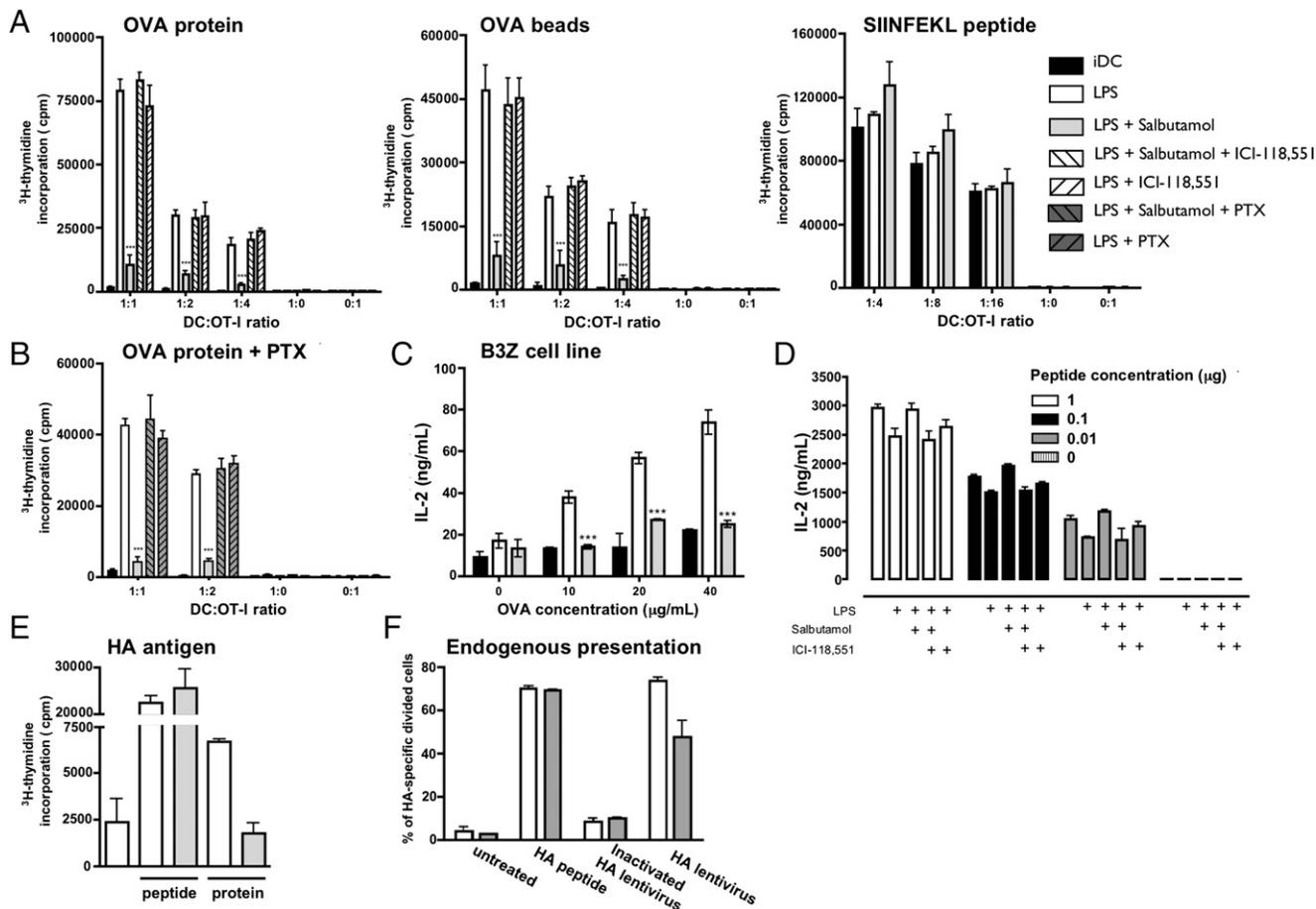


FIGURE 2. Salbutamol-treated DC are affected in their cross-presentation but not presentation of peptide capabilities in a $G_{\alpha i/o}$ -dependent fashion. $CD11c^+$ -sorted bmDC were left untreated (iDC), LPS matured with (LPS plus salbutamol group) or without (LPS group) β_2 -AR agonist for 3 h. (A) For each condition, $CD11c^+$ -sorted bmDC derived from C57/B6J mice were either coincubated with soluble OVA protein, particulate OVA-coated beads, or H2-K^b-restricted OVA_{257–264} peptide (SIINFEKL), thoroughly washed, and cocultured with anti-OVA CD8⁺ T cells from OT-I mice. β_2 -AR specificity was tested by addition of β_2 -AR antagonist (ICI-118,551). Radioactive thymidine incorporation was evaluated 36 h later for 18 h. One representative experiment out of three is presented. (B) $CD11c^+$ bmDC from C57BL/6J were pretreated with PTX ($G_{\alpha i/o}$ inhibitor) for 1 h before being treated and loaded with soluble OVA protein as described above. Radioactive thymidine incorporation was evaluated as in (A). (C and D) $CD11c^+$ bmDC from C57BL/6J mice were coincubated with soluble OVA protein (C) or SIINFEKL peptide (D) as described above, then thoroughly washed and cocultured with anti-OVA B3Z CD8⁺ T cell hybridoma. IL-2 secretion was evaluated after 24 h culture. (E) $CD11c^+$ -sorted bmDC derived from BALB/c mice were either incubated with HA Ags (inactivated influenza H1N1 PR8 virus produced on embryonated eggs) or H2-K^d-restricted HA_{512–520} peptide for 3 h, thoroughly washed, and cocultured with anti-HA CD8⁺ T cells isolated from CL4 TCR transgenic mice (36). Radioactive thymidine incorporation was evaluated as in (A). (F) $CD11c^+$ -sorted bmDC derived from BALB/c mice were infected either with an inactivated or a fully infectious lentivirus expressing HA Ag and cocultured 2 d later with anti-HA CD8⁺ T cells isolated from CL4 TCR transgenic mice. Uninfected or H2-K^d-restricted HA_{512–520} peptide-loaded DC were used as negative or positive controls, respectively. The mean value \pm SEM of one representative experiment performed in triplicates out of at least three is presented for (A) and (C)–(E), and two for (B) and (F). For statistical analysis a one-way ANOVA was performed. Only significant p values of the LPS plus salbutamol group compared with LPS group are shown. *** $p < 0.001$.

is not restricted to OVA Ag, as cross presentation of HA Ags from influenza virus is also inhibited following β_2 -AR stimulation of DC whereas exogenous peptide presentation is preserved (Fig. 2E). Interestingly, endogenous MHC-I presentation is also affected, as salbutamol-treated DC infected with a lentivirus expressing HA Ag display lower presentation capability to naive CD8⁺ T cells compared with untreated DC (Fig. 2F).

Collectively, these data establish that signaling through β_2 -AR inhibits cross-presentation as well as endogenous presentation without interfering with exogenous peptide presentation.

β_2 -AR signaling alters phagosomal degradation

Alteration of exogenous Ag cross-presentation by salbutamol-treated DC could result from impaired Ag uptake capacities. It was recently demonstrated that α_2 -AR stimulation enhances Ag uptake by DC (18). To investigate this possibility, LPS-treated DC

were incubated with salbutamol and Ag uptake was assessed using soluble OVA-Alexa 488 and particulate PE beads for endocytic and phagocytic pathways, respectively. As assessed by flow cytometry, Ag uptake of soluble pH-insensitive OVA fluorophore Alexa 488-OVA was unaffected upon β_2 -AR stimulation (Fig. 3A). Likewise, salbutamol did not change the ability of DC to phagocytose 1- μ m particulate beads coupled with PE (Fig. 3B). Moreover, no difference in the percentage of positive cells was observed at earlier or later time points either with Alexa 488-OVA or particulate beads coupled with PE (data not shown).

The next step of Ag processing following phagocytosis is protein degradation that occurs in the early phagosomal compartment (30). As before, DC were LPS matured, salbutamol treated with or without β_2 -AR antagonist before performing a well-standardized Ag degradation assay (26). OVA degradation at the surface of phagocytosed beads was significantly affected over time by β_2 -

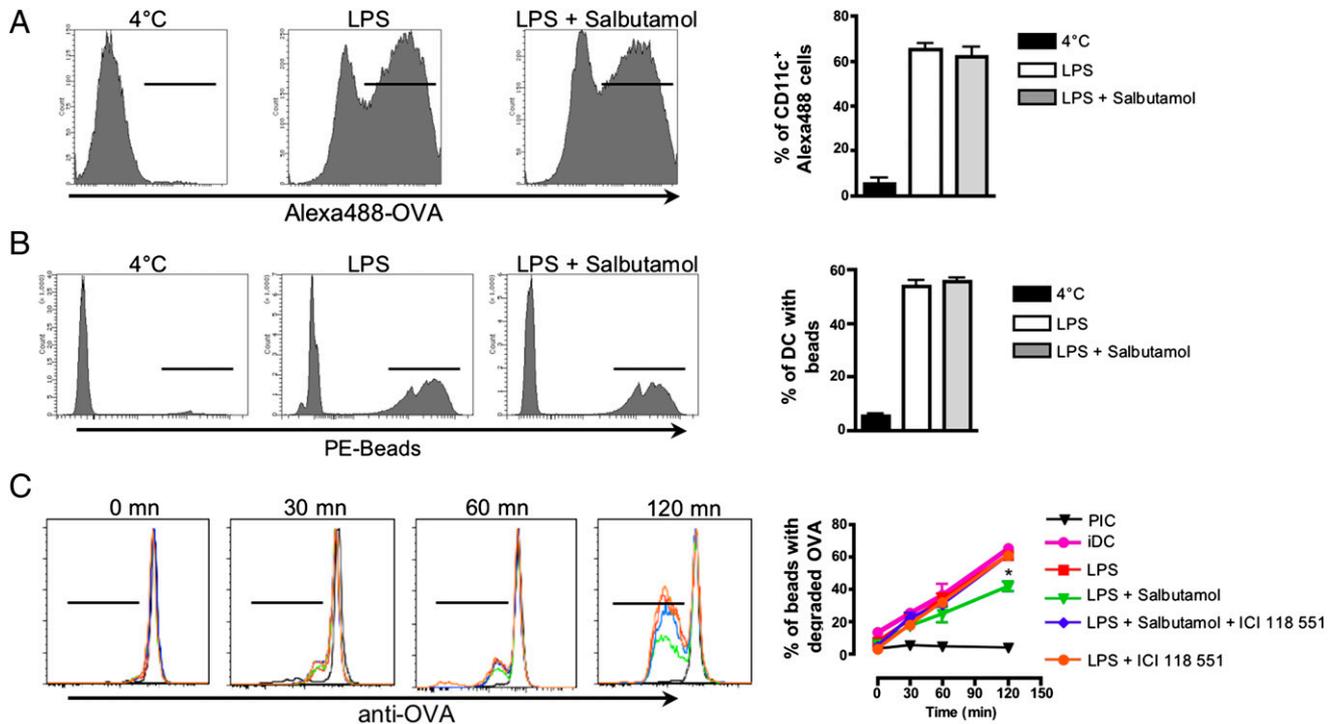


FIGURE 3. Salbutamol treatment does not affect Ag uptake but reduces phagosomal Ag degradation. CD11c⁺ bmDC were treated with LPS with or without salbutamol or antagonist for 3 h, as described in Fig. 1. Flow cytometric analysis was either performed during the chase phase after a 30 min pulse with Alexa 488-OVA-coupled protein (**A**) or directly after incubation with PE-coupled beads (**B**). Phagocytic degradation was kinetically determined after staining with anti-OVA Ab following a 30 min pulse period with OVA bead-coupled protein (**C**). A 4°C (**A**, **B**) or protease inhibitory mixture (PIC) (**C**) control was included at each time point to evaluate the percentage of nonspecific endocytosis, phagocytosis, or proteolysis. All flow cytometric graphs are representative of three (**B**), four (**C**), or five (**A**) independent experiments. For (**A**) and (**B**), a paired *t* test was applied between LPS and LPS plus salbutamol groups (none of which reached significance). Error bars represent SEM. For (**C**) a two-way ANOVA was performed to compare replicate means of the LPS plus salbutamol condition to the LPS condition [all tests are not significant for all time points, except in (**C**), where **p* < 0.05 at 120 min].

AR stimulation (Fig. 3C) whereas MHC-I Ag expression was unaffected (data not shown), demonstrating that phagosomal degradation was partially inhibited by β 2-AR signaling. This effect on LPS-matured DC was reversed using β 2-AR antagonist ICI-118,551.

Endosomal and phagosomal pathways can lead to cytosolic release of Ag fragments that are further processed by the proteasome before being either imported to the endoplasmic reticulum or reimported back to phagosomal or endosomal compartments essentially through TAP-mediated translocation (30). We looked for modulation of proteasomal activity following β 2-AR stimulation of DC using a Proteasome-Glo cell-based assay as a homogeneous, luminescent assay that measures the chymotrypsin-like, trypsin-like, and caspase-like activities associated with the proteasome complex in cultured cells. β 2-AR stimulation of bmDC did not affect any of the three proteasome activities tested after 1, 2, or 4 h incubation (Supplemental Fig. 2).

Taking advantage of the 25D1.16 Ab that specifically stains OVA-derived peptide bound to MHC-I H-2K^b (Supplemental Fig. 3A), we quantified the amount of MHC-I/peptide complexes in different conditions (Fig. 4A). We found that peptides loaded on MHC-I molecules following Ag processing were reduced in salbutamol-treated DC as compared with untreated DC, probably due to lower OVA degradation efficiency in β 2-AR agonist-treated DC. Moreover, using flow cytometry analyses with the 25D1.16 Ab, we demonstrated that surface OVA-derived peptide bound to MHC-I H-2K^b was also decreased by salbutamol treatment (Fig. 4B).

Collectively, these data demonstrate that β 2-AR stimulation of matured DC affects phagosomal degradation, which may con-

tribute to decreased MHC-I/peptide complex membrane expression.

Cross-talk between TLR4 and β 2-AR occurs at the NF- κ B level

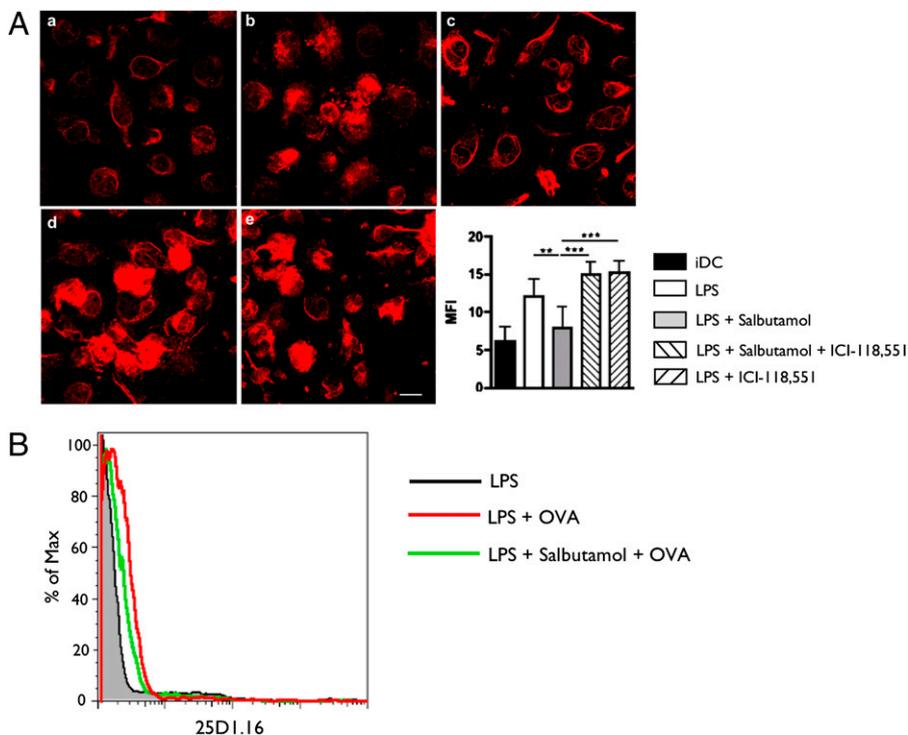
NF- κ B is one of the major transcriptional factors activated during TLR4-induced DC maturation process leading to proinflammatory cytokine release. To investigate whether β 2-AR signaling affects NF- κ B activation following TLR4 stimulation, we performed immunofluorescence assays on untreated and salbutamol-treated cells. Data showed that the nuclear translocation of both p65 (Fig. 5) and p50 (Supplemental Fig. 3B) NF- κ B subunits were significantly and specifically inhibited by salbutamol.

In vivo cross-presentation is inhibited by β 2-AR signaling

We next investigated whether β 2-AR signaling affects in vivo cross-presentation. Toward this aim, C57BL/6J recipient mice were injected with OT-I TCR transgenic T cells expressing CD45.1 congenic marker and immunized with OVA peptide SIINFEKL or soluble OVA in addition or not with salbutamol treatment. The proportion of OT-I donor cells was strongly decreased in salbutamol-treated compared with untreated mice in cross-presentation group (Fig. 6A, Supplemental Fig. 4A). In contrast, CD8⁺ T cell expansion to peptide was not inhibited (Fig. 6B, Supplemental Fig. 4A). Importantly, note that inhibition of CD8⁺ T cell expansion following salbutamol treatment is specifically mediated by β 2-AR, as β 2-AR-deficient mice did not show any inhibition of in vivo OVA protein immune response (Fig. 6C).

To investigate whether reduced cross-presentation of OVA by salbutamol-treated DC resulted in an inhibited proliferation of specific CD8⁺ T cells as observed in vitro, we transferred CFSE

FIGURE 4. Salbutamol inhibits MHC-I/peptide complex formation. CD11c⁺ bmDC were treated as described in Fig. 1 for 3 h in the presence of soluble OVA protein and then either fixed, permeabilized, and stained with 25-D1.16 Ab (A) or directly stained with 25-D1.16 Ab and processed for flow cytometry analysis (B). Slides were analyzed by confocal microscopy. (A) Representative images are presented for the different conditions. (a), iDC; (b), LPS; (c), LPS + salbutamol; (d), LPS + salbutamol + ICI 118,551; (e), LPS + ICI 118,551. Quantification of immunofluorescence of three independent experiments is also reported (bottom right). (B) A representative flow cytometry profile out of two independent experiments performed is presented. For statistical analysis a one-way ANOVA was performed. Scale bar, 10 μ m. Error bars represent SEM. Only significant *p* values are shown. ***p* < 0.01, ****p* < 0.001.



loaded CD45.1⁺CD8⁺ T cells from OT-I mice and followed CFSE dilution in donor C57BL/6J mice 5 d after immunization (Fig. 6D). Most donor cells in untreated mice had undergone more than five divisions whereas less than half of them had divided as much in salbutamol-treated mice. This strongly suggests that the defect in CD8⁺ T cell expansion was due to a defect of Ag cross-presentation by DC rather than to an increase in apoptosis-induced cell death or trapping of CD8⁺ cells into tissue.

To rule out a potential direct effect of salbutamol on CD8⁺ T cells, cardiovascular system, or metabolism rather than a direct effect on DC, we isolated splenic CD11c⁺ cells 3–5 h following OVA peptide or protein immunization and salbutamol treatment. These cells were in vitro cocultured with CFSE-loaded naive CD8⁺ T cells from OT-I mice for 36 h. As observed in vivo, ex vivo stimulation of anti-OVA

CD8⁺ T cells was decreased when mice were immunized with OVA protein and treated with salbutamol as compared with untreated control (Fig. 7). As previously observed, peptide immunization was as efficient to prime CD8⁺ T cells in salbutamol-treated compared with untreated mice (Fig. 7) even when nonsaturating peptide doses were used (Supplemental Fig. 4B).

Taken together, these results show that salbutamol inhibits in vivo cross-presentation by DC while retaining their exogenous peptide presentation capacities.

Discussion

Cross-presentation is often needed for the induction of CD8⁺ T cell responses and is critical to many immune mechanisms. However, ensuing CD8⁺ T cell priming depends on environmental context.

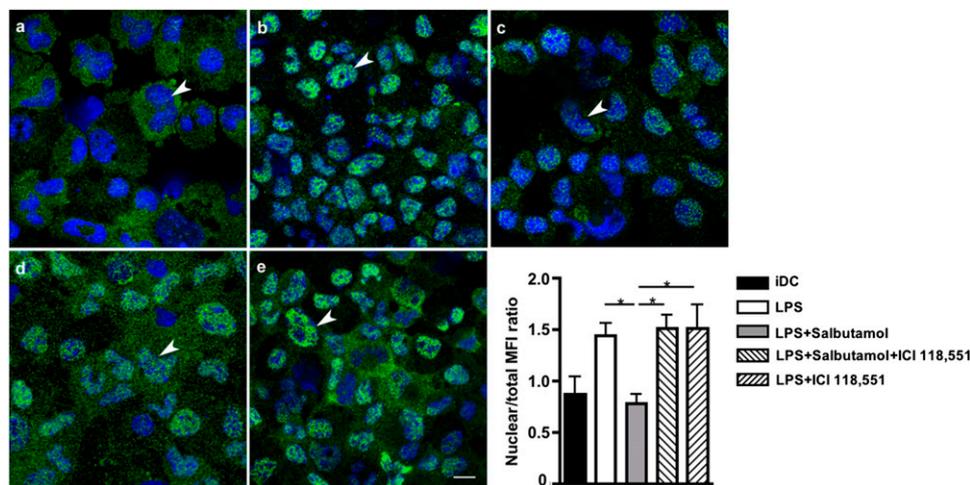


FIGURE 5. β 2-AR agonist affects NF- κ B translocation to the nucleus. CD11c⁺ bmDC were treated as described in Fig. 1 for 30 min, fixed, permeabilized, and stained using anti-p65 NF- κ B Ab. NF- κ B p65 nuclear translocation was analyzed by laser confocal microscopy. A representative image is shown in (a)–(e). (a), iDC; (b), LPS; (c), LPS plus salbutamol; (d), LPS plus salbutamol plus ICI-118,551; (e), LPS plus ICI-118,551. Quantification of immunofluorescence of three independent experiments is shown in the graph (bottom right). For statistical analysis a one-way ANOVA was performed. Scale bar, 10 μ m. Error bars represent SEM. Only significant *p* values are shown. **p* < 0.05.

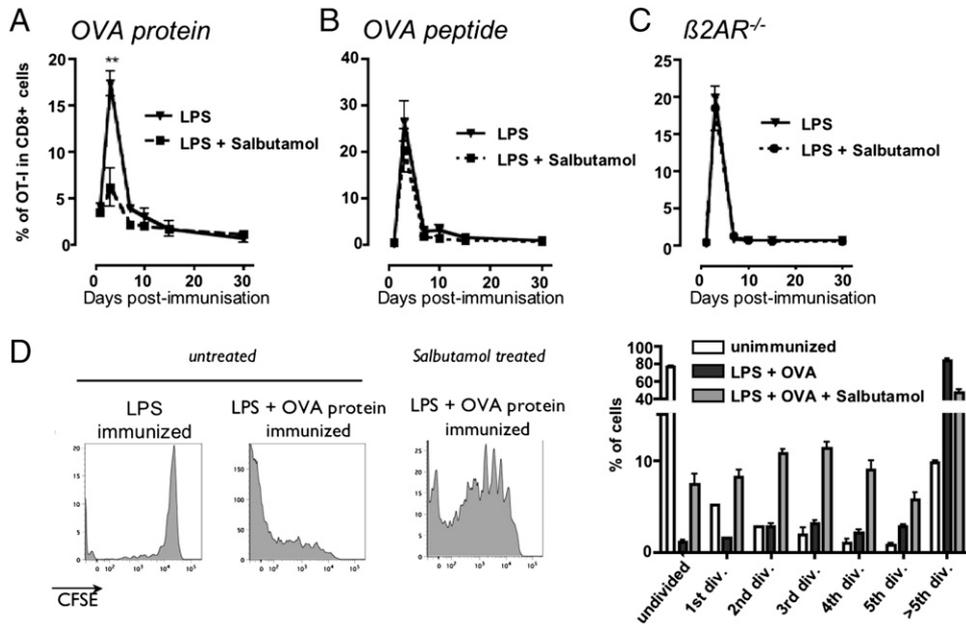


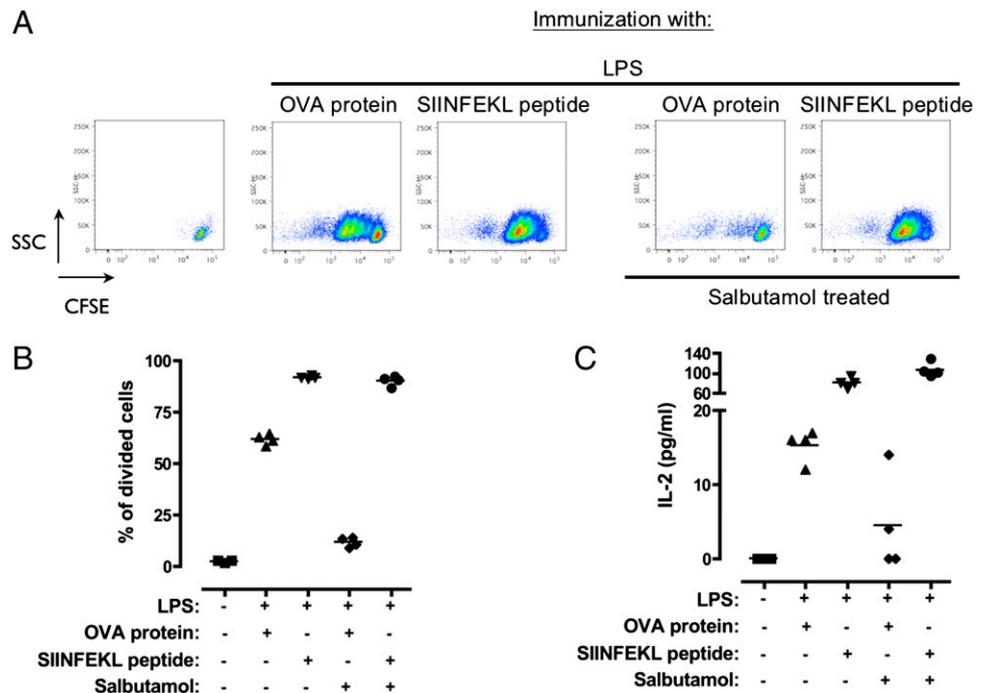
FIGURE 6. Salbutamol treatment of mice inhibits Ag immune response. C57BL/6J wt (A, B, D) or $\beta 2AR^{-/-}$ (C) mice received $4-8 \times 10^6$ anti-OVA CD8⁺ T cells i.v. from OT-I CD45.1⁺ mice at day 0. The day after, all mice (A–D) were challenged with LPS (80 μ g i.p./mouse) with or without salbutamol treatment (200 μ g i.p./mouse), and immunized either with OVA protein (250 μ g i.p./mouse) (A, C, D) or OVA peptide SIINFEKL (100 μ g i.p./mouse) (B). Then, mice were either followed for 1 mo (A–C) or sacrificed at day 5 (D). (D) Histograms of CD8 CD45.1 double-positive spleen cells are presented. The results shown are representative of at least three independent experiments with at least two animals per group for (A), (B), and (D) and two for (C). Error bars represent SEM. For (A)–(C) a two-way ANOVA was performed [all tests are not significant for all time points, except in (A), where $**p < 0.01$ for one time point].

Codelivery of inflammatory signals along with Ag by DC leads to cytotoxic CD8⁺ T cell generation whereas the absence of inflammatory signals leads to tolerized T cells. Modulating the initial steps of cross-presentation may be important, as it may help us to increase or prevent CD8⁺ T cell activation. In the present study, we demonstrate that $\beta 2AR$ signaling inhibits in vitro and in vivo cross-presentation by DC through the G_i protein pathway.

Interestingly, DC express $\beta 2AR$ at their surface (7, 15) and their stimulation by β -agonists leads to modification of their cy-

tokine secretion profile (18, 19). The G protein-coupled receptor $\beta 2AR$ was initially considered to be exclusively associated to G_s , thus increasing AC activity. However, it has recently become apparent that β -AR are also capable of transducing other signaling processes than those associated with the formerly recognized cAMP-related pathways. Studies have shown that some aspects of signaling via β -AR are inhibited by PTX, indicating that they might be mediated through G_i proteins (32). In this study, we demonstrate that $\beta 2AR$ inhibition of IL-12 secretion and cross-

FIGURE 7. Salbutamol treatment of mice inhibits splenic DC cross-presentation capabilities. C57BL/6J were challenged with LPS (80 μ g i.p./mouse) plus OVA protein (500 μ g i.p./mouse) or OVA peptide SIINFEKL (100 μ g i.p./mouse) and were treated or not with salbutamol (200 μ g i.p./mouse) the same day. Four hours after immunization, CD11c⁺ cells from the spleen were sorted out and cocultured with CFSE-loaded anti-OVA CD8⁺ T cells from OT-I mice. CFSE staining was assessed after 36 h culture. A representative flow cytometry analysis and quantification of divided cells and IL-2 production are reported in (A), (B), and (C), respectively. The results shown are representative of three independent experiments with at least three animals per group.



presentation is G_i -dependent. However, it does not disqualify the G_s signaling pathway, as it has been suggested that both α_1 - and β_2 -AR are able to switch their G protein-coupling specificity from G_s to G_i due to phosphorylation of the receptor by protein kinase A (33, 34). This new G_s/G_i switch-related aspect of β -AR signaling is G_i protein mediated and leads to activation of MAPKs (33, 34). In our case, this switch can be excluded concerning the effect of β_2 -AR on IL-12 release because G_s pathway modulators (forskolin, NF449, Rp-cAMP, and KT5720) did not restore IL-12 secretion.

We show that β_2 -AR stimulation inhibits transcription factor NF- κ B translocation to the nucleus. However, NF- κ B in matured DC is essential for at least three aspects of Ag-presenting function: upregulation of costimulatory molecules such as CD80, CD86, and CD40, immunostimulatory cytokines such as IL-12, and cross-presentation (35). From our data, we infer that the inhibition of nuclear NF- κ B translocation by β_2 -AR stimulation may explain all the effects on DC function observed following salbutamol treatment both in vitro and in vivo. The link between β -AR, the NF- κ B pathway, and cell function is not new in immune cells. β -AR also modulate the NF- κ B pathway in macrophages/monocytes and lymphocytes. β -AR agonists inhibit NF- κ B translocation and cytokine production in LPS-stimulated macrophages (36, 37) and in PMA/ionomycin activated human CD3⁺ T cells (38). However, signaling pathways in macrophages and DC seems very different, being cAMP-dependent for the former (37) but cAMP-independent for the latter. The link between NF- κ B and β_2 -AR is still missing, but it was recently suggested that I κ B inhibitory function could be upregulated by decreased interaction with β -arrestin-2 after β_2 -AR stimulation (39).

Our observation that in vitro cross-presentation of exogenous OVA by DC was reduced by >80% after exposure to β_2 -AR agonist indicates that a defect occurs within a subcellular process involved in this pathway. This observation is not limited to exogenous Ags, as endogenous presentation of Ags is also affected by salbutamol treatment (Fig 2F). Whereas soluble and particulate Ag uptakes are unaffected by β_2 -AR stimulation on DC, phagosomal Ag processing is significantly reduced as compared with untreated cells. Some authors have shown that Ag release kinetics in the phagosome are critical to cross-presentation efficiency (40). This delay in phagosomal degradation may be related to various effects of β_2 -AR activation on DC function, one of which is proteasome activity. However, we found that proteasome activity of salbutamol-treated DC was comparable to untreated DC. Other hypotheses include the effect of β_2 -AR stimulation on the regulation of phagosomal pH or protease activities. Interestingly, presentation of endogenously expressed Ags is also impaired, suggesting an additional yet unknown effect of β_2 -AR stimulation on protein cross-presentation.

To our knowledge, this is the first study describing a β_2 -adrenoreceptor-dependent inhibition of cross-presentation. We used experimental OVA and HA systems. In vivo, cross-presentation can result from processing of dead cells, apoptotic bodies, or heat shock protein-associated Ags. This cross-presentation pathway is crucial for tumor-associated Ag presentation or during bacterial or viral infection. Interestingly, a multitude of factors have been identified that influence whether tolerance or immunity is established against cell-associated Ags. Among these factors are how cells are dying, the recognition and uptake by phagocytic cells, and the resulting microenvironment. The microenvironment may possibly include adrenergic innervation. The impact of β_2 -AR signaling on cell-associated Ags needs to be further addressed along with the mechanisms involved, which may be different from those described in this paper.

Although the in vivo modulation of cross-presentation remains unclear, there is evidence for a positive effect of inflammatory stimuli (41, 42) upon engagement of TLRs that modulates endocytosis (43). Many authors have suggested that stress may regulate immune responses (44). The effect of stress on the immune system is not only glucocorticoid-mediated but also adrenergic-mediated (45, 46). Both systems have shown a capacity to modulate the cross-presentation capability of DC (7, 47, 48), consistent with the deleterious effect of chronic stress on immune responses. However, β -agonists and antagonists are among the most widely used drugs in clinical practice for a number of distinct pathologies, including heart failure, hypertension, asthma, and migraines. To our knowledge, our study demonstrates for the first time that administration of exogenous β_2 -AR agonist impairs CD8⁺ priming by cross-presenting DC in mice. However, Panina-Bordignon et al. (36) showed more than a decade ago that human DC, similar to mouse DC, are impaired in IL-12 secretion upon salbutamol treatment. We can reasonably speculate that β -agonist treatment would also affect cross-presentation in human DC and that the impact of administered exogenous β -agonists on the immune system was underestimated. These results also provide the proof of concept that β_2 -agonists may represent a new way for manipulating the immune system.

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Disclosures

The authors have no financial conflicts of interest.

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