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Probiotic *Escherichia coli* Nissle 1917 activates DC and prevents house dust mite allergy through a TLR4-dependent pathway

Emmanuelle Adam[1], Laurence Delbrassine[1], Céline Bouillot[1], Virginie Reynders[1], Anne-Catherine Mailleux[2], Eric Muraille[3] and Alain Jacquet[1]

[1] Laboratoire d'Allergologie Expérimentale, Université Libre de Bruxelles, Belgium
[2] Unité d'écologie et de biogéographie, Université Catholique de Louvain, Belgium
[3] Laboratoire de Parasitologie, Université Libre de Bruxelles, Belgium

Experimental animal and human studies have demonstrated that probiotic strains have beneficial effects on allergy. Here we report that the probiotic *Escherichia coli* Nissle 1917 strain (EcN) is able to activate DC, as shown by important cytokine synthesis together with up-regulation of membrane expression of CD40, CD80 and CD86. This EcN-induced DC activation was strictly dependent on the TLR4 signaling pathway and was also associated with stimulation of NF-κB and MAPK. We next investigated the prophylactic potential of i.n. co-administration of EcN with a recombinant form of Der p 1 (ProDer p 1) in a murine model of mite allergy. I.n. vaccinations with EcN plus ProDer p 1 prevented the subsequent allergic response following Der p 1 sensitization and airway challenge with aerosolized mite extracts through the induction of an allergen-specific IgG2a response, the prevention of specific IgE production and a strong reduction of IL-5 secretion by allergen-restimulated splenocytes. EcN alone or in combination with ProDer p 1 inhibited the development of airway eosinophilia and neutrophilia. This *in vivo* protective effect of EcN was, in part, mediated by TLR4 signaling. Our results suggest that EcN represents an efficient adjuvant to prevent allergic responses.

Key words: Adjuvant · *Escherichia coli* Nissle · House dust mite allergy · TLR4

Introduction

Probiotic bacteria are live non-pathogenic microorganisms, which exert beneficial effects on the host through their influence on the development of the mucosal immune system notably *via* TLR activation [1]. Indeed, at least TLR2, 4, 5 and 9 have been shown to be activated by several commonly used probiotics including lactobacilli and bifidobacteria [2–8]. These immunomodulatory properties of probiotics have also been shown to play an important role in the prevention and treatment of allergic diseases. Not only have epidemiological studies reported the positive influence of lactobacilli and bifidobacteria microflora on allergy incidence [9], but we and others have highlighted the protective effects of either probiotic co-administration with allergens or recombinant lactic acid bacteria expressing allergens in mouse models of allergy [2, 3, 10–14].

The non-pathogenic *Escherichia coli* Nissle 1917 strain (EcN) has been extensively characterized by, notably, the complete elucidation of its genome [15], and its therapeutic efficacy and safety have been convincingly proven for the treatment of gastrointestinal diseases such as inflammatory bowel disease, Crohn's disease and ulcerative colitis [16–18]. In contrast, EcN-based immunotherapeutic approaches against allergy remain poorly investigated.

Correspondence: Dr. Alain Jacquet
e-mail: alain.j@chula.ac.th

Recent data have highlighted that EcN is able to initiate maturation and cytokine production (IL-6, IL-10, TNF-α in low amount) in Peyer's patch DC, MLN DC and spleen DC [19], to induce γδ T cell apoptosis through caspase and FasL-dependent pathways [20], to trigger high production of IL-10 together with low secretion of TNF-α in PBMC [21] and to modulate the gastrointestinal epithelial barrier function by, notably, the induction of β-defensin 2 [22]. Nevertheless, the precise mechanisms underlying these protective and anti-inflammatory effects are still under investigation.

The current study first investigated the immunomodulatory properties of EcN at the level of the DC, as these professional antigen-presenting cells play a pivotal role in the Th2 polarization of the allergic response. The nature of the TLR signaling pathways triggered by EcN was, in particular, characterized. Second, we evaluated, in a murine model of house dust mite (HDM) allergy, the putative beneficial effect of i.n. co-administration of EcN with a recombinant form of the major mite allergen Der p 1 (ProDer p 1) on the prevention of the specific allergic response.

Results

EcN induces IL-10, IL-12 production and maturation in BMDC

BMDC were cultured with various CFU EcN (bacteria/DC ratio from 0.01 to 100) for 24 h. IL-6, IL-10, IL-12p40 and IL-12p70 levels were measured in the supernatants by ELISA whereas CD40, CD80 and CD86 cell surface expression was analyzed by flow cytometry. As it has been demonstrated that gram-negative bacteria release endotoxins during their growth, we incubated BMDC with washed EcN (containing cell wall-associated LPS) and, as control, with EcN bacteria culture supernatant containing the same amount of LPS (cell-free LPS). As shown in Fig. 1, washed EcN induced IL-6, IL-10, IL-12p40 and IL-12p70 secretion from BMDC when compared with control medium ($p<0.05$). The IL-6, IL-10 and IL-12p40 production was EcN dose-dependent, whereas the IL-12p70 secretion reached a plateau for a bacteria/DC ratio ranging from 0.1 to 100. Supernatants from EcN cultures used at the same LPS dose induced less IL-6, IL-12p70, IL-12p40 secretion but more IL-10 production in BMDC ($cytokine_{EcN}/cytokine_{free\ LPS}$ ratio of 6.5, 21.7, 1.3 and 0.09 for IL-12p40, IL-12p70, IL-6 and IL-10, respectively (data not shown), demonstrating that the EcN bacteria themselves display immunomodulatory properties. Similar levels of cytokine production were observed with the non-probiotic *E. coli* K12 MG1655 strain (data not shown). We consider that EcN, according to the observed IL-12/IL-10 ratio, preferentially induced Th1 polarization at the level of the DC.

EcN induced DC maturation as judged by the up-regulation of the cell surface markers CD40, CD86 and CD80 (Fig. 2B). A similar maturation was observed with the positive control LPS.

EcN activates BMDC through the TLR4 signaling pathway

To assess the importance of TLR signaling in the EcN-induced cytokine production in BMDC, we first compared cytokine secretion in response to 10^7 CFU EcN in WT and MyD88-deficient BMDC. As shown in Fig. 2A, cytokine production by BMDC stimulated by EcN was abolished in MyD88-deficient BMDC ($p<0.05$ *versus* WT), indicating the involvement of TLR in the DC response to this commensal bacteria. As TLR2, 4, 5 and 9 can specifically recognize PAMP of bacterial origin, respectively, peptidoglycan, LPS, flagellin and unmethylated genomic DNA, we next evaluated the responses of BMDC deficient in TLR2, TLR4 and TLR9 to EcN. Whereas TLR2- and TLR9-deficient DC responded as efficiently as WT DC to EcN, a drastic reduction

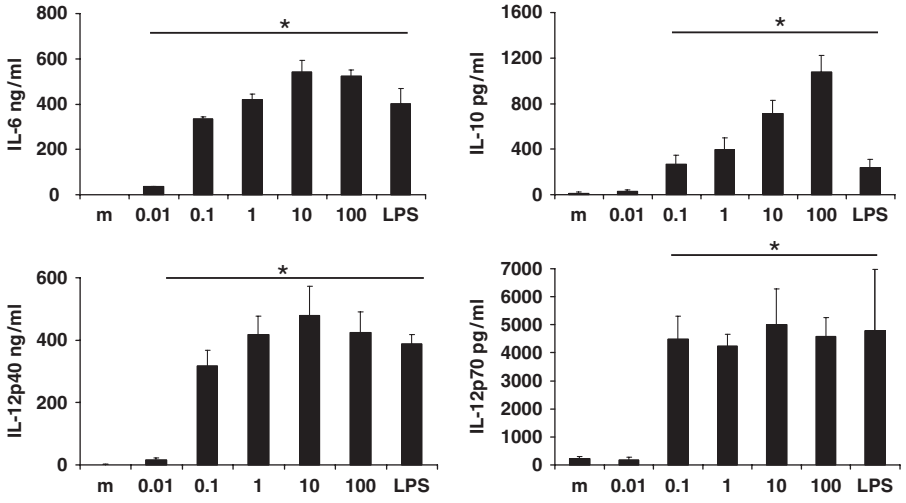

Figure 1. Activation of BMDC by washed EcN stimulates cytokine production. BMDC generated from WT mice were treated for 24 h with medium alone (m), LPS (500 ng/mL) or EcN (bacteria/DC ratio from 0.01 to 100). Data show cytokine concentrations as mean+SEM and are representative of three independent experiments (*$p<0.05$ *versus* medium alone, m).

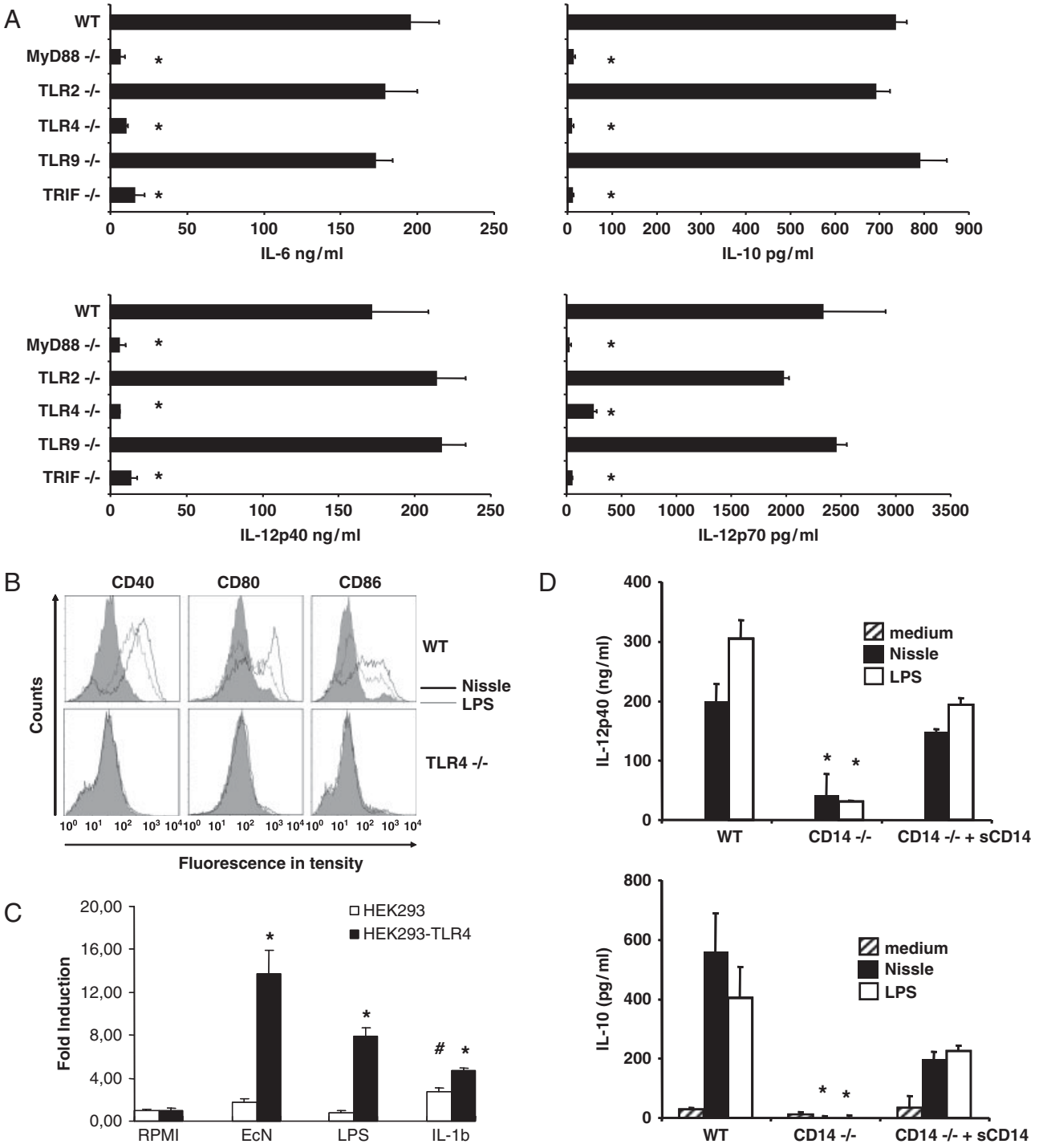

Figure 2. Stimulation of BMDC by EcN depends on TLR4 signaling. (A) BMDC generated from WT, TLR2-, TLR4-, TLR9-, MyD88-or TRIF-deficient mice (10^6 cells/well) were treated for 24 h with EcN (10^7 CFU). Data show cytokine concentrations as mean+SEM and are representative of three independent experiments. $^*p \leq 0.05$ (*versus* WT control). (B) BMDC from WT or TLR4-deficient C57BL/6 mice were treated for 24 h with LPS (500 ng/mL) or EcN (10^7 CFU). BMDC were analyzed by means of cytometry for CD40, CD80, CD86 expression. Data are representative of three independent experiments. (C) WT or TLR4/MD2-expressing HEK 293 cells, transiently transfected with a NF-κB reporter plasmid encoding firefly luciferase, were stimulated for 24 h with 10^7 CFU of EcN, RPMI medium, LPS (500 ng/mL) or IL-1β (0.1 U/mL). Data show mean fold-induction of luciferase activity+SEM and are representative of three independent experiments. $^*p \leq 0.01$ (HEK293-TLR4 compared with HEK293), $^\sharp p \leq 0.05$ (stimuli compared with medium). (D) BMDC from WT or CD14-deficient mice were treated for 24 h with medium, LPS or EcN under serum-free condition in the presence or absence of sCD14. Data show mean cytokine concentrations of (IL-12p40, IL-10)+SEM and are representative of three independent experiments. $^*p \leq 0.01$ (deficient mice compared with WT).

of cytokine secretion was observed in TLR4-deficient BMDC (Fig. 2A, $p<0.05$ *versus* WT). In a reciprocal experimental setting, when we used a flagellin-deficient EcN strain (EcN ΔfliC) to determine the role of the TLR5 signaling pathway in EcN-induced cytokine production, our results showed that this EcN mutant strain maintains its capacity to activate cytokine secretion in BMDC indicating that DC activation by EcN is TLR5 independent (data not shown).

Since TLR4-mediated signal transduction depends on both MyD88 and Toll/interleukin-1 receptor-domain-containing adaptor-inducing IFN-β (TRIF) adaptor proteins [23], we then evaluated the response of TRIF-deficient LPS2 mice to EcN. As shown in Fig. 2A, cytokine production was blunted in TRIF-deficient BMDC ($p<0.05$ *versus* WT), indicating that both MyD88-dependent and TRIF-dependent signaling pathways are engaged upon exposure to EcN and are required for efficient activation of BMDC. We next evaluated DC maturation induced by this commensal bacteria in TLR4-deficient BMDC. In a manner similar to LPS, EcN failed to induce up-regulation of CD40, CD80 and CD86 in TLR4-defective BMDC (Fig. 2B). It is noteworthy that a similar TLR4 dependence in cytokine production and DC maturation was evidenced for *E. coli* MG1655 (data not shown). We next confirmed that EcN use TLR4 to exert their stimulatory action by testing effect of EcN on WT and stably expressing human TLR4/MD2 HEK293 cells co-transfected with the NF-κB-dependent reporter plasmid as an TLR4 signaling pathway triggers NF-κB mobilization. As shown in Fig. 2C, NF-κB activation in response to EcN was detected only in HEK293 cells expressing TLR4/MD2 ($p\leq0.01$ *versus* HEK293). As expected, IL-1β used here as a positive control was able to activate HEK293 cells in a TLR4-independent manner. Finally, we evaluated the cytokine production induced by this commensal bacteria in CD14-deficient BMDC as CD14, a LPS receptor, is closely associated with TLR4 [23]. Because of the presence of soluble CD14 (sCD14) in the serum, BMDC were incubated with EcN in serum-free medium. IL-12p40 as well as IL-10 production by EcN- or LPS-activated BMDC was similarly and drastically reduced when CD14 was not expressed by these cells (Fig. 2D, $p<0.01$ *versus* WT). The addition of 5 μg/mL sCD14 in the serum-free medium partially restored the cytokine production, confirming the CD14-dependence of DC activation by EcN. Again, similar results were obtained with *E. coli* MG1655 (data not shown).

Collectively, our data highlighted the strict TLR4 dependence of the DC activation by EcN.

EcN stimulates ERK1/2, JNK and p38 MAPK in BMDC

Since MAPK activation is known to be correlated with TLR4 signal transduction, we next examined the effect of EcN on ERK, JNK and p38 MAPK activation. As shown in Fig. 3A, EcN induced an increase in the phosphorylation of ERK, p38 and JNK. The EcN-mediated activation of ERK was already maximal at 15 min and persisted for up to 120 min. EcN also induced a strong phosphorylation of p38 and JNK that was clearly evident after 15 min, was maximal at 30–60 min and began to decline after 120 min. Similar results were obtained with *E. coli* MG1655 (data not shown). The LPS-induced ERK activation followed the same kinetics as that obtained with EcN (or *E. coli* MG1655). In contrast, LPS caused a delayed phosphorylation of p38 and JNK compared with EcN, which was maximal at 60 min.

EcN-induced cytokine release is dependent on MAPK and NF-κB activation

Using specific pharmacological inhibitors, we then assessed the role of ERK, p38, JNK and NF-κB activation on the cytokine production from BMDC in response to EcN. The used inhibitor concentrations (20 μM for MAPK inhibitors and BAY11-7082, 10 μM for MG132) did not significantly affect cell viability (data not shown). Pretreatment of cells with the JNK (SP600125) or NF-κB (BAY 11-7082 and MG132) inhibitors for 1 h drastically reduced the EcN-induced cytokine production (Fig. 3B and C, $p<0.01$ *versus* untreated). On the other hand, the blockade of p38 or ERK pathways by SB203580 (a p38 MAPK inhibitor) or U0126 (a MEK1/2 inhibitor) reduced to a lesser extent cytokine production but failed to affect the secretion of IL-12p70 in response to EcN (Fig. 3B and C). Again, similar results were obtained with *E. coli* MG1655 (data not shown) and LPS activation with the exception of the strict p38 dependence of the LPS-induced IL-10 production compared with EcN and *E. coli* MG1655. This result suggests that activation of the NF-κB and MAPK pathways is essential for cytokine production in EcN-treated BMDC.

I.n. co-application of EcN with ProDer p 1 prevent the development of the Der p 1 allergic response

As EcN was shown to induce IL-12 and IL-10 secretions in BMDC, this probiotic could display anti-allergic immunomodulatory properties. Consequently, the prophylactic potential of EcN was assessed in a Der p 1 sensitization animal model. BALB/c mice were i.n. immunized eight times with recombinant ProDer p 1, the enzymatically inactive precursor form of Der p 1, in the presence or absence of 10^9 CFU EcN (Fig. 4A). As control, animals were vaccinated with saline or with EcN alone. Pretreated mice were subsequently intraperitoneally sensitized with natural Der p 1 adsorbed to the pro-Th2 adjuvant alum and exposed to aerosolized HDM extract to provoke airway inflammation [2, 3].

I.n. vaccinations of naïve mice with recombinant ProDer p 1 alone induced a weak but detectable anti-ProDer p 1 IgG1 response (Fig. 4A, $p<0.05$) but, strikingly, prevented the development of allergen-specific IgE antibodies (Fig. 4A, $p<0.05$). The presence of EcN during the allergen vaccinations significantly increased the specific IgG1 response but also reduced more drastically the allergen-specific IgE response ($p<0.05$ *versus* ProDer p 1 alone). Low titers of specific IgG2a were only detected

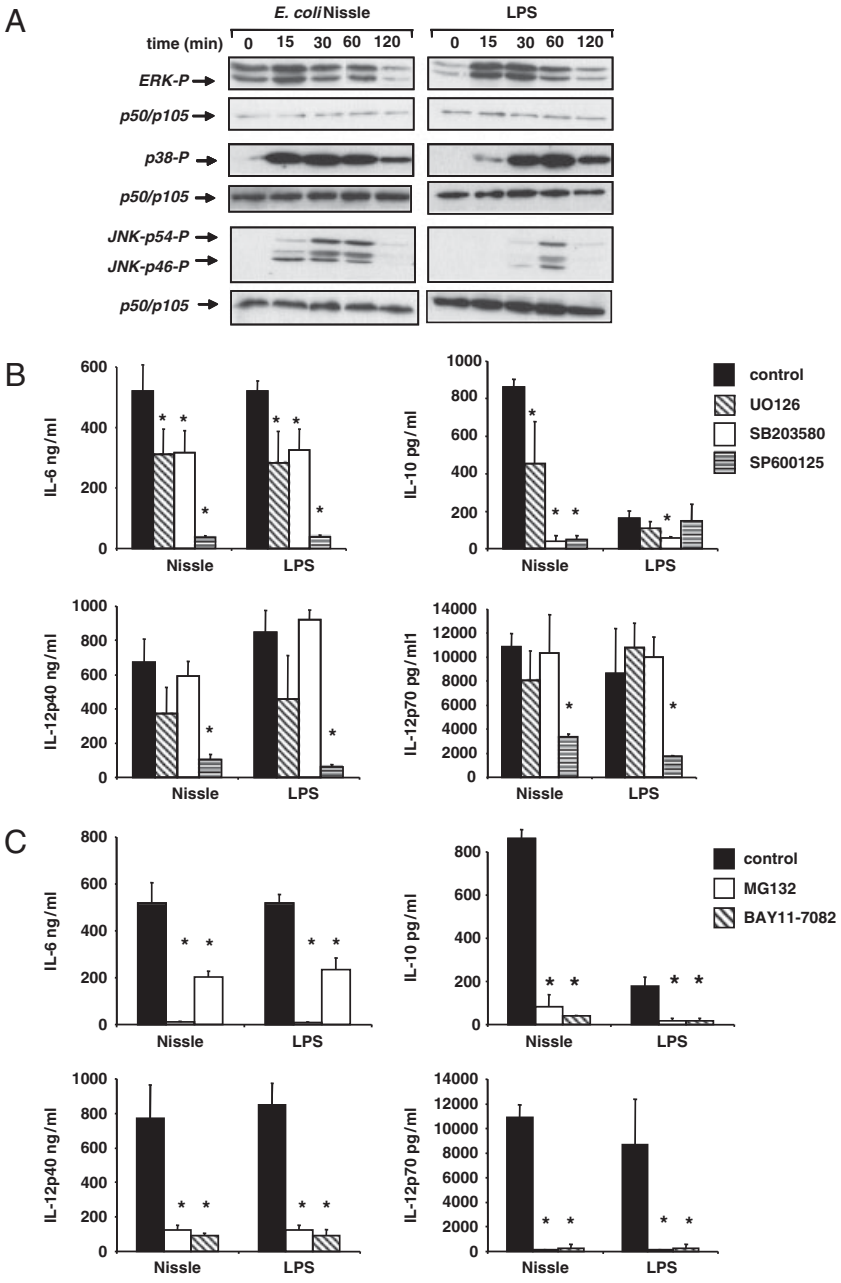

Figure 3. EcN activates MAPK in BMDC and cytokine production induced by EcN stimulation involves NF-κB and MAPK pathways. (A) BMDC (10^6 cells/well) were incubated with LPS (500 ng/mL) or EcN (10^7 CFU) for the indicated periods of time. MAPK activation was detected by Western blotting with polyclonal antibodies specific for phosphorylated forms of ERK 1/2, p38 or JNK 1/2. Blots were reprobed with anti-p50/p105 antibodies to assess equal loading. BMDC were incubated with (B) JNK, p38 or MEK1/2 (SP600125, SB203580, U0126) or (C) NF-κB pathway inhibitors (MG132, BAY 11-7082) before stimulation (6 h) by EcN (10^8 CFU) or LPS (500 ng/mL). Data show mean cytokine concentrations+SEM and are representative of three independent experiments. $^*p \leq 0.01$ (inhibitor treatment compared with sham treatment).

in animals pre-treated with EcN plus ProDer p 1 ($p<0.05$). The humoral response induced by immunizations with ProDer p 1 combined or not with EcN, although weak, was statistically significant as no detectable specific antibodies were measured in control animals vaccinated with saline or EcN alone.

Following the subsequent Der p 1 sensitizations and HDM aerosol challenges, saline- or EcN-vaccinated mice developed a strong Th2-biased allergen-specific response characterized by high specific IgG1 and IgE titers and the absence of anti-ProDer p 1 IgG2a antibodies. I.n. ProDer p 1 plus EcN co-administration prevented this allergic response as high specific IgG2a production together with a drastic reduction of the allergen-specific IgE antibodies were elicited after the sensitizations and the challenges (Fig. 4A, $p<0.05$ *versus* saline). Vaccinations with ProDer p 1 alone induced only the prevention of the specific IgE response. On the other hand, a similar increase of the specific IgG1 response was observed in all groups.

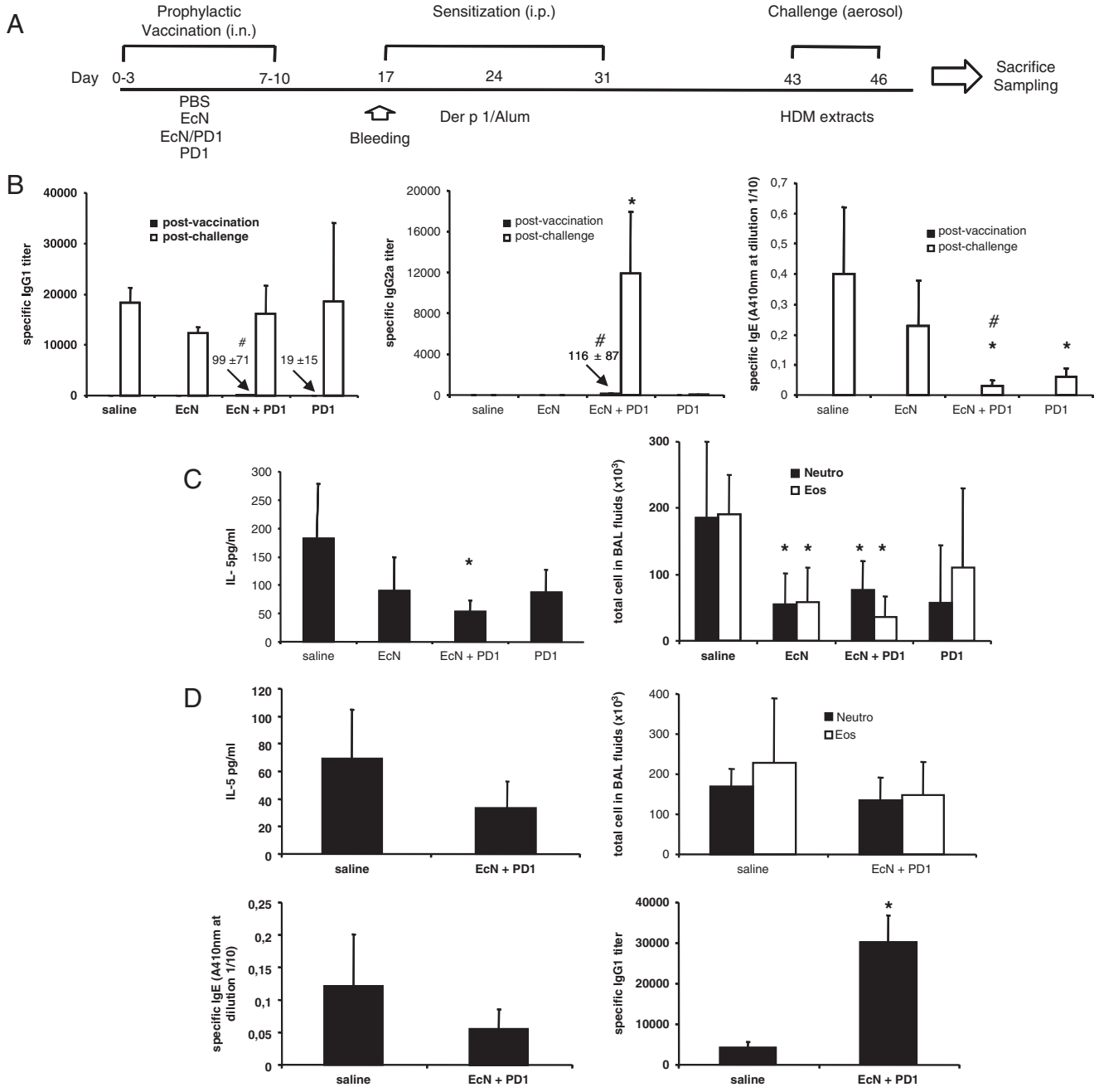

Figure 4. Prevention of experimental HDM allergy by co-administration of EcN+ProDer p 1 is TLR4-dependent. (A) Experimental setup. Naïve BALB/c mice (six *per* group of treatment) were pre-treated with saline (PBS), EcN, ProDer p 1 (PD1) or EcN+PD1, sensitized to Der p 1 and challenged. (B) Specific antibody titers (IgE, IgG1, IgG2a) and (C) splenocytes cytokine production (IL-5), airway neutrophil and eosinophil infiltrates were analyzed. (D) TLR4-deficient C57BL/6 mice (six *per* group of treatment) were pretreated with saline (PBS) or EcN+PD1 following the same experimental protocol described in (A). Specific antibody titers (IgE, IgG1), splenocytes cytokine production (IL-5), airway neutrophil and eosinophil infiltrates were analyzed. Data show mean ± SEM and are representative of three independent experiments. $^{*}p \leq 0.05$ (treatment compared to saline), $^{\sharp}p \leq 0.05$ (EcN+PD1 treatment compared with PD1 vaccinations alone).

We next analyzed IL-5, IL-10 and IFN-γ secretions by allergen-restimulated spleen cells. Der p 1-sensitized and HDM-challenged control mice developed high IL-5 production and vaccinations with EcN or ProDer p 1 alone did not modulate this cytokine secretion. Strikingly, i.n. co-administration of EcN plus ProDer p 1 resulted in a marked alteration in IL-5 secretion by spleen cells ($p<0.05$). However no detectable secretion of IFN-γ along with a very weak and similar IL-10 production (<20 pg/mL) were observed in each group despite the fact that high production of IFN-γ and IL-10 was detected after restimulation with anti-CD3 antibody (data not shown). Finally, animals immunized with Der p 1 and challenged with aerosolized HDM extracts developed an important airway eosinophilia and neutrophilia in BAL fluids (Fig. 4A). Vaccinations with ProDer p 1

alone did not impair inflammatory cell accumulation in the lungs. In contrast, pretreatments with EcN alone or combined with ProDer p 1 significantly decreased the eosinophil and neutrophil influx in the airways ($p<0.05$), confirming that EcN has beneficial effects on allergen-induced airway inflammation.

In the aim to demonstrate that the protective effect of EcN *in vivo* is TLR4-mediated, TLR4-deficient mice were treated with saline or EcN plus ProDer p 1 following the same vaccination protocol. The EcN+ProDer p 1 pretreatment reduced the production of IL-5, specific IgE, eosinophilia/neutrophilia but the differences with the allergic group (saline) were not statistically significant (Fig. 4B). These results emphasized the TLR4 dependence of the anti-allergic effect of EcN.

Discussion

In the current study, we determined whether the immunoregulatory potential of EcN can prevent the inflammatory allergic response induced by the major HDM allergen Der p 1. As the maturation status of DC as well as the cytokine production at the level of these APC is critical for the immune polarization, we first investigated whether EcN was able to trigger the release of the cytokines from BMDC and to up-regulate costimulatory molecules expression, with particular attention paid to a potential role for TLR. We clearly demonstrated that EcN induce high dose-dependent IL-6, IL-12p40, IL-12p70 as well as significant IL-10 production in BMDC. Based on the estimate that 10^5 *E. coli* correspond to 1 ng of LPS [24], we could consider that the IL-12, IL6-inducing potency of isolated LPS (from *E. coli* strain O111:B4) was similar with that of EcN containing the same amount of LPS. By contrast, EcN was a much stronger inducer of IL-10 than free LPS (O111:B4) at the same dose. Comparable IL-10 up-regulation was also recently observed in EcN-activated DC purified from different tissues [19]. However, it must be pointed out that other non-probiotic coliform strains as MG1655 (data not shown) and 2282 [25] similarly triggered significant IL-10 production. These data clearly suggested that LPS anchored into the bacterial cell would deliver a "strength of signal" mechanism, which could result in the hyper-induction of IL-10. EcN appears thus to be a probiotic strain that induces, in immature DC, production of IL-12 concomitant with high-level induction of IL-10.

Our data next highlighted that the signaling events mediated by EcN to trigger cytokine release in DC was MyD88-, TRIF- and TLR4-dependent but TLR2-, TLR5- and TLR9-independent. To our knowledge, this is the first study that clearly reports the activation of DC by EcN through TLR4 signaling. Moreover, CD14 was shown to be also important for the cytokine release induced by EcN, which strengthened the importance of TLR4 dependence for the immunostimulatory properties of this commensal bacteria. Our data suggest that LPS from EcN, although lacking O6 antigen repeating units compared with both non-pathogenic and pathogenic *E. coli* [26], is more likely the unique TLR ligand present in this bacterial strain involved in the DC activation. However, it would be interesting to evaluate whether LPS from EcN could also interact with SIGNR1, a mouse C-type lectin receptor homologous to human DC-SIGN. Indeed, SIGNR1 has been recently shown to bind the saccharide core portion of LPS in bacteria and to enhance subsequent pro-inflammatory cytokine production, possibly through an observed physical association between SIGNR1 and TLR4–MD-2 complex [27].

To confirm the importance of TLR4 in the EcN-induced DC activation, we compared the maturation status of WT and TLR4-deficient BMDC following incubation with this *E. coli* strain. The up-regulation of CD40, CD80 and CD86 expression triggered by EcN, already observed in other DC types [19] and in PBMC from allergic patients [28], was drastically prevented by the absence of TLR4 expression in BMDC. Taken together, our data strongly indicated that EcN elicits efficiently phenotypical changes at the level of BMDC for the development of adaptive immunity but also suggested that this probiotic strain is a potent DC activator that is able to drive a Th1 (IL-12)/Treg (IL-10) biased cytokine profile and, consequently, could modulate a Th2-biased allergic response.

Since TLR4 activates MAPK and NF-κB, leading to the production of cytokines involved in innate immunity [23], we evaluated the impact of EcN on these signaling pathways. We clearly demonstrated using luciferase and phosphorylation assays, specific NF-κB and MAPK signaling inhibitors that EcN induced NF-κB activation through TLR4 as well as rapidly activated the ERK1/2, JNK and p38 MAPK and that these both signaling events contribute to the induction of cytokines in BMDC by EcN. Similar results were obtained with the *E. coli* MG1655 (data not shown) and with LPS, except that p38 appeared to be the main MAPK required for the LPS-induced IL-10 production as previously described by others [29] while all three MAPK (especially p38 and JNK) contributed to IL-10 expression induced by EcN (and *E. coli* MG1655). In connection with that, it is interesting to note that cross-linking of SIGNR1 was recently shown to activate JNK and induce TNF-α production in the mouse macrophage-like RAW264.7 cell expressing SIGNR1 [30].

Although EcN has been recommended for the treatment of gastrointestinal diseases, anti-allergic treatments based on the use of EcN remained, in contrast, poorly investigated. According to the potent pro-Th1/Treg cytokine profile induced by EcN-activated BMDC, EcN could represent an efficient live adjuvant for the prevention of Th2-biased allergic responses. In view of our *in vitro* data, EcN appeared to be not the unique *E. coli* strain displaying anti-Th2 immunomodulatory properties. However, as EcN is currently the only *E. coli* strain fulfilling safety requirements needed to treat humans, it was not relevant to evaluate the effect of a non-probiotic *E. coli* strain in the prevention of allergy. In this context, we next evaluated the efficacy of the co-administrations of EcN plus ProDer p 1, a recombinant precursor form of the major mite allergen Der p 1, in the prevention of HDM allergy using a Der p 1-sensitization murine model. The i.n. route was chosen for these prophylactic vaccinations as the efficacy of this administration route was previously proven in animal models of allergy including ours with *L. plantarum*, another commensal bacteria [2, 3, 12, 13]. Moreover, TLR4-positive epithelial cells [31], TLR4- and CD14-positive DC are present in the sinonasal mucosa [32].

Our study demonstrated that musosal prophylactic treatments with ProDer p 1 alone, as previously reported with other allergens administrated i.n. [33, 34], efficiently prevented the induction of the specific IgE response. However, the allergen alone was inefficient for the modulation of the IL-5 production and the airway inflammation. In contrast, i.n. prophylactic vaccinations with EcN combined with ProDer p 1 inhibited more extensively the development of Der p 1-specific allergy as evidenced not only by very weak specific IgE titers but also by low IL-5 secretion upon splenocyte restimulation and the absence of airway eosinophilia and neutrophilia. The EcN/allergen co-administrations more likely prevented the typical Th2-biased allergic immune response by the induction of a Der p 1-specific Th1 profile characterized by high IgG2a titers. However, a specific production of IFN-γ could not be detected either in the proliferation supernatants or in the BAL fluids (data not shown). This finding is surprising since the induction of specific Th1 response correlates with IFN-γ production. Moreover, a very recent report demonstrated that EcN can inhibit the development of OVA-specific allergic responses when the bacteria are present at the site of Th2 cell priming through the induction of specific Th1 response characterized by IFN-γ production and the presence of increased numbers of IFN-γ-producing Th1 cells in BAL fluids [35]. However, EcN was shown to inhibit the production of IFN-γ in animal models of colitis [36] suggesting, together with the observed IL-10 production in EcN-activated BMDC, that the suppression of the Th2-biased allergic response could also likely be mediated by other cells than specific Th1 cells, notably by the generation of Treg cells.

It is interesting to note that, in this OVA allergic model and contrary to our data, no reduction of the specific IgE response was observed and the i.n. route of EcN administration was shown to be inefficient to prevent airway eosinophilia [35]. Such conflicting results, similarly reported from the use of gram-positive probiotics in clinical trials as well as in animal models of allergy [37], clearly confirmed that the efficacy of this *E. coli* strain in the primary prevention of allergic disease could not be only strain-specific but dramatically depends on the dose, the duration of the treatment and the timing of the administrations. Consequently, it appears from our results and from the data related on the effect of EcN in an OVA allergic model that EcN would represent a more promising immunomodulatory treatment in the primary prevention of allergy rather than in the immunotherapy of developing and/or established allergic diseases.

Finally, to correlate the TLR4-dependence of the immunomodulatory properties of EcN with the pro-Th1/Treg adjuvant effect of this bacterial strain, we performed the same vaccination protocol in TLR4-deficient mice. Our finding that EcN plus ProDer p 1 co-administrations reduced but not significantly eosinophilia, neutrophilia, IgE, IL-5 in TLR4-deficient mice clearly suggested that EcN LPS, through interactions with TLR4, mediates at least partly the protective effect of EcN+recombinant allergen immunizations *in vivo*.

Our data differ from a recent report, which demonstrated that TLR4 and EcN LPS are not involved in the reduced Th2 response following EcN administration [35]. This published result is somewhat surprising for the following reasons: (i) a previous study demonstrated the importance of TLR4 in the immunomodulation of experimental induced colitis in mice by EcN [38], (ii) LPS is present in EcN, although lacking O6 antigen repeating units compared with both non-pathogenic and pathogenic *E. coli*, (iii) our and others' recent findings showed than EcN induce similar DC and intestinal epithelial cells activation compared with free LPS and the non-pathogenic *E. coli* TG1 [39] and MG1655 (data not shown) strains, (iv) the DC activation was strictly mediated through TLR4 signaling pathways. It is now clear that the route, concentration, timing and duration of LPS exposure can drastically influence the effects of endotoxins on the airway allergen sensitization through probably different mechanisms. Notably, Th2 responses can be induced with an antigen (OVA) at low doses of LPS whereas high doses of LPS will skew OVA-specific responses towards a Th1 type [40]. An elegant study clearly demonstrated that both Th1 (high dose LPS) and Th2 (low dose LPS) responses elicited by i.n. antigen are dependent on MyD88. By contrast, Th2 responses after intraperitoneal antigen injections in the presence of low dose LPS are TLR4 and MyD88 independent [41].

Consequently, we speculate that conflicting results regarding the immune parameters (specific IgE, IL-5 and IFN-γ production, neutrophilia) and the TLR4 and LPS independence of the suppressive effect of EcN could be explained by differences at the level of the EcN dose, the timing and the route of bacteria administration between our Der p 1 sensitization and the OVA allergic models. Repeated i.n. administrations of EcN ($2 \times 4 \times 10^9$ CFU)+ProDer p 1 before sensitizations would mimic application of LPS at high dose, inducing, as previously reported [40, 41], protective mixed Th1/Treg anti-allergic response whereas EcN intraperitoneally administrated ($2 \times 2 \times 10^6$ CFU) during OVA/alum sensitizations would favor a more Th1-polarized response characterized by IFN-γ production and neutrophil infiltration in the airways [35].

In conclusion, the present study indicated that EcN activates DC through a strict TLR4 signaling pathway and that this TLR4 activation *in vivo* during allergen administration is able to prevent the subsequent development of the specific Th2-allergic response. Although a TLR2 dependence of the protective effect of EcN was evidenced in a murine model of colitis [38], our data could not evidence an important role for TLR2 in the immunomodulatory properties of EcN. Consequently, EcN-based and more specifically EcN LPS-based new allergen-specific immunotherapeutics could represent an efficient approach to prevent and/or to cure allergy.

Materials and methods

Animals

Female BALB/c and C57BL/6 (B6) mice (6 wk old) were obtained from Harlan (Horst, The Netherlands). TLR2-, TLR4-, TLR9-deficient mice with B6 background (6 wk old) were gifted from Dr S. Akira (RIMD, Osaka University, Japan). CD14- and MyD88-deficient mice were from Dr M. Moser (IBMM, ULB,

Belgium). TRIF-deficient mice were from B. Beutler (Scripps Research Institute, La Jolla, CA). Animal care and experimental procedures were carried out in accordance with local institutional guidelines (laboratory licence n LA 1500474).

Bacterial strains and growth conditions

EcN, the flagellin-deficient EcNΔfliC [22] (a gift of Dr K. Fellermann, Dr. Margarete Fischer Bosch Institute of Clinical Pharmacology, Stuttgart, Germany) and *E. coli* K-12 MG1655 (*E. coli* MG1655) were grown overnight at 37°C under gentle agitation at 220 rpm in Luria broth (LB) medium. To obtain bacteria in a linear growth phase, 100 μL of the bacterial suspension was added to 10 mL of fresh LB medium and grown under permanent shaking for 5 h. Bacteria were adjusted to a density of 10^{10} CFU/mL in PBS containing 20% glycerol for the *in vitro* experiments and to 10^{11} CFU/mL in PBS for i.n. vaccinations.

Antigens

Natural Der p 1 and recombinant native ProDer p 1 were purified from whole *Dermatophagoides pteronyssinus* cultures and CHO cell spent culture medium, respectively, as previously described [42].

Preparation and activation of mouse BM-derived DC

Mouse BM-derived DC (BMDC) were prepared as previously described [43]. BMDC (10^6 cells/well) were incubated with bacteria (ratio bacteria/DC from 0.01 to 100) in fresh BMDC medium for 24 h. As controls, BMDC were also incubated with ultrapure *E. coli* 0111:B4 LPS (500 ng/mL) (InvivoGen). When appropriate, BMDC were also incubated with sCD14 (recombinant mouse CD14-Fc, R&D Systems). In another set of experiments, BMDC were pre-treated at 37°C for 1 h with MAPK (U0126, 25 μM; SB203580, 25 μM; SP600125, 25 μM) or NF-κB (MG132, 10 μM; BAY-11–7082, 10 μM) specific inhibitors and then stimulated with 10^7 CFU of bacteria for 6 h. Culture supernatants of stimulated BMDC were collected and stored at −80°C until use. Mouse IL-12p40, IL-12p70, IL-10, IL-6 were assayed in culture supernatants by ELISA using BD OptiEIA kits (Becton Dickinson). For cell surface marker analysis, BMDC were stained for 30 min at 4°C with PE-conjugated anti-CD40, CD80 or CD86 monoclonal antibody (BD) before flow cytometry analysis.

Human cell transfection and luciferase assay

WT HEK293 or HEK293 T4Y cells (HEK293 cells stably expressing human TLR4/MD2 receptors kindly provided by Dr D.T. Golenbock, University of Massachussets Medical School, USA) (3×10^5 cells/mL) were transiently transfected with NF-κB reporter plasmid pTK-4 × NF-κB-luc [43] mixed with Fugene6 (Roche) according to manufacturer's instructions. Cells were then stimulated with bacteria at mentioned concentrations, LPS (500 ng/mL) or IL-1β (0.1 U/mL) for 24 h. Cells were then lysed (Promega) and assayed for luciferase activity (Promega). Luciferase activities were normalized with respect to total protein concentration measured by the detergent-compatible protein assay (Bio-Rad).

Western blotting experiments

BMDC were washed in cold PBS, lysed with SDS loadind buffer and analyzed by SDS-PAGE and Western blotting with polyclonal antibodies specific for phosphorylated forms of ERK 1/2, p38 or JNK MAPK (Cell Signaling) as described previously [43].

Vaccination protocol and measurement of allergen-specific response

BALB/c mice ($n = 6$) were i.n. pretreated on days 0–3 and 7–10 with 10^9 CFU (15 μL/nostril) of EcN in the presence or absence of recombinant ProDer p 1 (5 μg). As control, animals were vaccinated with saline or ProDer p 1 alone. Seven days after the last vaccination (day 17), animals were subsequently intraperitoneally sensitized for 3 wk at weekly intervals with 2 μg of natural Der p 1 (day 17, 24 and 31) formulated with alum (ratio allergen/adjuvant of 1/50). Sera were collected on day 38. To induce airway inflammation, mice were challenged 10 days after the last sensitization by exposition to aerosolized crude HDM extract (containing 10 μg/mL Der p 1) over a 30-min period on days 43, 44, 45 and 47. Anti-ProDer p 1 IgG1 IgG2a and IgE antibody assays and bronchoalveolar lavage fluids analysis were performed as previously described [2, 3]. The Der p 1-specific T-cell proliferative responses as well as the levels of IFN-γ and IL-5 production by ELISA were measured as previously described [2, 3].

Statistical analysis

Results are expressed as mean+SEM. Statistical analysis was performed by Student's *t*-test for comparison between groups.

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Abbreviations: **BMDC:** BM-derived DC · **EcN:** *Escherichia.coli* Nissle 1917 strain · **HDM:** house dust mite · **HEK:** Human embryonic kideney · **sCD14:** soluble CD14 · **TRIF:** Toll/interleukin-1 recptor-domain-containing adapter-inducing IFN-β

Full correspondence: Dr. Alain Jacquet, Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Road, Pathumwan, Bangkok 10330, Thailand
Fax: +66-2-6523100
e-mail: alain.j@chula.ac.th

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