

# MV140, a sublingual polyvalent bacterial preparation to treat recurrent urinary tract infections, licenses human dendritic cells for generating Th1, Th17, and IL-10 responses via Syk and MyD88

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Recurrent urinary tract infections (RUTIs) are one of the most common bacterial infectious diseases, especially in women. Antibiotics remain the mainstay of treatment, but their overuse is associated with antibiotic-resistant infections and deleterious effects in the microbiota. Therefore, alternative approaches are fully demanded. Sublingual immunization with MV140 (Uromune), a polyvalent bacterial preparation (PBP) of whole heat-inactivated bacteria, demonstrated clinical efficacy for the treatment of RUTIs, but the involved immunological mechanisms remain unknown. Herein, we demonstrated that MV140 endorses human dendritic cells (DCs) with the capacity to generate Th1/Th17 and IL-10-producing T cells by mechanisms depending on spleen tyrosine kinase (Syk)- and myeloid differentiation primary response gene 88 (MyD88)-mediated pathways. MV140-induced activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and p38 in human DCs is essential for the generated Th1/Th17 and IL-10 immune responses whereas c-Jun N-terminal Kinase (JNK) and extracellular-signal regulated kinase (ERK) contribute to Th1 and IL-10 responses, respectively. Sublingual immunization of BALB/c mice with MV140 also induces potent systemic Th1/Th17 and IL-10 responses *in vivo*. We uncover immunological mechanisms underlying the way of action of MV140, which might well also contribute to understand the rational use of specific PBPs in other clinical conditions with potential high risk of recurrent infections.

## INTRODUCTION

Urinary tract infections (UTIs) are one of the most common bacterial infectious diseases, especially in women.<sup>1,2</sup> Recurrent UTIs (RUTIs) are defined as three or more symptomatic UTIs during a year or two or more symptomatic UTIs within six months.<sup>3</sup> The pathogen most frequently isolated from patients with UTIs is *Escherichia coli* (80% of infections), followed by *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, *Proteus* spp and *Enterococcus* spp.<sup>1,3,4</sup> Antibiotics remain the mainstay of treatment for RUTIs,<sup>1</sup> but their overuse is associated with antibiotic-resistant infections and deleterious effects in the microbiota.<sup>5–7</sup> Therefore, there is an urgent need for the

development of new alternative approaches to treat patients suffering from recurrent infections.<sup>1</sup>

Different types of mucosal bacterial vaccines have gained a lot of attention for the treatment of RUTIs over the last years. Bacterial vaccines may contain soluble antigens (bacterial components or lysates)<sup>8,9</sup> or inactivated whole bacteria from one or more species or strains.<sup>10–12</sup> Mucosal bacterial vaccines for RUTIs were initially administered orally, but more recently the sublingual delivery route has been also assayed.<sup>1,8,12</sup>

The sublingual epithelium contains a dense network of dendritic cells (DCs) that play an essential role in linking innate and adaptive immune responses.<sup>13,14</sup> DCs are equipped with a

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wide battery of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) as sensors of pathogen-associated molecular patterns (PAMPs).<sup>15,16</sup> The role of TLRs and CLRs in shaping DCs innate immune responses and their capacity to polarize T cell responses has been intensely investigated.<sup>17-19</sup>

MV140 (Uromune) is a polyvalent bacterial preparation (PBP) composed of equal proportions of whole heat-inactivated bacteria producing the majority of RUTIs in Europe (*Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Enterococcus faecalis*). Two retrospective observational studies have recently demonstrated that patients treated with sublingual MV140 showed a significant reduction in the number of UTIs compared to patients treated with conventional antibiotics.<sup>11,12</sup> Despite relevant clinical benefits, the immunological ways of action of this PBP remain fully elusive.

In this study, we demonstrated for the first time that MV140 (Uromune) endorses human DCs with the capacity to generate Th1, Th17, and IL-10-producing T cells through mechanisms partially depending on spleen tyrosine kinase (Syk)- and myeloid differentiation primary response gene 88 (MyD88)-mediated signaling pathways. Sublingual immunization of BALB/c mice with MV140 also promotes the generation of systemic Th1, Th17, and IL-10 immune responses *in vivo*. Our data not only shed light into the immunological mechanisms underlying the clinical benefits of MV140 for the treatment of RUTIs but also might well pave the way for the use of sublingual specific PBPs in other clinical conditions at high risk of recurrent infections.

## RESULTS

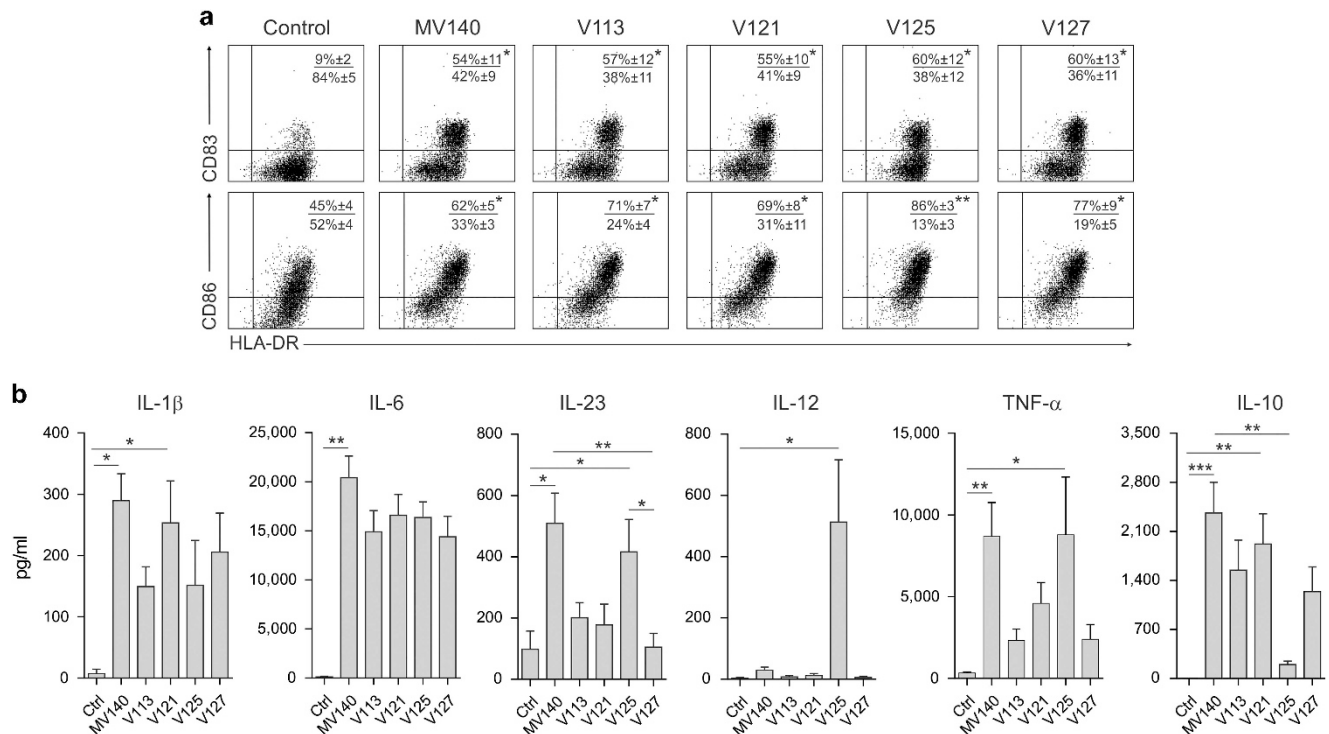
### MV140 induces maturation of human DCs and production of pro-inflammatory cytokines with high levels of IL-10

We assessed the capacity of the PBP MV140 (Uromune) to immunomodulate the phenotype and function of human DCs. MV140 significantly increased the expression of the co-stimulatory molecules CD83 and CD86 in human monocyte-derived DCs (hmoDCs) compared to control excipients, demonstrating that this PBP promotes the maturation of hmoDCs (Figure 1a). The individual gram-negative (*K. pneumoniae* V113, *E. coli* V121, and *P. vulgaris* V127) and the gram-positive (*E. faecalis* V125) bacteria also induced significantly higher levels of CD83 and CD86 than control without significant differences among them. The gram-positive V125 showed the highest induction of CD86 expression (Figure 1a). As expected for dendritic cells, almost all of them were HLA-DR positive and significant changes were not detected when comparing the different assayed treatments (Supplementary Figure 1a online). However, the analysis of MFI values revealed an increment in HLA-DR expression when hmoDCs were activated with MV140 or individual components compared to control treatment, reaching statistical significant only for V125 (Supplementary Figure 1b). HmoDCs activated with MV140 produced significantly higher levels of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-23, and TNF- $\alpha$  but not

IL-12 than control cells (Figure 1b). Interestingly, MV140-activated hmoDCs also produced significantly higher levels of the anti-inflammatory cytokine IL-10 than control. The individual bacterial components were also able to stimulate the production of pro-inflammatory cytokines but at a lower extent than MV140. The gram-positive component (V125) was the single bacterium able to induce significantly higher levels of IL-12 than control but without IL-10 production (Figure 1b). In contrast, IL-12 production was not observed in MV140-activated hmoDCs, which might be likely due to the high levels of IL-10 induced by MV140. V125 also induced significantly higher levels of TNF- $\alpha$  and IL-23 than control. The gram-negative *E. coli* (V121) induced significantly higher levels of IL-1 $\beta$  and IL-10 than control. When comparing MV140 and individual components among them, we only detected significant differences for IL-23 (MV140 vs V127 and V125 vs V127) and for IL-10 (MV140 vs V125). Additive effects were not observed in any case when individual bacteria were combined in MV140.

### MV140-activated hmoDCs generate Th1, Th17 and IL-10-producing T cells

To determine the capacity of MV140 to condition the ability of human DCs to polarize T cell responses, we cultured hmoDCs with allogeneic naive CD4<sup>+</sup> T cells in the presence of MV140 or control excipients for 3 days. MV140-activated hmoDCs induced a significantly higher percentage of proliferating allogeneic CFSE-labeled CD4<sup>+</sup> T cells than control-treated hmoDCs (Figure 2a). HmoDCs activated with MV140 generated T cells producing significantly higher levels of IL-17A, IFN- $\gamma$ , and IL-10 and lower IL-5 than control-activated hmoDCs (Figure 2b). Naive CD4<sup>+</sup> T cells stimulated alone with MV140 did not proliferate and did not produce detectable levels of any assayed cytokine (data not shown). To confirm these results, primed CD4<sup>+</sup> T cells were washed and restimulated with anti-CD3 and -CD28 mAbs for 24 h. After this polyclonal re-expansion, CD4<sup>+</sup> T cells generated by MV140-activated hmoDCs also produced significantly higher levels of IL-17A, IFN- $\gamma$ , and IL-10 and lower IL-5 than those primed by control-treated hmoDCs (Figure 2c). To further verify these data at the single cell level, we performed intracellular staining experiments. Primed CD4<sup>+</sup> T cells were restimulated with PMA/ionomycin in the presence of Brefeldin A. The percentages of IL-17A-, IFN- $\gamma$ - and IL-10-producing CD4<sup>+</sup> T cells generated by MV140-activated hmoDCs were significantly higher than those induced by control-treated hmoDCs (Figure 2d). We did not detect significant differences in IL-4-producing CD4<sup>+</sup> T cells. Representative dot plots are displayed in Figure 2e. Double intracellular staining experiments showed that a significantly higher population of CD4<sup>+</sup> T cells simultaneously producing IL-17A and IFN- $\gamma$  was generated by MV140-activated hmoDCs than control (Figure 2f). Interestingly, the induced CD3<sup>+</sup>CD4<sup>+</sup> IL-10-producing T cells did not simultaneously produce IL-17A or IFN- $\gamma$  and they did not express FOXP3 or GATA3 (Figure 2g). MV140-treated hmoDCs induced a significant increase in



**Figure 1** MV140 (Uromune) induces hmoDCs maturation and production of pro-inflammatory cytokines with high levels of IL-10. **(a)** Flow cytometry representative dot plots for the expression of the activation surface markers CD83, CD86, and HLA-DR on hmoDCs stimulated with MV140 ( $10^7$  bact. per mL), the individual bacteria ( $2.5 \times 10^6$  bact. per mL) or the control for 18 h. The percentages of positive cells for the indicated markers are displayed inside the plot. **(b)** Cytokine production after stimulation of hmoDCs with the indicated stimulus for 18 h quantified in cell-free supernatants by ELISA. Ctrl, control containing all excipients except the bacteria; MV140, PBP Uromune; V113, *K. pneumoniae*; V121, *E. coli*; V125, *E. faecalis*; V127, *P. vulgaris*. Results are mean  $\pm$  s.e.m. of 4 **(a)** or 5–8 **(b)** independent experiments. Friedman test for multiple comparisons, \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

FOXP3<sup>+</sup> IL-10<sup>-</sup> T cells but not GATA3<sup>+</sup> IL-10<sup>-</sup> T cells compared to control-treated hmoDCs (**Supplementary Figure 2**).

We performed the same type of coculture experiments with the individual bacteria. HmoDCs activated with gram-negative or -positive components alone also induced significantly higher percentages of proliferating allogeneic CD4<sup>+</sup> T cells than control-treated hmoDCs without significant differences among them or with respect to MV140 (**Figure 3a**). The gram-negative bacteria alone (V113, V121 or V127) also generated T cells producing IL-17A, IFN- $\gamma$ , and IL-10 but the levels were lower than those produced by T cells primed with MV140-activated hmoDCs and additive effects were not observed (**Figure 3b**). For IL-10 production, significant differences were detected when hmoDCs were activated with the individual gram-negative V113 and V121 components alone or MV140 compared to control and to V125. T cells primed with V125-activated hmoDCs produced significantly higher levels of IFN- $\gamma$  than control cells without IL-10 production (**Figure 3b**).

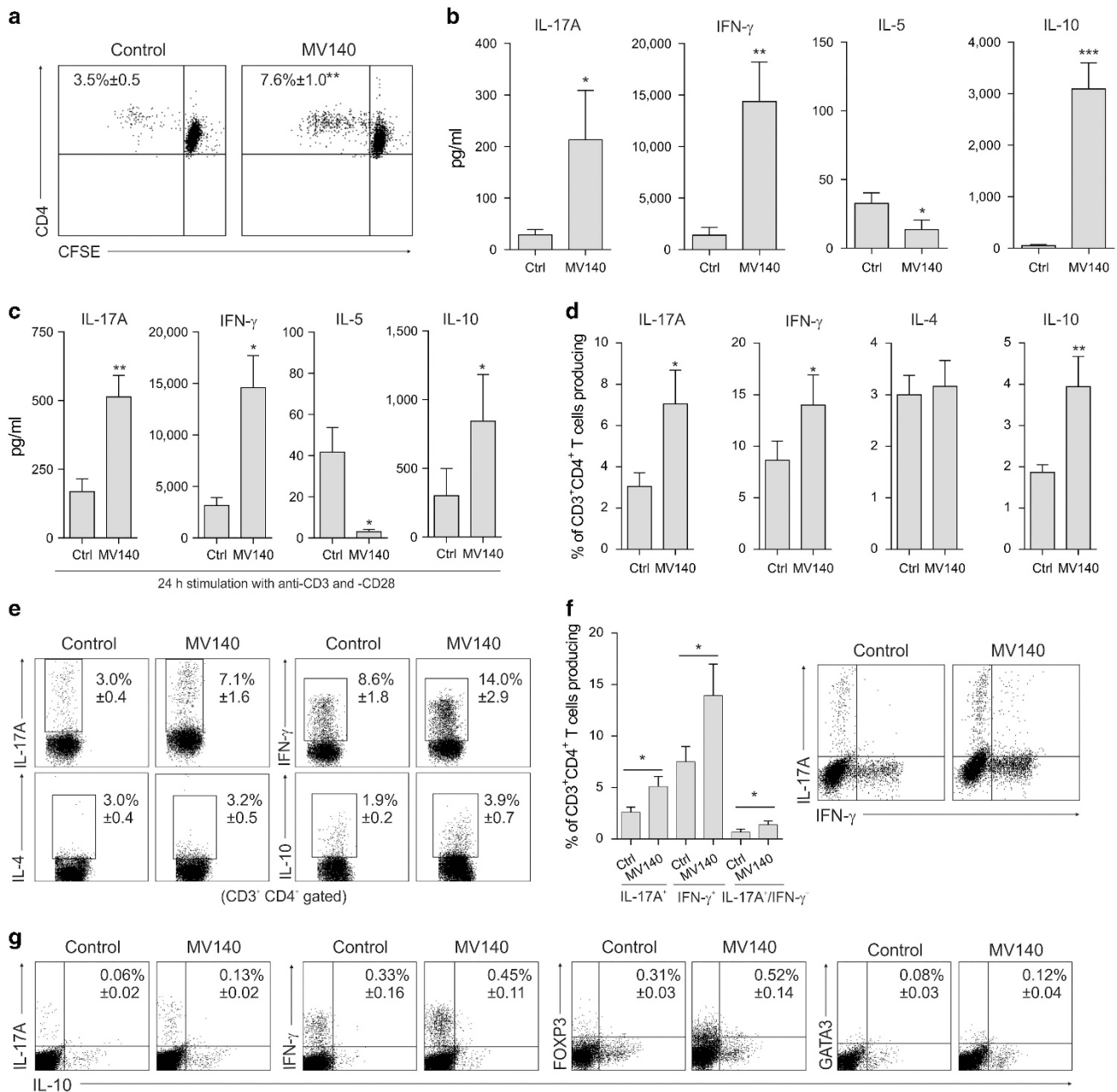
#### MV140-activated total blood DCs containing mDCs and pDCs produce pro- and anti-inflammatory cytokines and induce Th1, Th17, and IL-10-producing T cells

We isolated an enriched fraction of human total DCs containing both mDCs and pDCs from peripheral blood (**Figure 4a**). MV140-activated total DCs produced high levels

of the pro-inflammatory and Th17-polarizing cytokines IL-1 $\beta$ , IL-6 and IL-23, as well as the Th1-polarizing cytokine TNF- $\alpha$  but not IL-12 (**Figure 4b**). As shown in this figure, MV140-activated total DCs also produced high levels of IL-10. Interestingly, coculture experiments with allogeneic naive CD4<sup>+</sup> T cells demonstrated that MV140- but not control-activated total blood DCs also generated T cells producing high levels of IL-17A, IFN- $\gamma$ , and IL-10 (**Figure 4c**). Differences in IL-5 production were not observed. After restimulation with anti-CD3 and -CD28 mAbs for 24 h, CD4<sup>+</sup> T cells primed by MV140-activated total blood DCs also produced higher levels of IL-17A, IFN- $\gamma$ , and IL-10 than those primed by total DCs stimulated with control excipients (**Figure 4d**).

#### Syk-coupled CLRs in cooperation with MyD88-coupled TLRs are the main drivers of the immune responses imprinted by MV140 in human DCs

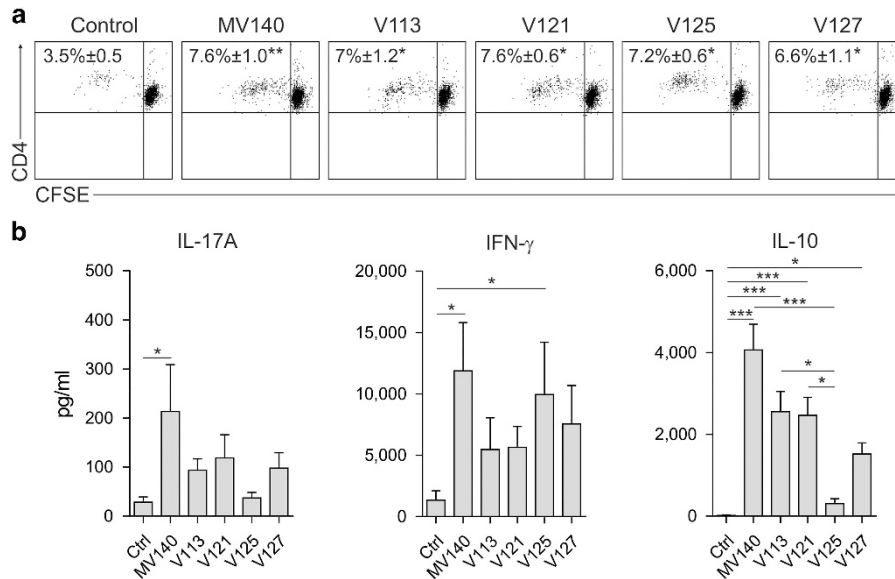
Syk and MyD88 are key molecules in the initiation of downstream signaling pathways after engagement of CLRs and TLRs, respectively.<sup>18,19</sup> To assess the contribution of these PRRs to the cytokine signature imprinted by MV140 in hmoDCs, we performed inhibition experiments using piceatannol (a selective pharmacological inhibitor of Syk) and/or pepinh-MYD (an intracellular peptide blocking MyD88-mediated signaling pathways). The production of the Th17-driven cytokines IL-1 $\beta$  and IL-23 by MV140-activated



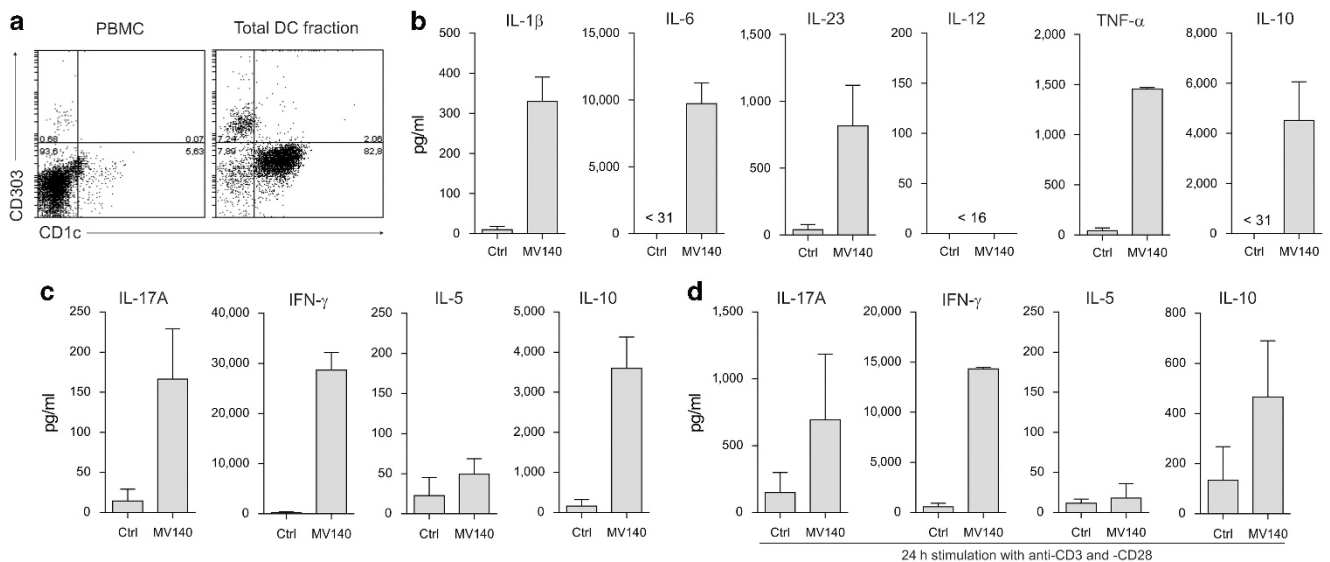
**Figure 2** MV140-activated hmoDCs induce T cell proliferation and the generation of Th1, Th17 and IL-10-producing T cells. (a) Representative dot plot of proliferating CFSE-labeled naive CD4<sup>+</sup> T cells after 3 days of coculture with control- or MV140-activated allogeneic hmoDCs. The frequency of proliferating cells is displayed inside the plot. (b) Cytokines produced by allogeneic naive CD4<sup>+</sup> T cells primed by control- or MV140-activated hmoDCs after 3 days quantified in cell-free supernatants by ELISA. (c) Cytokines produced after polyclonal stimulation with anti-CD3 and -CD28 mAbs for 24 h were quantified by ELISA in cell-free supernatants. (d) After 3 days of coculture, primed T cells were washed and stimulated for 6 h with PMA/ionomycin in the presence of Brefeldin A. Percentage of CD3<sup>+</sup> CD4<sup>+</sup> T cells producing the indicated cytokines after intracellular staining and flow cytometry analysis are shown. (e) Representative dot plots for the intracellular staining and flow cytometry analysis. (f) Percentage of CD3<sup>+</sup> CD4<sup>+</sup> T cells producing IL-17A, IFN-γ or IL-17A and IFN-γ simultaneously after intracellular staining and flow cytometry analysis. A representative dot plot is shown. (g) Representative dot plots for the intracellular staining and flow cytometry analysis of the indicated markers. Results are mean ± s.e.m. of 8 (a), 11–17 (b), 4–6 (c), 7–11 (d), 5 (f) or 3 (g) independent experiments. Ctrl, control; MV140, PBP Uromune. Paired *t*-test, \**P*<0.05, \*\**P*<0.01, and \*\*\**P*<0.001.

hmoDCs was significantly impaired (around 75% of inhibition) when cells were simultaneously preincubated with piceatannol and pepinh-MYD (Figure 5a). This effect was mainly driven by piceatannol for both cytokines (around 55% inhibition) with similar additive effects observed in the presence of pepinh-

MYD. For IL-6, the same tendencies were observed but with lower percentages of inhibition (around 45%) than for IL-1β and IL-23. These results point out Syk-coupled CLR as the main drivers of the Th17 responses induced by MV140-activated human DCs with cooperation of MyD88-coupled



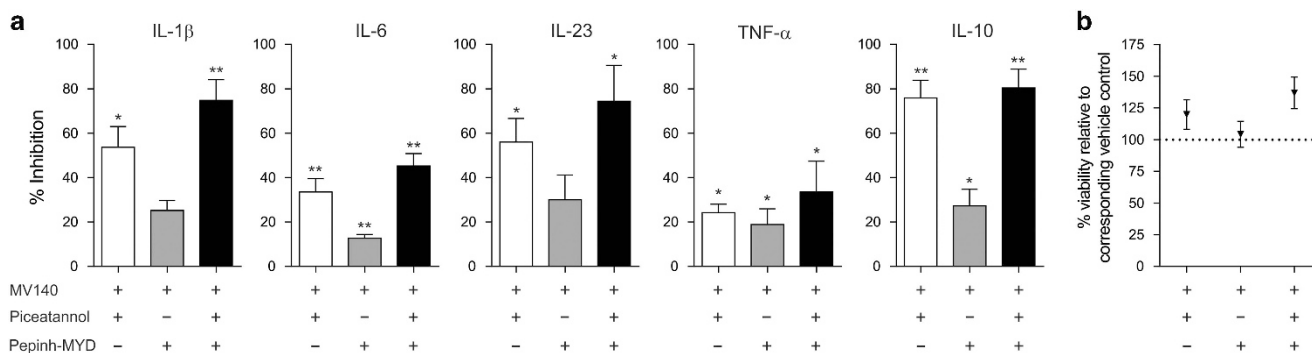
**Figure 3** Contribution of the individual bacterial components of MV140 to polarize T cell responses. **(a)** Representative dot plots of proliferating CFSE-labeled naive CD4<sup>+</sup> T cells after 3 days of coculture with allogeneic hmoDCs in the presence of MV140, the individual bacterial components alone or the control. The frequency of proliferating cells is displayed inside the plot. **(b)** Cytokines produced by allogeneic naive CD4<sup>+</sup> T cells primed by hmoDCs activated by the indicated stimulus after 3 days quantified in cell-free supernatants by ELISA. Results are mean  $\pm$  s.e.m. of 5–6 **(a)** or 5–10 **(b)** independent experiments. Ctrl, control; MV140, PBP Uromune; V113, *K. pneumoniae*; V121, *E. coli*; V125, *E. faecalis*; V127, *P. vulgaris*. Kruskal–Wallis for multiple comparison, \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .



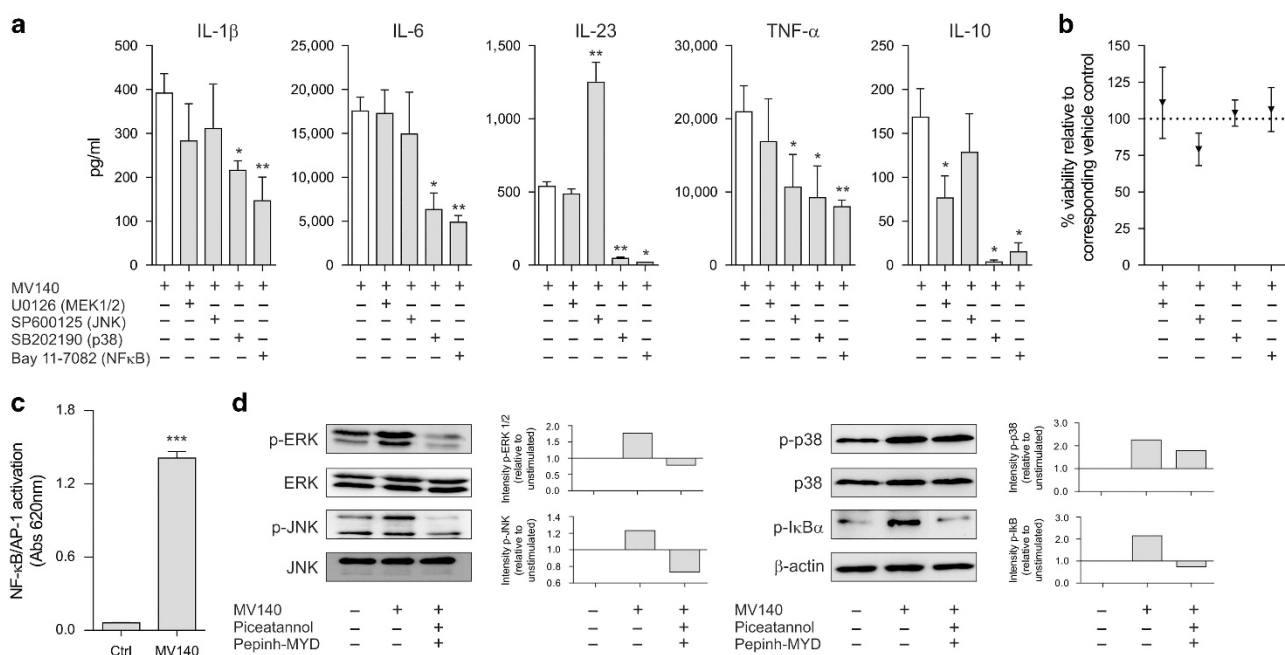
**Figure 4** MV140 immunomodulates the function of human total blood DCs to generate Th1, Th17, and IL-10-producing T cells. **(a)** Representative dot plots of the different DC subsets contained in PBMCs and the enriched total DC fraction gating on HLA-DR<sup>+</sup> CD19<sup>-</sup> cells. HLA-DR<sup>+</sup> CD303<sup>+</sup> pDCs and HLA-DR<sup>+</sup> CD1c<sup>+</sup> mDCs are shown. **(b)** Cytokine production by the enriched total DC fraction after 18 h stimulation with MV140 or control quantified by ELISA. **(c)** Cytokines produced by allogeneic naive CD4<sup>+</sup> T cells primed by control- or MV140-activated total DCs after 3 days quantified by ELISA. **(d)** After 3 days of coculture in the indicated conditions, primed T cells were washed and stimulated with anti-CD3 and -CD28 mAbs for 24 h. Cytokines produced after this polyclonal stimulation were quantified by ELISA. Results are mean  $\pm$  s.e.m. of two independent experiments. Ctrl, control; MV140, PBP Uromune.

TLRs. Interestingly, piceatannol significantly blocked also the production of IL-10 (around 76% inhibition). Pepinh-MYD induced significant inhibition of IL-10 production (around 30%) but additive effects were not observed (**Figure 5a**), indicating that Syk-coupled CLR are the main contributors to

IL-10 production. Around 25% inhibition of TNF- $\alpha$  production was observed when cells were incubated with piceatannol or pepinh-MYD (**Figure 5a**). Incubation with both inhibitors resulted in around 40% inhibition, suggesting that additional pathways might also contribute to the



**Figure 5** Involvement of Syk- and MyD88-mediated signalling pathways in the immune responses induced by MV140 in human DCs. (a) Inhibition of cytokine production by MV140-activated hmoDCs by the indicated inhibitors related to vehicle controls. (b) Percentage of viability for each assayed condition respect to each vehicle control by using trypan blue exclusion. Results are mean  $\pm$  s.e.m. of 5–10 (a) or 5 (b) independent experiments. Paired *t*-test \* $P < 0.05$ , and \*\* $P < 0.01$ .



**Figure 6** MAPKs and NF- $\kappa$ B differentially contribute to the mechanisms of action of MV140. (a) Cytokine production by MV140-activated hmoDCs in the presence of the indicated inhibitors. (b) Percentage of viability for each assayed condition respect to each vehicle control by using trypan blue exclusion. (c) Control- or MV140-induced NF- $\kappa$ B/AP-1 activation in THP1-XBlue cells after 18 h of incubation. (d) Western blot analysis of protein extracts from THP1 cells stimulated for 15 min in the indicated conditions and quantification of the reactive phosphorylated bands by scanning densitometry. One representative example out of 3 is shown.  $\beta$ -actin was used as a loading control. Results are mean  $\pm$  s.e.m. of 4–6 (a and b) or 6 (c) independent experiments. Ctrl, control; MV140, PBP Uromune. Paired *t*-test, \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

production of TNF- $\alpha$ . Cell viability was not significantly affected in any of the assayed conditions (Figure 5b). A slight cell viability increase was observed in the presence of piceatannol both when incubated alone or together with pepinh-MYD.

#### CLRs- and TLRs-mediated activation of MAPKs and NF- $\kappa$ B differentially contribute to the mechanisms of action of MV140 in human DCs

To gain further insights into the molecular mechanisms involved in the mode of action of MV140, we assayed the cytokine signature of MV140-activated hmoDCs in the

presence of specific pharmacological inhibitors for mitogen-activated protein kinases (MAPK) (extracellular-signal regulated kinase (ERK), c-Jun N-terminal Kinase (JNK) and p38) and nuclear factor  $\kappa$ B (NF- $\kappa$ B). U0126, a specific pharmacological inhibitor of MEK 1/2 (upstream of ERK 1/2), significantly inhibited the production of IL-10 without affecting the production of any other Th17- or Th1-polarizing cytokines (Figure 6a). The JNK inhibitor SP600125 significantly impaired the production of TNF- $\alpha$  without modifying the levels of IL-10, IL-1 $\beta$  or IL-6. Remarkable, the inhibition of JNK resulted in the significant increment of IL-23 production by MV140-activated hmoDCs, suggesting a potential blocking

effect of this pathway in Th17 cell generation. The inhibition of p38 significantly reduced the production of Th17- and Th1-polarizing cytokines as well as IL-10 by MV140-activated hmoDCs. Interestingly, the production of IL-23 and IL-10 was nearly abolished by SB202190 p38 inhibitor (**Figure 6a**). Bay 11-7082, a pharmacological inhibitor of NF- $\kappa$ B, significantly impaired the production of all the assayed cytokines and nearly abolished the induction of IL-23 and IL-10 (**Figure 6a**). Cell viability was not significantly affected in any case and only a slight decrease (around 20%) in cell viability was observed for JNK inhibitor SP600125 (**Figure 6b**).

Next, we studied the capacity of MV140 to induce the activation of MAPKs and NF- $\kappa$ B at the protein level in THP1 cells and the contribution of CLR and TLRs. MV140-induced significant activation of the transcription factors NF- $\kappa$ B/AP-1 in the THP1-XBlue reported cell line (**Figure 6c**). MV140 rapidly induced activation of the MAPKs ERK1/2 (phosphorylation of Thr202/Tyr204), JNK (phosphorylation of Thr183/Tyr185), and p38 (phosphorylation of Thr180/Tyr182), as well as NF- $\kappa$ B as determined by the phosphorylation of the inhibitor I $\kappa$ B $\alpha$  at Ser32/36 in THP1 cells (**Figure 6d**). The activation of ERK, JNK, and NF- $\kappa$ B was completely abolished by simultaneous inhibition with piceatannol and pepinh-MYD. In contrast, the activation of p38 was only partially impaired by these inhibitors (**Figure 6d**).

#### Sublingual immunization of BALB/c mice with MV140 induces the generation of systemic Th1, Th17, and IL-10 immune responses *in vivo*

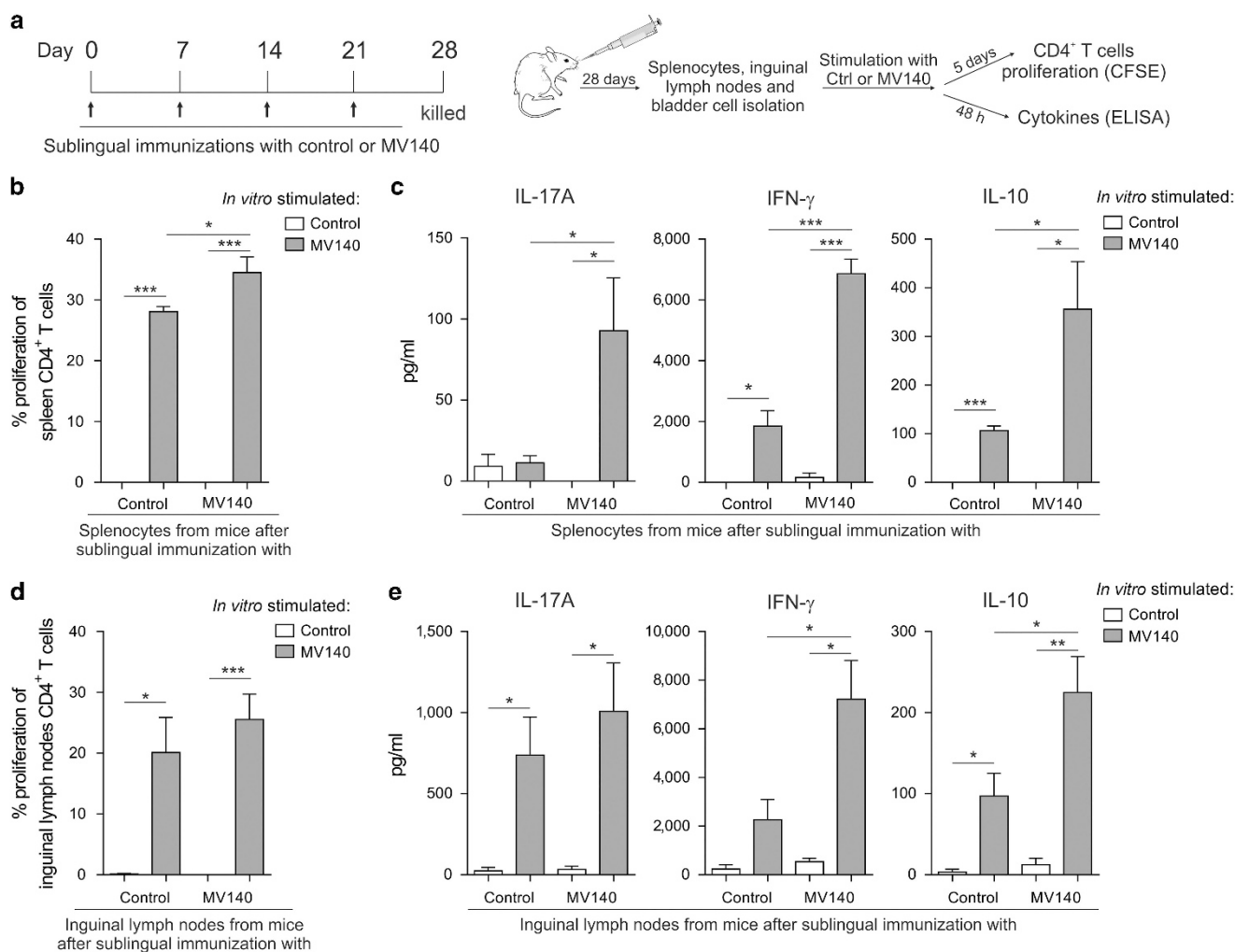
To assess the *in vivo* relevance of our findings we subjected BALB/c mice to sublingual immunization with MV140 or control excipients following the protocol showed in **Figure 7a**. The responses induced systemically were analyzed after *in vitro* stimulation of splenocytes isolated from each group (**Figure 7a**). Spleen CD4<sup>+</sup> T cells from mice immunized sublingually with MV140 or control excipients showed significantly higher proliferation rates after *in vitro* stimulation with MV140 than control (**Figure 7b**). Splenocytes from mice immunized with MV140 or control excipients produced significantly higher levels of IFN- $\gamma$  and IL-10 after *in vitro* stimulation with MV140 than control (**Figure 7c**). IL-17A production was only observed after MV140 *in vitro* stimulation of splenocytes from MV140 immunized mice (**Figure 7c**). Interestingly, the proliferation rates and the levels of IL-17A, IFN- $\gamma$  and IL-10 produced by splenocytes from mice sublingually immunized with MV140 were significantly higher than those produced by splenocytes from control mice after *in vitro* stimulation with MV140 (**Figure 7b,c**). IL-5 production was not detected in any assayed condition (data not shown). When analyzing cells isolated from inguinal lymph nodes we observed similar responses to those reported for spleen (**Figure 7d,e**). Although we did not detect significant proliferation or IL-17, IFN- $\gamma$  and IL-10 production in isolated bladder cells (**Supplementary Figure 3**), a significant production of TNF- $\alpha$  was detected in bladder cells from

MV140 immunized mice compared to control group after MV140 stimulation (**Supplementary Figure 3b**).

#### DISCUSSION

In this study, we shed light into the immunological mechanisms by which MV140 (Uromune) might exert beneficial clinical effects for the treatment of RUTIs. We demonstrated that MV140 primes human DCs to generate Th1, Th17 and IL-10-producing T cells. None of the individual bacterial components included in this PBP fully mimicked the final outcomes imprinted by MV140, suggesting that all the components are required to drive the observed immune responses. MV140 initiates Syk- and MyD88-mediated downstream signaling pathways that culminate in the activation of MAPKs and NF- $\kappa$ B in human DCs. Syk-coupled CLR seems to be the main driver of Th17 and IL-10 immune responses in cooperation with MyD88-coupled TLRs. Both CLR and TLRs contribute to the production of Th1-driving cytokines by human DCs but other PRRs might well also play a role. MV140-induced activation of NF- $\kappa$ B and p38 is essential for the generation of Th1/Th17 and IL-10 immune responses by human DCs whereas JNK and ERK significantly contribute to Th1 and IL-10 responses, respectively. We also showed that sublingual immunization of BALB/c mice with MV140 promotes the generation of systemic Th1, Th17 and IL-10 immune responses *in vivo*.

RUTIs are among the most prevalent bacterial infections and represent the second leading cause of community-acquired and nosocomial infections. Currently, antibiotics are still the main therapeutic option used for the treatment of RUTIs.<sup>1,2</sup> However, the overuse of antibiotics in humans has been associated with the increase in antibiotic-resistant infections, which represents a major public health concern worldwide that menaces the effective prevention and treatment of a wide range of infections caused by bacteria, parasites, viruses and fungi.<sup>20,21</sup> Other negative factors associated to the overuse of antibiotics include horizontal gene transfer, limitation in terms of diversity of action spectra and deleterious effects in microbiota, which enhance pathogen invasion and subsequent fungal superinfections.<sup>6,7</sup> Therefore, alternative approaches for the treatment of recurrent infections in general, and RUTIs in particular are fully demanded. Among these alternatives, the development of mucosal bacterial vaccines has received a lot of attention over the last years.<sup>1</sup> Different studies suggest that formulations based on PBPs of inactivated whole cells represent suitable vaccines able to induce more potent and effective immune responses than bacterial lysates.<sup>22,23</sup> In contrast to oral administration, sublingual immunization protects the PBPs from gastrointestinal degradation and induces strong and durable immune responses not only locally but also in peripheral lymphoid organs and distant mucosal tissues, such as the urinary tract.<sup>24,25</sup> Sublingual MV140 is a PBP composed of whole heat-inactivated bacteria showing clinical efficacy for the treatment and prevention of RUTIs.<sup>11,12</sup> Despite these relevant clinical benefits, the immunological ways of action and the molecular mechanism by which MV140 could modulate the



**Figure 7** Induction of Th1, Th17 and IL-10 immune responses after *in vivo* MV140 sublingual immunization of BALB/c mice. **(a)** Scheme of the sublingual immunization protocol and analysis of induced systemic responses. **(b)** Proliferation of CFSE-labeled CD4<sup>+</sup> T cells from splenocytes or **(d)** inguinal lymph nodes isolated from mice immunized sublingually with MV140 or control after *in vitro* stimulation with MV140 or control. **(c)** Cytokine production (IL-17A, IFN- $\gamma$  and IL-10) by splenocytes or **(e)** inguinal lymph node cells isolated from mice immunized sublingually with MV140 or control and stimulated *in vitro* with MV140 or control. Results are mean  $\pm$  s.e.m. of 7–8 **(b)** or 4 **(c, d, and e)**. Ctrl, control containing all excipients except the bacteria; MV140, PBP Uromune. Unpaired *t*-test, \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

function of DCs and their capacity to polarize T cell responses remained fully unknown prior to this study.

Herein, we showed that MV140 directly acts on human DCs to induce maturation and production of pro-inflammatory cytokines and high levels of IL-10. Among pro-inflammatory cytokines, Th17- (IL-1 $\beta$ , IL-6, and IL-23) or Th1-driving (TNF- $\alpha$ ) cytokines were produced, which could justify the capacity of MV140-activated human DCs to generate mixed Th1, Th17, and IL-10-producing T cell responses. Depending on the encountered antigens and surrounding environment, DCs can integrate different signaling pathways and polarize distinct effector Th or regulatory T (Treg) cells using a wide battery of soluble and membrane-bound factors.<sup>26–30</sup> This function is of paramount importance to protect the organism against potentially dangerous pathogens while keeping homeostasis and tolerance to innocuous and self-antigens.<sup>29,31–33</sup> MV140-induced Th1 and Th17 cells could contribute to clear

intracellular and extracellular pathogens whereas the IL-10-producing T cells could control excessive immune responses, avoid deleterious consequences for the tissues and contribute to pathogen clearance by keeping homeostasis.<sup>34–36</sup> Our data suggest that the generated IL-10-producing T cells could phenotypically correspond to induced type 1 regulatory T cells (Treg1). Our experiments with the individual bacterial components of MV140 uncovered that none of them fully mirror the tuned immune responses imprinted by this PBP. There were no additive effects in any case, suggesting that the final outcomes arise from the integration of the signals that all the bacterial components of MV140 simultaneously trigger in human DCs. At this regard, compelling experimental evidence showed cross-talk and pathways interactions among different pattern recognition receptors, including TLRs and CLRs.<sup>19,37</sup> In addition, for the particular case of CLRs (a main contributors of the reported effects for MV140) it is described that the strength



and duration of signaling and induced functional responses not only depend on the type of targeted CLR but also on the specific features of the ligand, such as low or high affinity, soluble or particulate and particle size.<sup>18,38</sup> For example, the gram-positive bacterium (*E. faecalis* V125) was the only individual component inducing the production of significant levels of the Th1-polarizing cytokines IL-12 and TNF- $\alpha$  without IL-10 production in hmoDCs. In contrast, MV140-activated hmoDCs only produced TNF- $\alpha$  but not IL-12, which could be justified by the high levels of IL-10 induced by MV140. Supporting these data, it has been previously reported that IL-10 is able to inhibit IL-12 production by different mechanisms.<sup>39</sup> Significant high levels of IFN- $\gamma$ -producing T cells were only induced by MV140- or V125-activated hmoDCs but not by individual gram-negative bacteria, supporting a relevant role of V125 for Th1 polarization.

CLRs and TLRs are PRRs that play very important roles in shaping proper immune responses in human DCs.<sup>18,19</sup> Syk-coupled CLRs signaling induces PI3K/Akt, MAPK, NF- $\kappa$ B and inflammasome activation.<sup>37,40,41</sup> After engagement of TLRs, MyD88-dependent downstream signaling pathways activate MAPK and NF- $\kappa$ B.<sup>17,19</sup> Our Syk and MyD88 inhibition experiments in MV140-activated human DCs pointed out CLRs as the main drivers of Th17 and IL-10 immune responses in cooperation with TLRs. In addition, other PRRs might be involved in the generation of Th1 immune responses. ERK, JNK and p38 MAPKs differentially regulate phenotypic and functional maturation, cytokine production and T cell polarization capacity of human DCs.<sup>42,43</sup> Similarly, NF- $\kappa$ B is also a crucial transcription factor in the control of immune responses in DCs.<sup>44,45</sup> We showed that in MV140-activated human DCs NF- $\kappa$ B and p38 are essential for Th1/Th17 and IL-10 immune responses, whereas ERK and JNK significantly contribute to IL-10 and Th1 responses, respectively. These data are in accordance with previous studies showing that p38 and to a lesser extent JNK signaling pathways positively regulate functional maturation and cytokine production in human DCs, whereas ERK might favor IL-10 production.<sup>42,46–48</sup> Our data also showed that MV140-mediated ERK/JNK and NF- $\kappa$ B activation should be fully induced by CLRs and TLRs. Although additional signaling pathways might also contribute to p38 activation, our data on IL-10 and IL-23 inhibition by piceatannol suggest that Syk-mediated pathways might be indispensable for the functional activation of this MAPK.

In humans, circulating blood mDCs and pDCs represent specialized subsets of DCs that display a different repertoire of receptors and cytokine signature after antigen stimulation.<sup>49</sup> mDCs and pDCs play distinct but complementary roles in the initiation and maintenance of proper immune responses.<sup>31</sup> We showed that an enriched fraction of human total blood DCs containing both mDCs and pDCs activated with MV140 also generates Th1, Th17, and IL-10 immune responses, suggesting the potential *in vivo* relevance of these findings. Supporting these data, *in vivo* sublingual immunization of mice with MV140 also promotes the generation of potent systemic Th1, Th17, and IL-10 immune responses, indicating that these

immunological mechanisms might well play a very important role in the reported beneficial therapeutic effects of this PBP to treat RUTIs in humans.<sup>11,12</sup> Although responses were not observed in bladder after *in vitro* stimulation, sublingual immunization with MV140-induced potent local immune responses in pelvic lymph nodes, suggesting that proper local responses might well be mounted upon bladder reinfection. Moreover, the fact that a significant production of TNF- $\alpha$  was detected in bladder cells from MV140 immunized mice compared to control group after MV140 stimulation raises the possibility of mucosal trained immunity mechanisms after MV140 immunization.<sup>50</sup>

In conclusion, our data shed light into the immunological mechanisms underlying the clinical benefits reported for MV140 for the treatment of RUTIs. This study uncovers the potential way of action of MV140, which might well also contribute to understand the rational use of specific PBPs in other clinical conditions with potential high risk of recurrent infections.

## METHODS

**Media and reagents.** RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum, 100  $\mu$ g ml<sup>-1</sup> normocin, 50  $\mu$ g ml<sup>-1</sup> penicillin-streptomycin, 1% non-essential aminoacids, 1% MEM-vitamins and 1 mM sodium pyruvate (cRPMI). For experiments with THP1 cells, also glucose up to 4.5 g l<sup>-1</sup> was added. In addition, 200  $\mu$ g ml<sup>-1</sup> zeocin (InvivoGen) was added when using THP1-XBlue. PBP MV140 (Uromune) composed of heat-inactivated bacteria (25% *E. coli*, 25% *P. vulgaris*, 25% *K. pneumoniae*, and 25% *E. faecalis*), negative control (containing all excipients without bacteria) and each individual bacterium were provided by Immunotek S.L. Inhibitors for Syk (piceatannol) (Enzo), MyD88 (Pepinh-MYD), p38 (SB202190), JNK (SP600125), and MEK 1/2 (U0126) (InvivoGen) were used for the inhibition experiments.

**Generation of hmoDCs, purification of naive CD4<sup>+</sup> T cells and isolation of total DC fraction.** Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors by Ficoll density gradient centrifugation (800g, 20 min). Immature hmoDCs were generated from blood monocytes obtained from total PBMCs using anti-CD14 microbeads and cultured for 6 days with cRPMI medium containing 100 ng ml<sup>-1</sup> of IL-4 and GM-CSF (PeproTeck, Rocky Hill, NJ). The purity and phenotype of monocytes and generated immature hmoDCs were analyzed by flow cytometry with lineage-specific markers. Peripheral blood naive CD4<sup>+</sup> T cells were isolated using the “Naive CD4<sup>+</sup> T Cell Isolation Kit” (Miltenyi Biotec, Bergisch Gladbach, Germany) and total dendritic cell fraction was obtained from PBMC with “Blood Dendritic Cell Isolation Kit II” (Miltenyi Biotec), according to manufacturer’s protocol.

**Cell cultures.** Immature hmoDCs or human total blood DC enriched fraction (10<sup>6</sup> cells per mL) were stimulated with control, MV140 (10<sup>7</sup> bact. per mL), or the individual bacterial components (2.5 · 10<sup>6</sup> bact. per mL) for 18 h. Subsequently, cells were collected and centrifuged. Cell pellets were used to analyze their phenotype by flow cytometry and cell-free supernatants were used to quantify IL-1 $\beta$ , IL-6, IL-23, IL-12, TNF- $\alpha$  and IL-10 by ELISA. For inhibition experiments, hmoDCs were preincubated for 2 h with Pepinh-MYD (25  $\mu$ M) or for 30 min with piceatannol (25  $\mu$ M), U0126 (1  $\mu$ M), SB202190 (1  $\mu$ M) or SP600125 (10  $\mu$ M) (or their vehicle controls, Pepinh-Control or DMSO) prior to activation. Then, the cells were stimulated with MV140 (10<sup>7</sup> bact. per mL) for 4 h in the presence of the corresponding inhibitors to quantify IL-1 $\beta$ , IL-6, IL-23, TNF- $\alpha$ , and IL-10 in cell-free supernatants by

ELISA. Cell viability was analyzed in all the cases by trypan blue exclusion with a light microscope.

**Flow cytometry.** The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR and anti-CD1c; allophycocyanin (APC)-conjugated anti-HLA-DR and anti-CD83; phycoerythrin (PE)-conjugated anti-CD86 and anti-CD303; peridinin-chlorophyll-protein (PerCP)-conjugated anti-CD14 and anti-CD4 (Myltenyi Biotec). APC-conjugated anti-CD3; Alexa Fluor 488-conjugated anti-IFN $\gamma$  and anti-IL-4 (BD Pharmingen, San Jose, CA). PE-conjugated anti-IL-10 and anti-IL-17A; PE/Cy7-conjugated anti-CD19; Alexa Fluor 488-conjugated anti-IL-17A, anti-FOXP3 and anti-GATA3 (Biolegend, San Diego, CA). Cells were washed with PBS/EDTA 2 mM/0.5% BSA and stained for 15 min at room temperature in the darkness. The corresponding isotype controls were included in each staining (IgG2A-FITC, IgG1-PE, IgG2A-PerCP, or IgG1-APC). Flow cytometry analysis was performed in a FACSCalibur in the Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid.

**Cytokine quantification.** Concentrations of IL-1 $\beta$ , IL-6, IL-12p70, TNF- $\alpha$ , IL-10, IFN- $\gamma$ , and IL-5 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences, San Jose, CA). IL-23 and IL-17A levels were quantified by Human IL-23 ELISA Ready-SET-Go! (e-Biosciences) and quantikine Elisa Kit (RD Systems), respectively. In all cases, manufacturer's instructions were followed with minor modifications.

**Coculture experiments.** Immature hmoDCs or human total blood DC enriched fraction were cocultured with purified allogeneic naive CD4<sup>+</sup> T cells (DC/T cell ratio of 1:5) for 3 days in the presence of the control, MV140 (10<sup>7</sup> bact. per mL) or the individual bacterial components (2.5  $\times$  10<sup>6</sup> bact. per mL). IL-17A, IFN- $\gamma$ , IL-5 and IL-10 were quantified in cell-free supernatants by ELISA. For polyclonal restimulation, T cells were washed and incubated with 1  $\mu$ g ml<sup>-1</sup> of plate-bound anti-CD3 and 1  $\mu$ g ml<sup>-1</sup> of soluble anti-CD28 mAb at a concentration of 10<sup>6</sup> cells per mL for 24 h. For intracellular cytokine production, the primed CD4<sup>+</sup> T cells were washed and restimulated with 25 ng ml<sup>-1</sup> PMA plus 1  $\mu$ g ml<sup>-1</sup> ionomycin for 6 h. 10  $\mu$ g ml<sup>-1</sup> brefeldin A was added during the last 4 h. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. The cells were stained with the combination of fluorochrome-labeled mAbs to IL-17A, IFN- $\gamma$ , IL-4, and IL-10. For analysis of FOXP3 and GATA3 expression in human T cells primed with DCs, a double permeabilization was performed and cells were stained with anti-human FOXP3-Alexa Fluor 488 or GATA3-Alexa Fluor 488 (BioLegend) according to the manufacturer's instructions. For proliferation assays, purified allogeneic naive CD4<sup>+</sup> T cells were labeled with CFSE (Molecular Probes, Europe) according to standard protocols prior to coculture with immature hmoDCs. Proliferation was assessed by measuring CFSE dilution on labeled CD4<sup>+</sup> T cells by flow cytometry.

**THP1 and THP1-XBlue.** Human monocytic THP1 and THP1-XBlue cell lines (InvivoGen) were maintained in supplemented RPMI medium. THP1-XBlue cells were cultured (10<sup>6</sup> cells per mL) with control or MV140 (10<sup>7</sup> bact. per mL) for 18 h. To determine NF- $\kappa$ B/AP-1 activation, 20  $\mu$ L of the supernatant were added to 180  $\mu$ L of QUANTI-Blue. The mixture was incubated at 37  $^{\circ}$ C and the optical density was read with an ELISA EZ Read 400 Microplate Reader (Biochrom).

**Western blot analysis.** THP1 cells were used for western blotting analysis. THP1 cells (10<sup>6</sup> cells per mL) were incubated with control or MV140 (10<sup>7</sup> bact. per mL) for 15 min prior to cell lysis and protein extraction. Cell lysis was carried out with PBS/Triton 1% in the presence of 1 mM PMSF (Sigma), 1  $\mu$ g ml<sup>-1</sup> Leupeptin (Bachem) and Aprotinin (Roche) for 30 min at 4  $^{\circ}$ C with vortex every 10 min. Lysates were clarified by centrifugation at 10,000g for 15 min at 4  $^{\circ}$ C. Protein

quantification was performed with Micro BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions and samples with equal amounts of total protein were resolved in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes. For western blotting, after 1 h of incubation in blocking buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5 (TBS-T) with 5% BSA), membranes were incubated overnight at 4  $^{\circ}$ C with the corresponding primary antibodies. Then, membranes were washed with TBS-T and incubated with the corresponding secondary antibody for 1 h at room temperature. After washing, the signal was developed with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and detected in a Fujifilm LAS-3000 developer (Tokyo, Japan). The following primary anti-human antibodies were from Cell Signaling (Danvers, MA) and used at a 1:1,000 dilution: mouse anti-phospho-I $\kappa$ B $\alpha$  (Ser32/36), rabbit anti-phospho-ERK1/2 (Thr202/Tyr204), rabbit anti-ERK1/2, rabbit anti-phospho-p38 MAPK (Thr180/Tyr182), rabbit anti-p38 MAPK, mouse anti-phospho-SAPK/JNK (Thr183/Tyr185), rabbit anti-SAPK/JNK. Mouse anti-human anti- $\beta$ -actin (Sigma-Aldrich, St Louis, MO) was used at a 1:15,000 dilution. Horseradish peroxidase-conjugated goat anti-mouse (1:2,500, Pierce) or goat anti-rabbit (1:3000, BIORAD) were used as secondary antibodies.

**Sublingual immunization of mice with MV140 or control, quantification of CD4<sup>+</sup> T cells proliferation and cytokine signature of *in vitro* stimulated splenocytes, bladder and lymph nodes cells with MV140 or control.** BALB/c mice (6-weeks-old) were immunized four times with 10  $\mu$ L of MV140 (10<sup>9</sup> bact./mL) or control (all excipients without bacteria) by sublingual administration every 7 days and killed 7 days after the last immunization. Sublingual administration was performed under anesthesia (ketamine, 100 mg kg<sup>-1</sup> and xylazine, 5 mg kg<sup>-1</sup>) to ensure proper delivery. Spleens were used to prepare single cell suspensions following conventional protocols. Pelvic lymph nodes (inguinal) and bladder were treated apart in RPMI containing penicillin/streptomycin and HEPES buffer and digested with 67  $\mu$ g ml<sup>-1</sup> Liberase (Roche, Mannheim, Germany) and 20  $\mu$ g ml<sup>-1</sup> DNase I (Sigma) for 20 min while rotating. The digestion was then stopped with 10% fetal bovine serum (FBS) and 5  $\mu$ M EDTA. Finally, lymph nodes were disaggregated by mashing through 100  $\mu$ m nylon cell strainer. Cells were labeled with CFSE and stimulated *in vitro* with MV140 (10<sup>8</sup> bact. per mL) or control for 5 days. Proliferation of CFSE-labeled CD4<sup>+</sup> T cells was monitored by flow cytometry. Cells were *in vitro* stimulated for 48 h with MV140 (10<sup>7</sup> bact. per mL) or control and cytokine production (IL-17A, IFN- $\gamma$ , IL-5, and IL-10) measured by flow cytometry in cell-free culture supernatants using a commercial kit (CBA: cytometric bead array) from BD Biosciences following manufacturer's recommendations. Animals were maintained in Biolab S.L. under the conditions stabilized by the R.D. 53/2013 and in accordance with the international GLP normative, RD 223/83 and ISO 10993-2:2007. The tests are performed in accordance with official methods, European Pharmacopoeia, USP Pharmacopoeia, OECD Guideline, UNE EN-ISO, and specific methodologies developed for R&D.

**Statistics.** All the data are expressed as mean  $\pm$  s.e.m. of the indicated parameters. Statistical differences were determined with the paired or unpaired Student's *t*-test as indicated in each figure using GraphPad Prism software, version 6.0. Friedman test or Kruskal-Wallis test were used for multiple comparison. Significance was defined as \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at <http://www.nature.com/mi>

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## AUTHOR CONTRIBUTIONS

Conceived and designed the study: O.P. Performed all the human experiments: C.B.-V. and C.C. Designed and performed the animal experiments: J.L.S. and C.M.D.-R. Analyzed and discussed the data: C.B.-V., C.M.D.-R., J.L.S., and O.P. Contributed reagents/materials/analysis tools: M.C., J.L.S. and O.P. Wrote the paper: O.P. and C.B.-V. All the authors read and approved the final manuscript.

## DISCLOSURE

J.L.S. is the founder of Immunotek SL and shareholder together with M.C. The rest of authors declare no conflict of interests.

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