Journal of Reproductive Immunology 138 (2020) 103085

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

Vaginal dysbiosis associated-bacteria *Megasphaera elsdenii* and *Prevotella timonensis* induce immune activation via dendritic cells

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ARTICLE INFO

Keywords:
Vaginal microbiome
Genital inflammation
Dendritic cells
Maturation markers
Pro-inflammatory cytokines
T helper cells

ABSTRACT

Dysbiosis of the vaginal microbiome as a result of overgrowth of anaerobic bacteria leads to bacterial vaginosis (BV) which is associated with increased inflammation in the genital mucosa. Moreover, BV increases susceptibility to sexual transmitted infections (STIs) and is associated with adverse pregnancy outcomes. It remains unclear how specific vaginal aerobic and anaerobic bacteria affect health and disease. We selected different vaginal bacteria ranging from true commensals to species associated with dysbiosis and investigated their effects on activation of dendritic cells (DCs). Commensal *Lactobacilli crispatus* did not induce DC maturation nor led to production of pro-inflammatory cytokines. In contrast, BV-associated bacteria *Megasphaera elsdenii* and *Prevotella timonensis* induced DC maturation and increased levels of pro-inflammatory cytokines. Notably, DCs stimulated with *Prevotella timonensis* suppressed Th2 responses and induced Th1 skewing, typically associated with preterm birth. In contrast, *Lactobacillus crispatus* and *Megasphaera elsdenii* did not affect Th cell polarization. These results strongly indicate that the interaction of vaginal bacteria with mucosal DCs determines mucosal inflammation and we have identified the anaerobic bacterium *Prevotella timonensis* as a strong inducer of inflammatory responses. Specifically targeting these inflammation-inducing bacteria might be a therapeutic strategy to prevent BV and associated risks in STI susceptibility and preterm birth.

1. Introduction

The female genital tract is a niche where many bacteria reside resulting in commensal relationships between the human host and indigenous microbial communities. This complex ecosystem is established via years of co-evolution between the genital tract's environment and the associated microbial community and this process is influenced by genetic, ethnic, environmental and behavioural factors (Kenyon et al., 2013). In this ecosystem, the nutrient rich environment of the genital tract stimulates growth of selected commensals and these established microbial communities provide colonization resistance to invasive pathogens. The last few decades the importance of the vaginal microbiome in genital health has become increasingly clear and major efforts have been made to understand the interaction between the host and the vaginal microbiome.

Employment of 16S ribosomal RNA gene sequencing had a great impact on the field of vaginal microbiota (Fredricks et al., 2005; Martin et al., 2012; Ravel et al., 2011; Srinivasan et al., 2012). This culture-independent approach revealed the complexity of the vaginal microbiome and identified bacterial clusters associated with genital health or inflammation. A healthy vaginal microbiome is dominated by *Lactobacilli* species (e.g. *Lactobacillus jensenii*, *Lactobacillus gasseri* or *Lactobacillus crispatus, collectively called Lactobacilli species or* spp.) (Fredricks et al., 2005; Martin et al., 2012; Ravel et al., 2011; Srinivasan et al., 2012). *Lactobacilli* spp. contribute to the hosts defence by producing lactic acid, bacteriocins, and hydrogen peroxide, which inhibit growth of pathogenic bacteria and fungi (Witkin and Linhares, 2017). In contrast, an increased diversity in the composition of the vaginal microbiome harbouring many (facultative) anaerobic bacterial species (e.g. *Gardnerella vaginalis*, *Atapobium vaginae, Megasphaera elsdenii, Prevotella*

Abbreviations: BV, bacterial vaginosis; STIs, sexually transmitted infections; HSV-2, herpes simplex virus type 2; HPV, human Papillomavirus; HIV-1, human immunodeficiency virus; DCs, dendritic cells; Th, T helper; PPROM, preterm premature rupture of membranes

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https://doi.org/10.1016/j.jri.2020.103085
Received 15 August 2019; Received in revised form 6 December 2019; Accepted 20 January 2020

bivia and *Prevotella timonensis*) causes a decrease in health-promoting *Lactobacilli* spp. and is referred to as dysbiosis of the vaginal microbiome (Fredricks et al., 2005; Martin et al., 2012; Ravel et al., 2011; Srinivasan et al., 2012).

Dysbiosis of the vaginal microbiome is the cause of the clinical diagnosis bacterial vaginosis (BV) (Fredricks et al., 2005; Ling et al., 2010; Nugent et al., 1991). Although BV can be asymptomatic, it is often characterized by vaginal discharge in combination with malodour. BV is the most common vaginal condition among women of reproductive-age with a prevalence ranging from 7 % to 33 % dependent on age, hygiene, ethnicity, education, and economical status (Kenyon et al., 2013). BV is associated with increased susceptibility to sexually transmitted infections (STIs) including bacterial infections like *Chlamydia trachomatis* and *Neisseria gonorrhoeae* as well as viral infections like herpes simplex virus type 2 (HSV-2), human Papillomavirus (HPV), and human immunodeficiency virus (HIV-1) (Cohen et al., 1995; Kaul et al., 2007; King et al., 2011; Wiesenfeld et al., 2003). Besides increased STI acquisition, BV is an independent risk factor for adverse pregnancy outcomes including; reduced fertility, early and late miscarriages, preterm premature rupture of membranes (PPROM) and preterm birth (Donders et al., 2009; Hay et al., 1994; Llahí-Camp et al., 1996; Ralph et al., 1999; Spandorfer et al., 2001). Previous reports have suggested that inflammation of the genital tract caused by specific inflammatory immune responses directed towards members of the vaginal microbiome associated with dysbiosis, is the underlying cause of BV-associated afflictions (Anahtar et al., 2015). Indeed, cohort studies have found elevated levels of pro-inflammatory mediators including IL-1β, IL-6, IL-8 (alternatively known as CXCL8), IL-12 and TNFα in the vaginal microenvironment of women with BV compared to those with *Lactobacilli*-dominated vaginal microbiomes (Jespers et al., 2017). However, little is known about the cellular mechanisms underlying genital inflammation caused by bacteria associated with BV.

Dendritic cells (DCs) are important players at the frontline of defence against mucosal infections that survey the vaginal mucosa and sub-mucosa. DCs operate at the interface of innate and acquired immunity by recognizing invading pathogens and inducing differentiation of pathogen-specific naïve T cells residing in lymphoid structures (Geijtenbeek and Gringhuis, 2016). DCs are pivotal in dictating the differentiation of naïve T helper (Th) cells into different Th responses such as Th1 or Th2 responses via upregulation of co-stimulatory molecules and the production of distinct cytokines profiles. Th1 induction results in a cell-mediated immune response characterized by production of pro-inflammatory cytokines and antimicrobial action (Berger, 2000; Geijtenbeek and Gringhuis, 2016). Excessive Th1 responses lead to uncontrolled inflammation resulting in an influx of immune cells and tissue damage (Berger, 2000). In contrast, Th2 differentiation leads to a humoral immune response associated with the production of antigen-specific antibodies and a Th2 response can counteract the detrimental effect of an inflammatory Th1 responses. A misbalance between Th1 and Th2 might be an underlying cause of genital inflammation. Therefore, achieving an optimal Th1/Th2 balance is paramount to ensure homeostasis and mount a protective immune response to aberrant microbiota (Berger, 2000; Geijtenbeek and Gringhuis, 2016). The ability of DCs to skew the Th1/Th2 balance positions them as potent regulators of genital health and inflammation.

Little is known about the cellular mechanisms underlying genital inflammation caused by bacteria associated with dysbiosis. We hypothesized that DCs induce bacteria-specific genital inflammation upon interaction with BV-associated bacteria. In this study, we characterise the initial interaction between DCs and different UV-inactivated vaginal bacteria including commensals (*Lactobacillus crispatus*) and BV-associated species (*Lactobacillus iners*, *Gardnerella vaginalis, Atopobium vaginae*, *Megasphaera elsdenii*, *Prevotella timonensis*). Notably, bacteria associated most strongly with BV, *M. elsdenii* and *P. timonensis,* induced both DC maturation and pro-inflammatory cytokines which is in contrast with the commensal *L. crispatus*. Moreover, *P. timonensis* suppressed Th2 and induced Th1 differentiation. Therefore, these data suggest that dysbiosis-associated species *M. elsdenii* and particularly *P. timonensis* contribute to DC-mediated inflammatory immune responses that can resulting in genital inflammation, the underlying cause of increased susceptibility to STIs and adverse pregnancy outcomes.

2. Methods

2.1. Bacteria

Lactobacillus crispatus DSMZ 20584, *Lactobacillus iners* DSMZ 13335, *Gardnerella vaginalis* (DSMZ 4944), *Atopobium vaginae* DMSZ 15829, *Megasphaera elsdenii* DSMZ 20460, and *Prevotella timonensis* DSMZ 22865 were grown to log phase, washed three times with PBS, and set to an optical density at 600 nm OD600 of 1. 1 mL suspension was pipetted in a 6-wells plate and UV irradiated in a UV crosslinker using 5 rounds of 100.000 μJ/cm2. Loss of viability was verified by plating UV-inactivated microbiota. Bacterial suspensions were aliquoted and stored at -20 C.

2.2. Cells

This study was performed in accordance with the ethical guidelines of the Amsterdam UMC. All blood donors gave informed consent and remained anonymous throughout all experimental procedures. Peripheral blood monocytes from healthy individuals were isolated and differentiated into DCs in 6–7 days in presence of IL-4 (500 U/mL, Biosource) and GM-CSF (800 U/mL, Invitrogen) as described before (Mesman et al., 2014). Naive CD4 + T cells were isolated from buffy coats of healthy individuals (Sanquin), using a human CD4 + T Cell Isolation Kit II (Miltenyi Biotec).

2.3. Stimulations

DCs were placed in a round bottom 96-well plate (100.000 DCs/well) and stimulated with selected vaginal microbiota at different ratios or left untreated. LPS (10 ng/ml) was used to validate donor differences. DCs were stimulated with vaginal microbiota with or without the presence of anti-TLR4 antibodies (7E3; 10 μg/ml).

2.4. Cytokines

DCs were stimulated with selected vaginal microbiota at different ratios, positive control LPS (10 ng/mL), or left untreated, for 6 h at 37 °C. Quantitative PCR was used to measure cytokine mRNA levels as described previously (Gringhuis et al., 2014, 2012). Expression of target genes was normalized to GAPDH [$N_t = 2^{Ct\ (GAPDH)\ -\ Ct\ (target)}$] and set at 1 in LPS stimulated DCs for each donor. Supernatant of stimulated DCs was collected 24 h after stimulation at 37 °C and cytokine production was determined by means of ELISA (eBioscience).

2.5. DC maturation

DCs were stimulated with selected vaginal microbiota, positive control LPS (10 ng/mL), or left untreated, for 24 h at 37 °C. After stimulation, DC maturation was measured by staining with PE-conjugated CD80 (1:25, 557227, BD Pharmingen), APC-conjugated CD83 (1:25, 551073, BD Pharmingen) and FITC-conjugated CD86 (1:25, 555657, BD Pharmingen). Expression was analyzed using a FACS Canto II (BD Biosciences). Mean fluorescent intensity (MFI) of CD80, CD83 and CD86 was normalized to LPS stimulated DCs and set to 100 % for each donor.

2.6. T helper cell differentiation

DCs were stimulated with LPS 10 ng/mL, LPS 10 ng/mL in

combination with IFNy 100 U/mL, AbD Serote or PGE2 1 μM, Sigma as positive controls for a Th1 and Th2 skewing respectively, or selected microbiota in a 1:10 ratio, for 2 days at 37 °C. Next, DCs were diluted in IMDM complete and combined with allogeneic naive CD4 + T cells 5,000 DCs : 20,000 T cells in the presence of *Staphylococcus aureus* enterotoxin B (10 pg/ml; Sigma) for 10–14 days. Every two days, cells were refreshed in IMDM complete supplemented with IL-2 (10 U/mL, Chiron). After 10–14 days cells were stimulated with PMA (100 ng/mL, Sigma) and ionomycin (1 μg/mL, Sigma) for 6 h and during the last for 4 h BrefeldinA (10 μg/mL, Sigma) was added. Th cell polarization was determined by flow cytometry by staining for intracellular IFNγ (Th1) and IL-4 (Th2) expression. Cells were stained in saponin buffer (1 % BSA + 0,1 % saponin + 0,02 % sodiumazide in PBS) containing APC-conjugated IL-4 (1:25, 554486, BD Phammingen) and FITC-conjugated IFNy (1:5, 340449, BD Pharmingen). Differentiation of Th cell population was analyzed using a FACSCanto II (BD Biosciences).

2.7. Statistics

Statistical analyses were performed using GraphPad 6.0 software. Mann-Whitney *U* test to test statistical significance to NS for unpaired observations was performed. For significance we employed *p < 0.05, **p < 0.01, *** p < 0.001 and **** p < 0.0001

3. Results

3.1. Vaginal bacteria species differentially affect dendritic cell maturation

First, we investigated whether vaginal microbiota affect DC maturation. DCs were incubated with UV-inactivated vaginal bacteria ranging from species associated with vaginal health (*L. crispatus*) to species associated with BV (*L. iners*, *G. vaginalis, A. vaginae*, *M. elsdenii*, *P. timonensis*) (Fredricks et al., 2005; Ling et al., 2010; Martin et al., 2012; Ravel et al., 2011; Srinivasan et al., 2012; Witkin and Linhares, 2017). DC maturation was determined by analyzing expression of co-stimulatory molecules CD80, CD83, and CD86 by flow cytometry and maturation was normalized to that induced by LPS. At a low stimulation ratio, all bacteria except *L. crispatus* induced expression of CD80, CD83 or CD86 (Fig. 1A–D). Notably, both *M. elsdenii* and *P. timonensis* induced he strongest upregulation of all maturation markers (Fig. 1A–D). With high stimulation ratio, all vaginal microbiota induced expression of maturation markers, whereas *M. elsdenii* and *P. timonensis* induced the highest expression of all maturation markers. Notably, *P. timonensis* induced CD83 about 3-fold higher when compared to LPS (Fig. 1C). TLR4 inhibition partially blocked upregulation of CD80, CD83 and CD86 by *P. timonensis* and *M. elsdenii* (sup. Fig. 3), suggesting that both bacteria trigger TLR4. Thus, all vaginal bacteria induced DC maturation. However, *M. elsdenii* and *P. timonensis*, which are hallmark bacteria of BV, induced the most enhanced DC maturation.

3.2. Megasphaera elsdenii and Prevotella timonensis induce pro-inflammatory cytokine production

Next, we investigated the production of pro-inflammatory cytokines by DCs exposed to vaginal bacteria. DCs were stimulated at a 1:10 ratio and cytokines were measured at mRNA and protein level. Both *P. timonensis* and *M. elsdenii* induced the highest expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-12p40, and TNFa at mRNA as well as on protein level (Figs. 2, 3 and sup. Fig. 1). TLR4 inhibition blocked *CXCL8* expression for both bacteria (sup. Fig. 3), suggesting that TLR4 induced these cytokines after engagement with *P. timonensis* and *M. elsdenii*. Notably, even though most microbiota induced *IL1B* mRNA (Fig. 2A) only *M. elsdenii* and *P. timonsis* induced secretion of IL-1β protein (Fig. 3A), suggesting that these bacteria not only induce *IL1B* mRNA transcription but also trigger processing of pro-IL-1β. Additionally, *L. iners*, *G. vaginalis,* and *A. vaginae* induced moderate mRNA and protein levels of IL-6 and IL-8 and *L. iners* also induced IL-10 and IL-12p40 (Figs. 2B–F and 3 B–E). The aerobic commensal *L. crispatus* did not induce any cytokine expression nor production significantly (Figs. 2, 3 and sup. Fig. 1). These data strongly suggest that *M. elsdenii* and *P. timonensis* are potent inducers of pro-inflammatory cytokines that affect both innate and adaptive immunity.

3.3. Prevotella timonensis skews DC-mediated T helper differentiation towards Th1 polarization

Next, we investigated whether vaginal bacteria induced specific Th differentiation. Therefore, DCs exposed to different vaginal bacteria were co-cultured with naïve CD4 T cells and the induction of Th cell responses was determined by intracellular staining for IFNγ (Th1) and IL-4 (Th2). As controls, DCs were stimulated with Th1 and Th2 inducing reagents LPS/IFNy and LPS/PGE2, respectively. Although both *P. timonensis* and *M. elsdenii* induced strong pro-inflammatory cytokines, only *P. timonensis* induced Th1 skewing and inhibited Th2 skewing similar to Th1 inducing stimuli LPS and IFNγ, whereas both *L. crispatus* and *M. elsdenii* induced a mixed Th1/ Th2 response similar to LPS alone (Fig. 4A–B). An increase of all maturation markers was observed upon stimulation with all control stimuli, *M. elsdenii* and *P. timonensis* while expression levels remained unchanged upon stimulation with *L. crispatus* (sup Fig. 2A–C). Thus, our data demonstrate that *P. timonensis* is a potent inducer of Th1 differentiation.

4. Discussion

Dysbiosis of the vaginal microbiome is associated with increased susceptibility to STIs and adverse pregnancy outcomes presumably caused by bacteria-specific induced inflammation in the genital tract (Anahtar et al., 2015; Cohen et al., 1995; Donders et al., 2009; Hay et al., 1994; Kaul et al., 2007; King et al., 2011; Llahí-Camp et al., 1996; Ralph et al., 1999; Spandorfer et al., 2001; Wiesenfeld et al., 2003). However, little is known about the cellular mechanisms underlying genital inflammation caused by specific members of the vaginal microbiome. Here we have investigated the influence of commensal aerobic bacteria and anaerobes associated with vaginal dysbiosis on DC-induced immunity. We investigated the effect of the different bacterial species on DC maturation and activation, production of cytokines and priming of Th cell responses. Our data strongly suggest that *M. elsdenii* and *P. timonensis* are potent inducers of DC-mediated genital inflammation. Both *M. elsdenii* and *P. timonensis* are strongly associated with BV and induced strong maturation and pro-inflammatory cytokines responses in DCs. Additionally, *P. timonensis* induced Th1 polarization. This is in contrast with the commensal aerobe *L. crispatus* which did not induce an inflammatory response in DCs or Th skewing. These data indicate that specific vaginal bacteria are potent inducers of inflammatory responses and Th1 polarization.

Upregulation of maturation markers CD80, CD83, and CD86 by DCs is essential in directing T-cell-mediated immune responses. Our data demonstrate that vaginal microbiota induce DC maturation in a dose-dependent and bacteria specific manner where commensal bacteria induce no to a moderate maturation whereas bacteria associated with BV induce high expression of CD80, CD83, and CD86 on DCs. To mount a full adaptive immune response the upregulation of maturation markers must be accompanied by the secretion of distinct cytokine profiles. Accordingly, we observed large differences in the production of IL-1β, IL-6, IL-8, Il-12, and TNFα by DCs stimulated with the different bacteria with *M. elsdenii* and *P. timonensis* being the most potent inducers for all these cytokines. Our data show that most of the tested bacteria induce some levels of *IL1B* mRNA but only *M. elsdennii* and *P.timonensis* induce high levels of *IL1B* mRNA as well as secretion of IL-1β. This is an important observation as IL-1β expression and secretion is carefully controlled and high IL-1β levels cause strong inflammation with detrimental effects (Netea et al., 2010). As secretion of IL-1β requires the

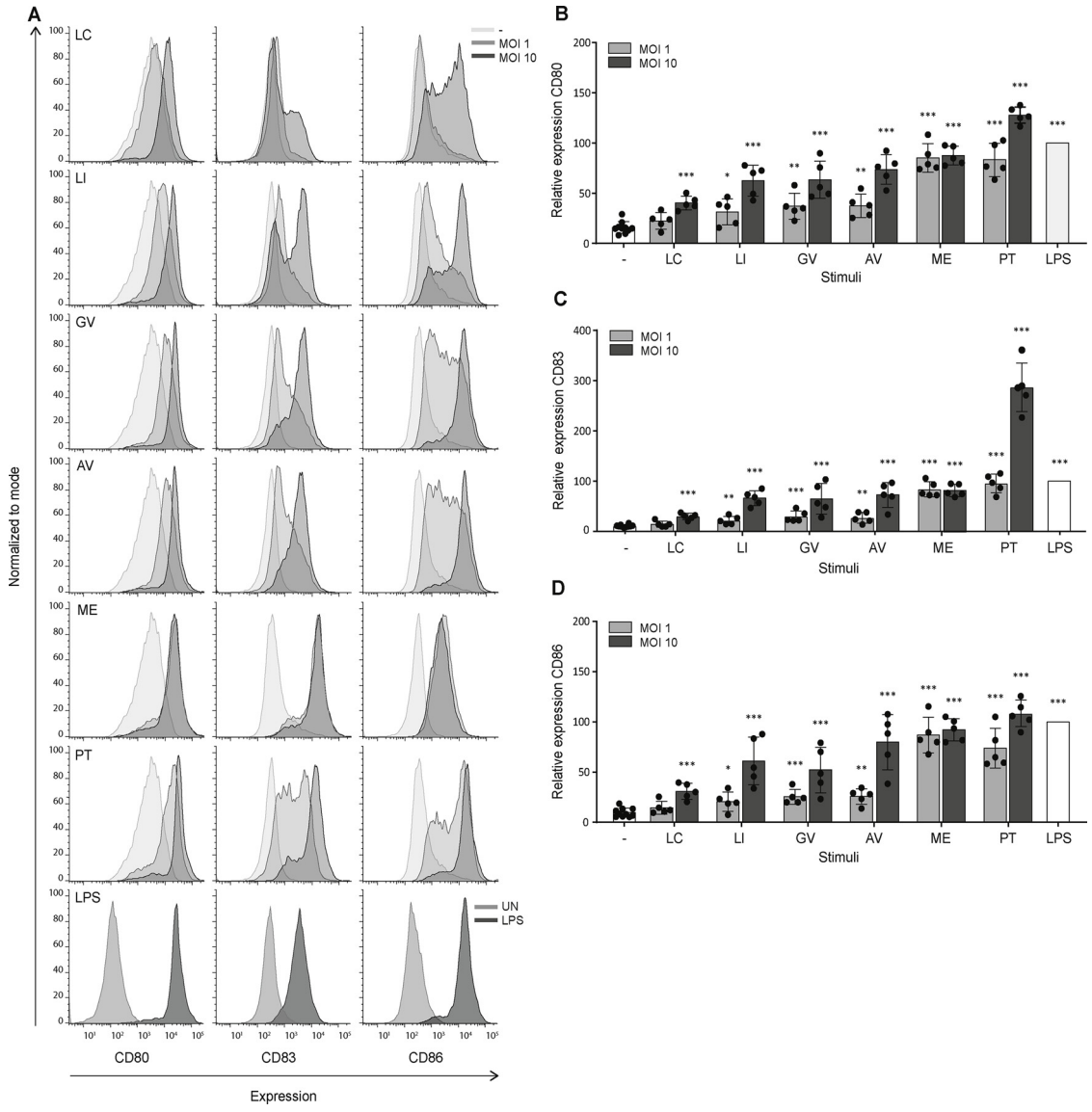

Fig. 1. *Megasphaera elsdenii*and *Prevotella timonensis*induce strong DC maturation.
(A–D) DCs were stimulated with selected vaginal microbiota including *Lactobacillus crispatus* (LC), *Lactobacillus iners* (LI), *Gardnerella vaginalis* (GV), *Atopobium vaginae* (AV), *Megasphaera elsdenii* (ME), and *Prevotella timonensis* (PT) at ratio 1:1 or ratio 1:10, LPS, or left untreated (-) for 24h. Expression of CD80, CD83, and CD86 was measured by flow cytometry. (A) Expression of markers for one representative donor. Lower plots show an unstained control (UN) and LPS control. (B–D) Relative expression based on mean fluorescent intensity of CD80 (B), CD83 (C), and CD86 (D) normalized to LPS-stimulated DCs and set to 100 % for each donor. The graphs represent collated data (mean ± SD) from five donors for ratio 1:1(MOI 1; light grey) and ratio 1:10 (MOI 10; dark grey). An unpaired Mann-Whitney *U* test was performed to test statistical significance to NS (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

processing of pro-IL-1β by caspases upon inflammasome activation (Netea et al., 2010), our data suggest that in particular *M. elsdennii* and *P. timonensis* are able to induce inflammasome activation. Additionally, the production of IL-1β in combination with TNFα further enhance the inflammatory response in an autocrine and paracrine fashion (Blanco et al., 2008). These data underscore the possible contribution of *M. elsdenii* and *P. timonensis* to BV-associated inflammation in the vaginal mucosa.

Several studies have linked BV with an increased production of pro-inflammatory cytokines IL-1β, IL-6, IL-8, Il-12, and TNFα in the vaginal microenvironment (Jespers et al., 2017). Production of pro-inflammatory cytokines IL-1β and IL-8 is associated with increased risk for HIV acquisition caused by an impaired barrier function and influx of CD4+ HIV target cells (Arnold et al., 2016; Masson et al., 2015; Rebbapragada et al., 2008; Shen et al., 2011). Moreover, increased amounts of TNFα is also directly linked to enhanced HIV-1 replication and increased viral dissemination (De Jong et al., 2008). Our data show that *M. elsdenii* and *P. timonensis*-stimulated DCs secrete significant amounts of IL-1β, IL-8 and TNFα and therefore suggest that *M. elsdenii* and *P. timonensis* have the potential to increase HIV acquisition via induction of these cytokines. Recent studies have shown that fluids and small particles are able to move between the vaginal tract and the uterine cavities (Zervomanolakis et al., 2007). Therefore it is very likely that inflammatory mediators could also translocate from the upper vaginal tract to the uterus. Production of pro-inflammatory cytokines also plays an important role in mediating adverse pregnancy outcomes as elevated levels of cytokines IL-1β, IL-6 and IL-8 at the maternal interface are shown to be predictive for spontaneous preterm delivery (Bosquet et al., 2005; Torbé and Czajka, 2004). As both *M. elsdenii* and *P. timonensis* are capable of inducing IL-1β, IL-6, and IL-8 production, we hypothesize that the presence of these bacteria might contribute to pre-term labour.

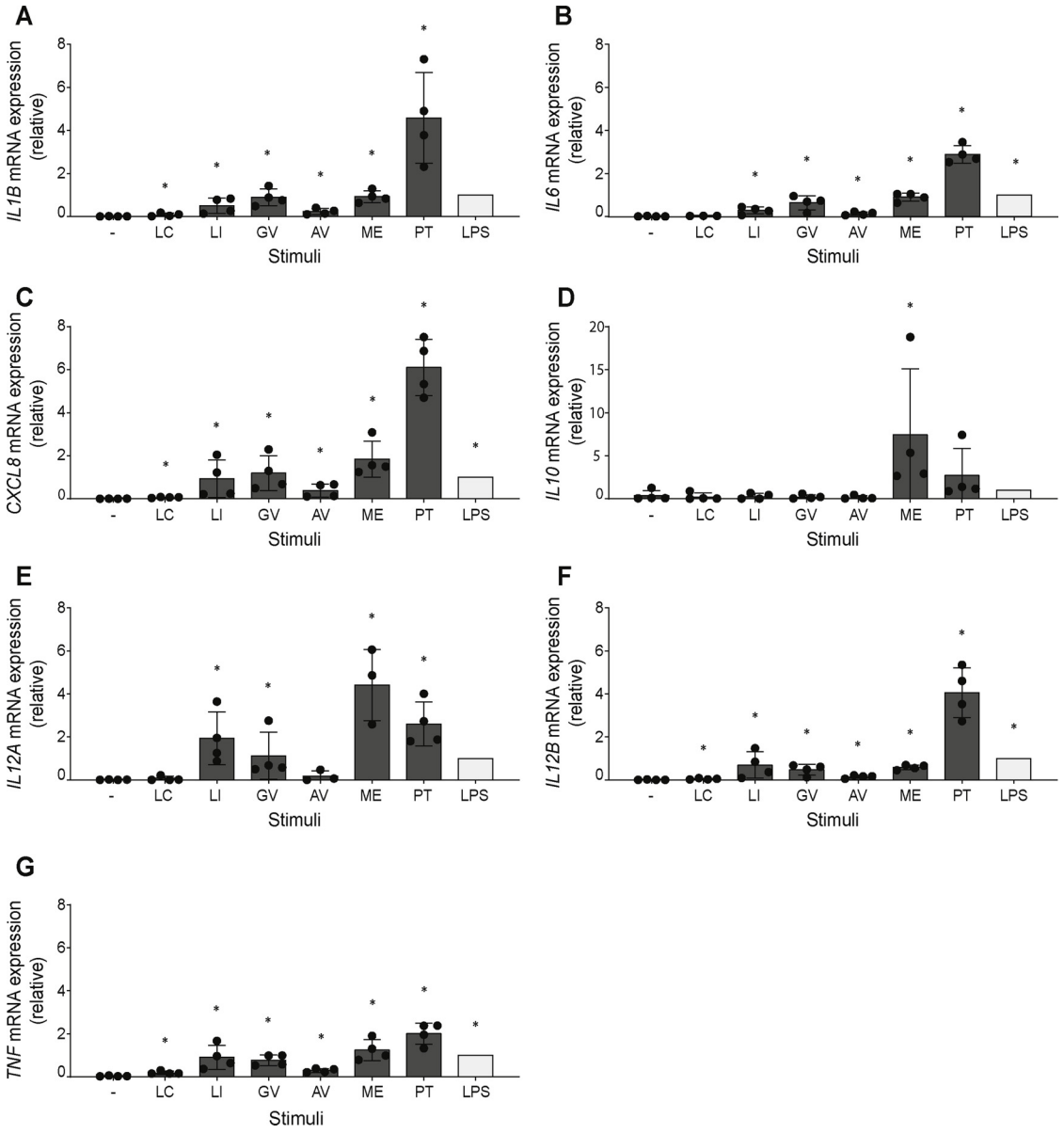

Fig. 2. *Megasphaera elsdenii*and *Prevotella timonensis*induce mRNA levels of inflammatory cytokines.
DCs were stimulated with selected vaginal microbiota as described for Fig. 1 at ratio 1:10, LPS, or left untreated (-) for 6h. mRNA expression of *IL1B* (A), *IL6* (B), *CXCL8* (C), *IL10* (D), *IL12A* (E), *IL12B* (F) and *TNFα* (G) was determined via quantitative PCR. Ct values of target genes were normalized to GAPDH and set at 1 in LPS stimulated DCs for each donor. Graphs represent collated data (mean ± SD) from four donors. A unpaired Mann-Whitney *U* test was performed to test statistical significance to untreated (*$p < 0.05$, ** $p < 0.01$,*** $p < 0.001$, **** $p < 0.0001$).

Both co-stimulation by CD80, CD83 and CD86, and production of distinct cytokine profiles are crucial in the skewing of specific Th subsets. CD80, CD83, and CD86 are important for T cell activation and differentiation as CD80 and CD86 are co-stimulatory molecules that provide co-stimulatory signals necessary for T cell priming while CD83 is important for T cell survival (Freeman et al., 1993; Peach et al., 1995). The production of distinct cytokines profiles by DCs skews naïve T cells either to a Th1 or Th2 polarization. IL-6 and IL-12, are critical for skewing Th1 differentiation while Th2 polarization is mediated by a decrease in IL-6 and IL-12, and an increase in IL-10 (Gringhuis et al., 2009). The induction of DC maturation and pro-inflammatory cytokines by *M. elsdenii* and *P. timonensis* suggests that these anaerobes might be important factors in genital inflammation and Th cell differentiation. Here, we show that exposure of DCs to *P. timonensis* induce a strong Th1 polarization in contrast to *L. crispatus* exposed DCs which do not induce T cell skewing. As other *lactobacilli spp*. have been shown to induce Th1, our data suggest that different *lactobacilli* spp. might have distinct effects (Mohamadzadeh et al., 2005). The Th1 response after *P. timonensis* exposure is consistent with the increased expression of CD80, CD83 and CD86 and the increased cytokine production of IL-6 and IL-12 we observe for this bacterium. The lack of Th1 polarization in *M. elsdenii* may be explained by the fact that the expression of maturation markers CD80 and CD83 is lower in *M. elsdenii* stimulated DCs compared to *P. timonensis* stimulated DCs. Additionally, production of IL-6 and Il-12p40 induced by *M. elsdenii* is lower compared to *P. timonensis* while IL-10 levels remain similar for both *M. elsdenii* and *P. timonensis.*

A Th1 response is associated with an inflammatory phenotype caused by the production of pro-inflammatory mediators like IFNγ, IL-2, and TNFα while a Th2 response characterized be the production of IL-4 and IL-10 (Zhu et al., 2010). Especially in pregnancy the Th1/Th2 dichotomy is of great importance. Normally, the production of IL-4 and IL-10 by Th2 cells at the maternal fetal interface is associated with

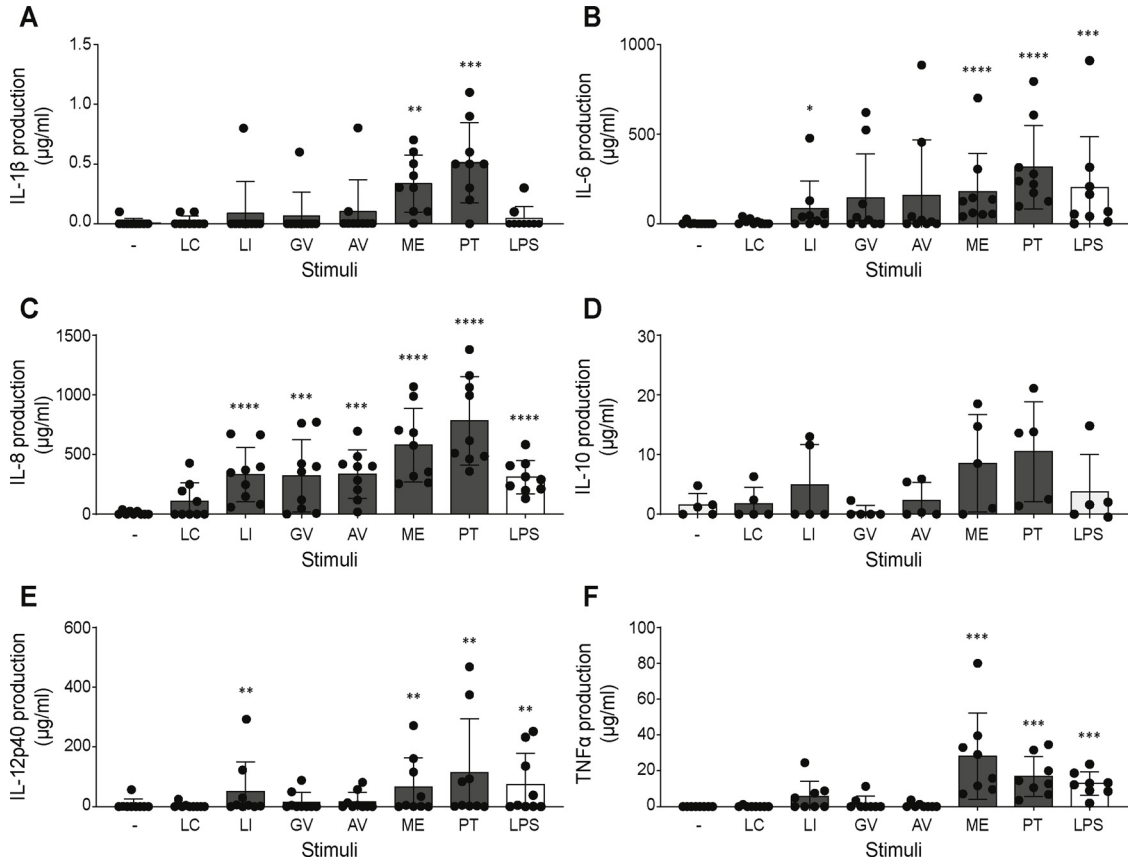

Fig. 3. *Megasphaera elsdenii*and *Prevotella timonensis*induce inflammatory cytokines.
DCs were stimulated with selected vaginal microbiota as described for Fig. 1 at ratio 1:10, LPS, or left untreated (-) for 24h. Production of IL-1β (A), IL-6 (B), IL-8 (C), IL-10 (D), IL-12p40 (E) and TNFα (F) in µg/ml was determined by ELISA. Graphs show collated data (mean ± SD) from nine donors (IL-1β, IL-6, IL-8, IL-12p40 and TNFα) or five donors (IL-10). A unpaired Mann-Whitney *U* test was performed to test statistical significance to NS (*p < 0.05, ** p < 0.01,*** p < 0.001, **** p < 0.0001).

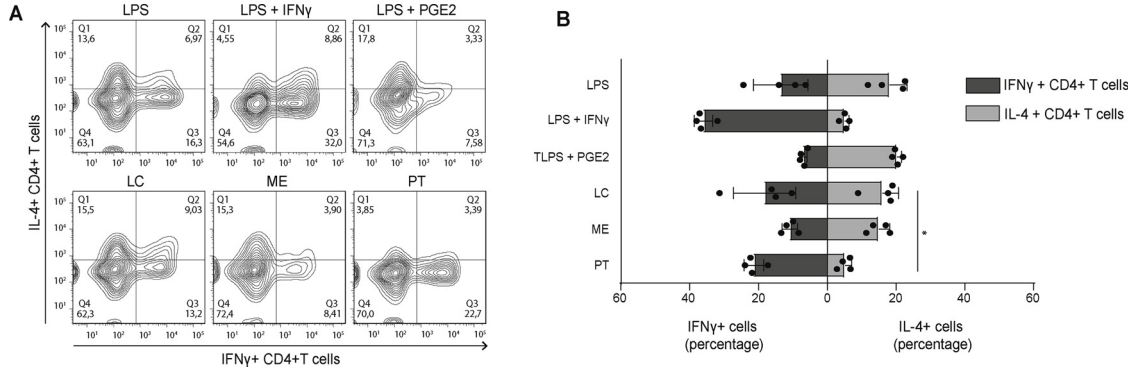

Fig. 4. *Prevotella timonensis*induces DC-mediated T helper 1 skewing.
DCs were stimulated with LPS alone or in combination with IFNγ or PGE2, vaginal bacteria *Lactobacillus crispatus* (LC), *Megasphaera elsdenii* (ME), or *Prevotella timonensis* (PT) at ratio1:10 for 24h and co-cultured with naïve T cells. After co-culture of exposed DCs with naïve CD4+ T cells the percentage IFNγ (dark grey) or IL-4 (light grey) positive CD4+ T cells was determined by flow cytometry. Data show a representative T cell donor (A) or are collated data (mean ± SD) from four possible DC/T cell donor combinations (B). A unpaired Mann-Whitney *U* test was performed to test statistical significance to LC (*p < 0.05, ** p < 0.01,*** p < 0.001, **** p < 0.0001).

successful pregnancy as the Th2 response is necessary to protect the fetus from a potentially harmful immune response elicited by the mother (Piccinni, 2010). Unexplained pregnancy loss is associated with reduced production of IL-4 and IL-10 by Th2 cells caused by an early switch during the pregnancy towards a Th1 cytokine response including IFNγ, TNFα but also IL-1β (Piccinni et al., 1998). Therefore, the skewing of Th1 cells by DC stimulated *P. timonensis* might be causative for the induction of pre-term labor. Moreover, an increased Th1 environment could also facilitate growth of other anaerobic bacteria thereby enhancing inflammation of the genital tract.

Differential upregulation of costimulatory molecules and production of inflammatory cytokines between healthy and unhealthy microbiota might be explained by distinct differences in the cell wall of these bacteria. *L. crispatus*, *L. iners*, and *A. vaginae* are gram positive while *G. vaginalis* can appear as gram-positive or variable displaying a cell wall that contains only very low concentration of LPS (Catlin, 1992; Sadhu

et al., 1989). In contrast, *M. elsdenii* and *P. timonensis* are purely gram negative bacteria expressing LPS (Marx et al., 2011; Roux et al., 2015). Indeed, our data strongly suggest that both *M. elsdenii* and *P. timonensis* induce DC maturation and cytokines via TLR4 activation.

Our data demonstrate that the interaction between DCs and anaerobic vaginal bacteria *M. elsdenii* and *P. timonensis* induces inflammatory processes. BV-associated bacteria have the ability to adherence to epithelial cells and some BV-associated spp. are able to form biofilms. These mechanisms facilitate the contact between bacteria and epithelial cells as well as DCs. Additionally, DCs have been shown to send dendrites through epithelial layers and interact with invading bacteria in (sub)mucosal layers (Rescigno, 2003; Rescigno et al., 2001), which allows sensing of the bacteria. Here we have used inactivated bacteria to investigate the first interaction between DCs and bacteria and our data strongly suggest that the cell-wall components of the anaerobic *P. timonensis* and *M. elsdenii* induce strong immune responses via DCs.

Overall, this study has identified *M. elsdenii* and *P. timonensis* as inflammatory commensals or "pathobionts" that can induce DC-mediated inflammatory processes like increased pro-inflammatory cytokine production. In addition, *P. timonensis* stimulation of DCs lead Th1 polarization that might result in more generalized inflammation of the female genital tract. Therefore, this study provides a DC-dependent mechanism by which vaginal dysbiosis can lead to genital inflammation resulting in increased susceptibility to STI acquisition and adverse pregnancy outcomes. Therapeutic interventions preventing *M. elsdenii* and *P. timonensis* colonization could reduce genital inflammation and therefore prevent the onset of associated afflictions.

Author contributions

NHvT and LCH designed, performed and interpreted most experiments and prepared the manuscript. EMZW performed and analyzed some experiments. JLvH performed and analyzed some experiments. KS provided *Lactobacillus crispatus*, *Lactobacillus iners*, *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera elsdenii* and *Prevotella timonensis*. KS, CMSR, EZW, JLvH and THBG participated in discussion of the data and writing the manuscript. TBHG supervised all aspects of the project.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

This work was supported by the AMC PhD Scholarship, Dutch Research Council (NWO-ZonMw) VIDI grant 91718331 and the European Research Council (ERC) Advanced Grant 670424. We thank Cees van 't Veer for providing the TLR4 inhibitors.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2020.103085.

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