

Nonpathogenic Bacteria Alleviating Atopic Dermatitis Inflammation Induce IL-10-Producing Dendritic Cells and Regulatory Tr1 Cells

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The beneficial effects of nonpathogenic bacteria are increasingly being recognized. We reported in a placebo-controlled study with atopic dermatitis (AD) patients that cutaneous exposure to lysates of nonpathogenic bacteria alleviates skin inflammation. To now unravel underlying mechanisms, immune consequences of sensing nonpathogenic bacterium *Vitreoscilla filiformis* lysate (Vf) were characterized analyzing (1) differentiation of dendritic cells (DCs) and, consecutively, (2) effector functions of DCs and T helper (Th) cells *in vitro* and in a murine model of AD in NC/Nga mice *in vivo*. Topical treatment with Vf significantly reduced AD-like inflammation in NC/Nga mice. Importantly, cutaneous exposure to Vf in combination with the allergen FITC significantly also reduced subsequent allergen-induced dermatitis indicating active immune modulation. Indeed, innate sensing of Vf predominantly induced IL-10-producing DCs, which was dependent on Toll-like receptor 2 (TLR2) activation. Vf-induced IL-10⁺ DCs primed naive CD4⁺ T helper cells to become regulatory IFN- γ ^{low} IL-10^{high} Tr1 (type 1 regulatory T) cells. These IL-10^{high} Tr1 cells were also induced by Vf *in vivo* and strongly suppressed T effector cells and inflammation. In conclusion, we show that innate sensing of nonpathogenic bacteria by TLR2 induces tolerogenic DCs and regulatory Tr1 cells suppressing T effector cells and cutaneous inflammation. These findings indicate a promising therapeutic strategy for inflammatory skin diseases like AD.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with increasing prevalence rates, affecting up to 10–20% of the children in western countries (Bieber, 2008; Boguniewicz and Leung, 2010). Although the detailed mechanisms underlying inflammation of AD skin are not fully understood, a defect in skin barrier function as well as an immune dysbalance play a crucial role, leading to T helper cell type 2 (Th2)-biased immune responses (Palmer *et al.*, 2006; Bieber, 2008; Irvine *et al.*, 2011). Increased hygiene standards, less infectious diseases, and lowered family sizes are claimed to lead to microbial deprivation during early years of life that facilitate misdirected effector immune responses rather than the induction of immune tolerance (“hygiene hypothesis”),

contributing to increase and development of allergies and atopic diseases (Strachan, 1989; Bach, 2002; von Mutius and Vercelli, 2010). Surface organs like the skin are constantly colonized with bacteria in the absence of detectable inflammation, but the mechanisms that inhibit inflammation or even induce tolerance to the local microbiota are still enigmatic (Grice *et al.*, 2009). A reduced genetic diversity of Gram-negative gammaproteobacteria in the environment of atopic individuals was observed that profoundly influenced the skin microbiota, leading to a decrease in Gram-negative bacterium *Acinetobacter* and the anti-inflammatory cytokine IL-10, demonstrating that resident microbes shape cutaneous immune homeostasis (Hanski *et al.*, 2012). These data ideally complement analyses in mouse models that first demonstrated the potential of Gram-negative bacterium *Acinetobacter* to prevent allergies (Debarry *et al.*, 2007; Conrad *et al.*, 2009). In contrast to prevention of allergic sensitizations, most therapeutic strategies reporting the oral use of nonpathogenic or the so-called “probiotic” bacteria failed to show significant effects in the treatment of AD (Lee *et al.*, 2008; Boyle *et al.*, 2009). Recently, we performed a proof-of-concept study in patients demonstrating that immune recognition of the nonpathogenic microbe *Vitreoscilla filiformis* is a promising strategy to treat AD when directly applied onto patients’ skin and not orally (Gueniche *et al.*, 2008). Based on these clinical findings we hypothesized that immune recognition of Gram-negative

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Abbreviations: AD, atopic dermatitis; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; LPS, lipopolysaccharide; Th cell, T helper cell; TLR, Toll-like receptor; Tr1, type 1 regulatory T cell; Vf, *Vitreoscilla filiformis* lysate

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nonpathogenic bacterium *V. filiformis* exploits an important mechanism of microbial immune sensing, finally alleviating inflammation by induction of tolerance.

Pattern recognition receptors such as Toll-like receptors (TLRs) play a key role in detecting “pathogen-associated molecular patterns” (Takeuchi and Akira, 2010; Volz et al., 2010; Volz et al., 2012). Especially within surface organs, dendritic cells (DCs) are equipped with numerous pattern recognition receptors and act as sentinels to sense microbes, leading to DC maturation and cytokine production (Reis e Sousa, 2004; Joffre et al., 2009). Activated DCs are the most potent directors of immune phenotypes in T cells, determining T-cell polarization to the different Th subtypes. During this process of Th cell differentiation, DC cytokines are most important possibly also shaping regulatory T cells (Kapsenberg, 2003; Bettelli et al., 2008; Volz et al., 2012). Intriguing studies demonstrated that these pathways are also critical for induction of tolerance to the microbiota (Round and Mazmanian, 2010; Geuking et al., 2011).

Given the positive results from our proof-of-concept study in AD patients, we investigated the underlying mechanisms. AD-prone NC/Nga mice with Th2-dominated cutaneous hypersensitivity to FITC showed, similar to AD patients, alleviated dermatitis when their skin was exposed to *V. filiformis* signals. Importantly, this therapeutic effect was even more pronounced when the skin was exposed to *V. filiformis* signals previous to allergen challenge, indicating effective immune modulation. Indeed, *V. filiformis* signals induced high levels of IL-10 in DCs via TLR2. These DCs orchestrated the induction of IL-10^{high}, IFN- γ ^{low}-producing Tr1 (type 1 regulatory T) cells. This regulation was also detected in AD mice after cutaneous exposure to Vf with dominant IL-10 production by T cells from skin-draining lymph nodes and consecutively reduced T-cell proliferation and proinflammatory cytokine production.

Thus, immune recognition of the Gram-negative nonpathogenic bacterium *V. filiformis* by DCs induces IL-10-producing DCs and regulatory Tr1 cells. This pathway may generally be functional when discriminating between “pathogenic” and “nonpathogenic” bacteria and could be exploited to alleviate cutaneous inflammation such as in AD.

RESULTS

Exposure to nonpathogenic bacteria attenuates cutaneous inflammation in a murine model of AD

We recently reported effective treatment of AD lesions by topical treatment of *V. filiformis* lysate (Vf) in a double-blind, placebo-controlled clinical trial (Gueniche et al., 2008). Thus, we first asked whether Vf solely suppressed cutaneous inflammation in AD at the time and site of application. To this end, mice of the NC/Nga strain that have been shown to develop AD-like skin lesions and clinical features most closely resembling human AD were investigated (Matsuda et al., 1997). NC/Nga mice sensitized to the allergen FITC clearly developed dermatitis lesions as measured by a strong increase in ear thickness following allergen challenge (Figure 1a). All appropriate controls without sensitization and subsequently challenged with FITC with or without Vf exposure showed ear

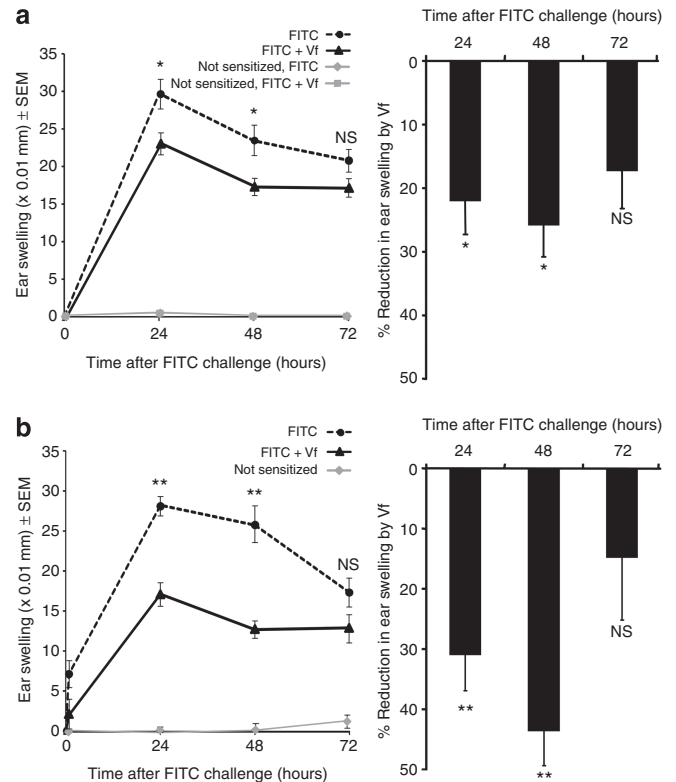


Figure 1. Cutaneous exposure to *Vitreoscilla filiformis* (Vf) attenuates skin inflammation. (a) FITC-sensitized NC/Nga mice showed ear swelling responses peaking at 24 hours after challenge and declining thereafter. Cutaneous exposure of mice to Vf exclusively during challenge significantly reduced ear swelling at 24 and 48 hours. Not sensitized but FITC-challenged mice (gray lines) did not display marked ear swelling responses irrespective of exposure to Vf. Percentage of reduction of ear swelling of FITC-sensitized Vf-exposed mice compared with FITC-challenged mice not receiving Vf is shown at the right. (b) Cutaneous exposure of mice to FITC together with Vf only previous to the final challenge significantly reduced ear swelling 24 and 48 hours after FITC challenge compared with the positive control group. Reduction of ear swelling was up to 50% compared with mice not being exposed to Vf, as depicted in the right panel. One out of two independent experiments is shown, mean $\pm$ SEM, $n = 5$ mice per group. NS, not significant. * $P < 0.05$ and ** $P < 0.01$.

swelling responses that were always < 0.03 mm. Dermatitis was reduced by $> 25\%$ in mice that received topical treatment of ear skin with Vf during FITC challenge, indicating direct anti-inflammatory properties of Vf (Figure 1a). However, these effects remained somewhat limited. Therefore, we next asked whether exposure to Vf could also orchestrate immune modulation or tolerance mediating long-term effects in addition. Therefore, one group of FITC-sensitized mice was exposed to Vf in combination with FITC and the effects of this exposure were determined 1 week later by challenge with the allergen only. Compared with the FITC-sensitized control group, mice previously exposed to Vf together with FITC showed significantly reduced ear swelling after allergen challenge alone ($P < 0.01$), with almost 50% reduction at the peak of skin inflammation (Figure 1b). Importantly, we determined antigen-specific immunoglobulin levels in all mice sensitized to FITC with or without exposure to Vf and

found elevated antigen-specific immunoglobulin levels when compared with naive control mice. We detected no difference in FITC-specific IgG1 and IgG2a levels when comparing Vf exposed with unexposed mice and only a slight reduction in antigen-specific IgE (Supplementary Figure S1 online). Thus, Vf attenuated cutaneous T cell-mediated inflammation in a murine model of AD by modes of immune modulation, indicating that innate sensing of nonpathogenic *V. filiformis* regulates even already established adaptive immunity.

Signals from nonpathogenic bacterium *V. filiformis* induce maturation of human and murine DCs

Immune modulation or tolerance can be induced by immature or semimature DCs not fully activated. To investigate DC activation, murine bone marrow-derived dendritic cells (BMDCs) and human monocyte-derived dendritic cells were stimulated with either lipopolysaccharide (LPS) as positive

control or Vf for 24–48 hours. DC maturation was assessed by FACS analysis.

Both Vf and LPS unequivocally induced maturation of BMDCs as detected by upregulation of maturation markers such as CD80, CD86, CD83, and major histocompatibility complex class II as compared with untreated cells (Figure 2a). After activation of human monocyte-derived dendritic cells with Vf or LPS, CD83, the most reliable surface marker for detection of human DC maturation, was also clearly upregulated (Supplementary Figure S2a online). This shows that signals of *V. filiformis* orchestrate the development of phenotypically mature DCs as determined by FACS analysis.

Signals from nonpathogenic bacterium *V. filiformis* orchestrate the induction of IL-10^{high} DCs

Next we analyzed cytokine production of DCs after activation with either Vf or LPS. As expected, LPS induced high amounts

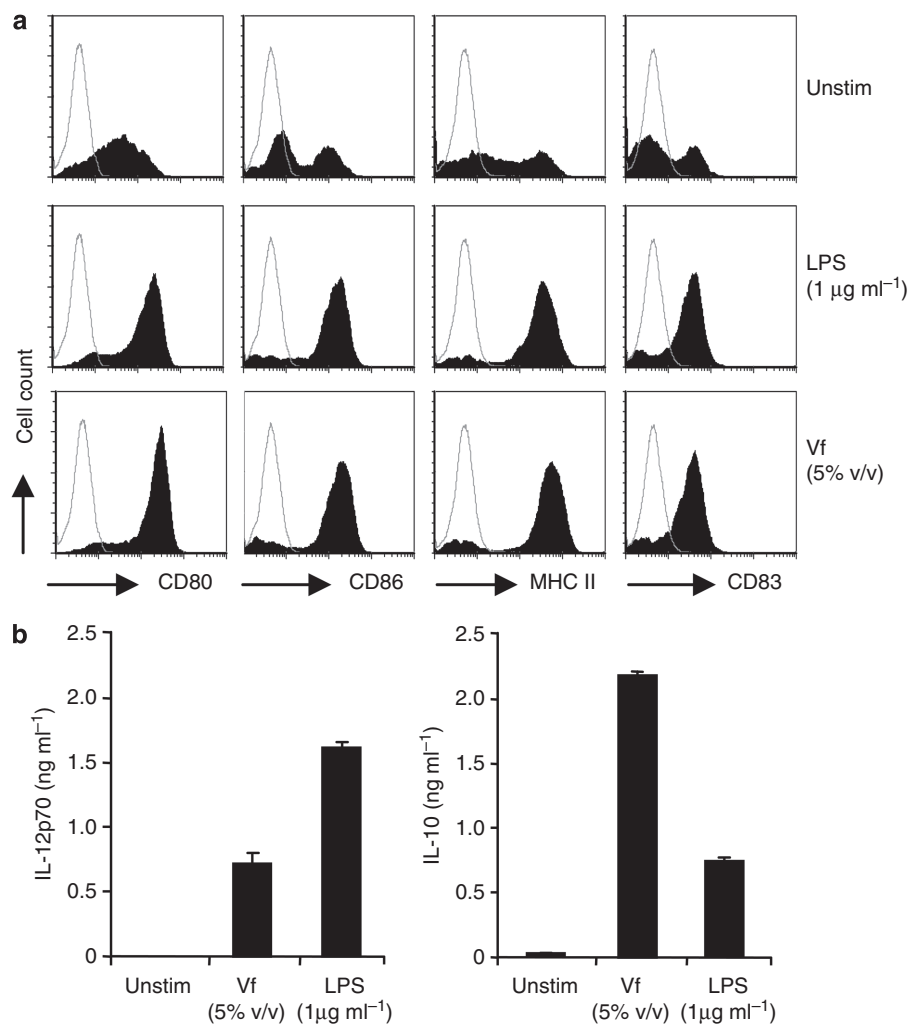


Figure 2. Signals of *Vitreoscilla filiformis* (Vf) predominantly induce IL-10-producing mature dendritic cells (DCs). DCs were exposed to Vf or lipopolysaccharide (LPS). (a) Vf and LPS unequivocally stimulated murine bone marrow-derived dendritic cells (BMDCs) to upregulate CD80, CD86, major histocompatibility complex class II (MHC II), and CD83 (filled area) compared with untreated cells indicating full DC maturation. Gray lines indicate isotype controls. Unstim, unstimulated. (b) BMDCs activated with Vf produced high levels of IL-10 but only low amounts of IL-12p70, characteristic for IL-10⁺ DCs. In contrast and, as expected, LPS induced high IL-12p70 but only low IL-10 levels in BMDCs. Representative data from one out of four independent experiments are shown.

of IL-12p70 and low levels of IL-10. In sharp contrast, stimulation with Vf led to a DC cytokine profile dominated by the anti-inflammatory cytokine IL-10, whereas IL-12p70 levels remained low for all *V. filiformis* strains investigated (Figure 2b and Supplementary Figure S3 online). This IL-10 production induced by Vf was dose dependent in both human and mouse DCs, indicating ligand(s) that trigger pattern recognition receptors (Supplementary Figure S2b online). Moreover, investigating numerous synthetic and bacterial-derived TLR2 ligands, Vf was always superior in the induction of IL-10 in DCs (Supplementary Figure S4 online). These data demonstrate that innate immune sensing of nonpathogenic bacteria governs DCs to predominantly produce the anti-inflammatory and potentially tolerogenic cytokine IL-10.

Innate immune pathways for DC activation and IL-10 production following encounter of signals from nonpathogenic bacterium *V. filiformis*

To gain further insight into innate immune pathways activated by Vf, we first investigated WT and *MyD88*^{-/-} DCs. DC

maturation was induced in WT DCs in response to TLR2 ligand Pam2Cys (data not shown), TLR4 ligand LPS, and Vf as detected by upregulation of major histocompatibility complex class II, CD80, CD83, and CD86 (Figure 3a). In contrast, but as expected, Pam2Cys was unable to induce maturation of *MyD88*^{-/-} DCs (data not shown) and DC maturation in response to LPS was partially hampered. Strikingly, lack of *MyD88* did not affect DC maturation in response to Vf, indicating that *MyD88*-independent pathways are at least in part functional for DC maturation induced by nonpathogenic bacteria (Figure 3a).

In sharp contrast to DC maturation, production of IL-10 and IL-12p70 was almost completely abolished in *MyD88*^{-/-} DCs (data not shown). Thus, DC maturation and cytokine production operate via distinct innate immune pathways.

To investigate the pathways that lead to DC cytokine production following contact to nonpathogenic bacteria, the consequences of Vf exposure were investigated in BMDCs lacking either TLR2 or TLR4. Strikingly, IL-10 production was almost completely dependent on TLR2, as IL-10 was

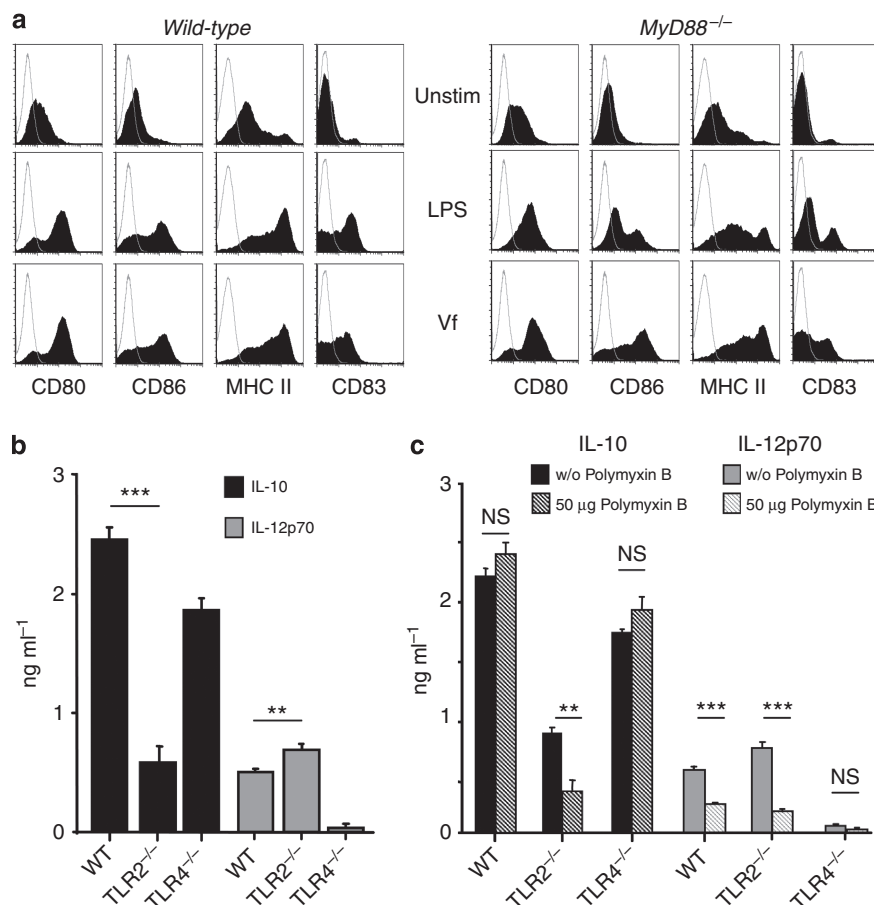


Figure 3. Distinct innate immune pathways control dendritic cell (DC) maturation and cytokine production induced by *Vitreoscilla filiformis* lysate (Vf). (a) Exposure to Vf also induced DC maturation in DCs lacking Toll-like receptor (TLR) adaptor protein *MyD88*, whereas it partially reduced lipopolysaccharide (LPS)-induced DC maturation. (b) Vf-induced IL-10 secretion was significantly reduced in *TLR2*^{-/-} but only marginally affected in *TLR4*^{-/-} DCs. In contrast, IL-12p70 production is almost completely abrogated in *TLR4*^{-/-}. (c) Sustained Vf-induced IL-10 production in wild-type (WT) or *TLR4*^{-/-} DCs following pretreatment with LPS-scavenger Polymyxin B but reduced Vf-induced IL-12p70 production in WT and *TLR2*^{-/-} DCs. Representative data (mean ± SD) from one out of three independent experiments are shown. MHC II, major histocompatibility complex class II; NS, not significant; Unstim, unstimulated. ***P* < 0.01 and ****P* < 0.001.

significantly reduced in TLR2^{-/-} DCs (Figure 3b). This indicates that ligand(s) of TLR2 within Vf is/are responsible for induction of high IL-10 levels. In sharp contrast to TLR2^{-/-} DCs, IL-10 production was only marginally reduced in TLR4^{-/-} DCs (Figure 3b). The low IL-12p70 levels induced by Vf were also further investigated. Lack of TLR2 significantly increased IL-12p70 production, presumably because of the diminished IL-10 levels, whereas the absence of TLR4 abrogated the induction of the proinflammatory cytokine IL-12p70. Thus, we conclude that during DC exposure to Vf, at least two different dominant pathogen associated molecular patterns are functional: TLR2 ligand(s) inducing high IL-10 levels and TLR4 ligand(s) responsible for induction of IL-12p70. The latter is presumably LPS.

To further characterize the nature of the different pathogen associated molecular patterns inducing either IL-10 or IL-12p70 WT, TLR2^{-/-}, and TLR4^{-/-} DCs were activated in the presence or absence of Polymyxin B that neutralizes LPS. Low IL-12p70 levels that were detected in WT and TLR2^{-/-} DCs following exposure to Vf were nearly completely suppressed after preincubation of Vf with Polymyxin B, demonstrating that *V. filiformis* LPS is responsible for IL-12p70 induction (Figure 3c). Importantly, Polymyxin B treatment did not alter IL-10 production in WT, TLR2^{-/-}, and TLR4^{-/-} DCs compared with DCs stimulated with Vf only (Figure 3c). Thus, we conclude that innate immune signals from nonpathogenic bacteria like *V. filiformis* activate TLR2 and induce high IL-10 levels independently from LPS and the TLR4 pathway.

Priming of IL-10-producing CD4⁺ Tr1 cells by Vf-activated DCs

To assess the consequences on the adaptive immune system of the innate immune sensing of Vf, cocultures of DCs with Th cells were set up. First, DCs were activated with different doses of Vf, pulsed with ovalbumin, and subsequently cocultivated with naive CD4⁺ CD62L⁺ OT-II Th cells. Increasing concentrations of Vf in the previous DC culture reduced Th cell proliferation as determined by [³H]-thymidine incorporation in a dose-dependent manner. This indicates direct suppressive effects on Th cells mediated by DCs activated with Vf (Figure 4a), which is in accordance to the observed direct immunosuppressive effect *in vivo* (Figure 1a). To determine immunomodulatory consequences on Th cell polarization, DCs were activated with either LPS or Vf and subsequently cocultivated for priming with Th cells as described before. Primed Th cells were then expanded with IL-2 for 10 days, stimulated, and analyzed for cytokine production. Th cells primed by DCs that were activated with LPS produced high IFN- γ levels and no IL-4, indicating Th1 polarization (Figure 4b). In contrast, IFN- γ levels secreted by Th cells primed with Vf-exposed DCs were markedly reduced compared with LPS-DCs and, again, IL-4 production was undetectable (Figure 4b). Most importantly, Vf-exposed DCs primed Th cells to secrete several fold higher levels of IL-10 compared with controls (Figure 4b). IL-10 production in T cells was dependent on DC-derived IL-10 and TLR2 signaling as both IL-10^{-/-} and TLR2^{-/-} DCs failed to induce high IL-10 levels in T cells (Figure 4c). Thus, innate immune

sensing of Vf induced IL-10^{high}, IFN- γ ^{low} IL-4⁻ Th cells in an IL-10-dependent manner, indicating the induction of Tr1 cells.

Regulatory function of Tr1 cells induced by Vf-exposed DCs

To assess the regulatory function of Tr1 cells primed by Vf-exposed DCs, suppression assays with proliferating CD4⁺ effector T cells were carried out. To this end, effector CD4⁺ Th cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and activated with anti-CD3/28 in the presence of unlabeled Tr1 cells previously primed with Vf-exposed DCs. To balance cell numbers, control experiments were performed by adding unlabeled unpolarized CD4⁺ Th cells to CFSE-labeled CD4⁺ effector Th cells. CFSE dilution was analyzed 72 hours after activation. Although addition of unpolarized Th cells could not alter proliferation of CFSE-labeled effector Th cells, Tr1 cells previously primed with Vf-exposed DCs suppressed Th cell proliferation in a cell number-dependent manner (Figure 4d and e).

Cutaneous exposure to signals of nonpathogenic bacterium

V. filiformis leads to enhanced T-cell IL-10 and inhibits T-cell proliferation *in vivo*

To assess functional consequences of innate immune sensing of nonpathogenic bacterium *V. filiformis* and consecutive shaping of the adaptive immune system *in vivo*, NC/Nga mice were again investigated. As described in Figure 1b, NC/Nga mice were sensitized to FITC and were or were not exposed to Vf. At 1 week after the last application, mice were challenged at the ear skin with FITC alone. Whole lymph node cells from the ear-draining lymph nodes isolated 8 hours after challenge were restimulated with antigen (FITC) or anti-CD3/CD28 antibodies *ex vivo*. Only T cells from mice previously exposed to Vf displayed antigen-specific production of IL-10, whereas control mice failed to do so (Figure 5a). Using anti-CD3/CD28 antibodies, immune modulation and induction of IL-10 production in T cells *in vivo* by previous exposure to *V. filiformis* signals was confirmed (Figure 5b). Consistently, exposure to *V. filiformis* signals reduced T-cell proliferation in draining lymph nodes *in vivo* as measured by [³H]-thymidine uptake *ex vivo* following FITC restimulation (Figure 5c). To analyze the effect of enhanced IL-10 production on effector T-cell responses *in vivo*, IFN- γ , the hallmark cytokine of chronic AD, was analyzed *ex vivo* (Grewe *et al.*, 1995; Biedermann, 2006). Consistent with the reduced T-cell proliferation, antigen-specific IFN- γ production by T cells was significantly reduced in mice previously exposed to *V. filiformis* signals (Figure 5d).

Together, these data show that signals of nonpathogenic bacterium *V. filiformis* induce IL-10^{high} T cells *in vivo* and inhibit antigen-specific T-cell proliferation and cytokine production, thus demonstrating the immunomodulatory role of nonpathogenic innate immune signals *in vivo*.

DISCUSSION

Modulating immune responses at surface organs using nonpathogenic bacteria is a promising strategy to treat inflammatory diseases as reported in clinical trials on inflammatory

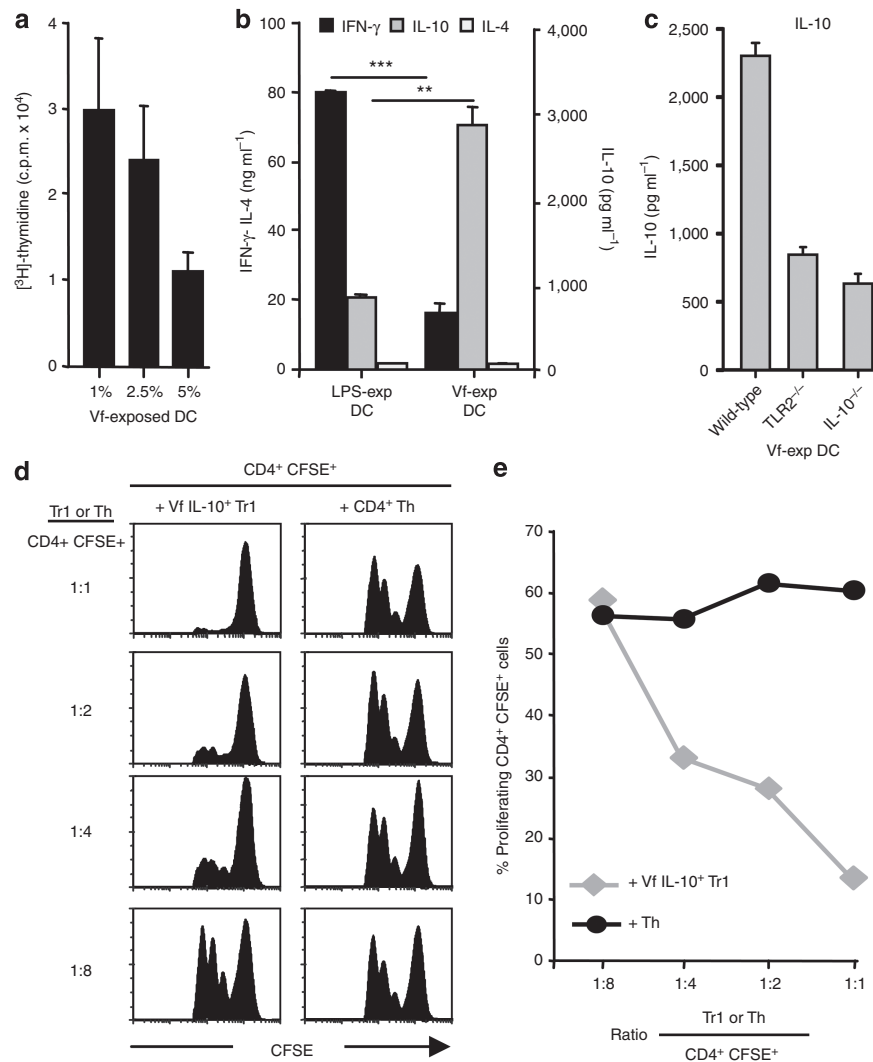


Figure 4. *Vitreoscilla filiformis* (Vf)-exposed dendritic cell (DCs) orchestrate induction of IL-10⁺ Tr1 (type 1 regulatory T) cells effectively suppressing T effector cells. (a) Direct and Vf-mediated dose-dependent reduction of antigen-specific CD4⁺ Th cell proliferation after activation with Vf-exposed DCs. (b) T-cell cytokine profile following previous stimulation with Vf-exposed (Vf-exp) or lipopolysaccharide (LPS)-exposed DCs. Induction of either Tr1 cells producing high IL-10 and low IFN-γ levels or Th1 cells with high IFN-γ and low IL-10 levels, respectively. (c) Vf-exposed IL-10 deficient and Toll-like receptor 2 (TLR2)-deficient DCs failed to induce high IL-10 levels in CD4⁺ T cells. (d) In contrast to T helper (Th) control cells, Vf-induced IL-10^{high} Tr1 cells inhibited proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ T cells in a cell ratio-dependent manner. (e) Quantifying proliferating CFSE⁺ cells, shown at each cell-to-cell ratio. Representative data (mean ± SD) from one out of three independent experiments are shown. ***P* < 0.01 and ****P* < 0.001.

bowel disease (Rembacken *et al.*, 1999). We recently demonstrated that nonpathogenic bacteria are also functional when applied to the skin, alleviating cutaneous inflammation in AD patients (Gueniche *et al.*, 2008). To analyze the underlying mechanism and to elucidate how nonpathogenic bacteria may shape and modulate immune responses, we investigated consequences of innate immune sensing of *V. filiformis* *in vitro* and *in vivo*. NC/Nga mice sensitized to FITC are characterized by a predominance of Th2 cells and high IgE levels and develop AD-like skin inflammation as measured by increase of ear thickness after FITC challenge (Matsuda *et al.*, 1997; Dearman and Kimber, 2000; Matsuoka *et al.*, 2003). Cutaneous treatment of these NC/Nga mice with Vf during elicitation of skin inflammation

significantly decreased allergen-specific dermatitis, indicating direct immunosuppressive effects of Vf. Importantly, cutaneous exposure to Vf and FITC antigen before the final allergen challenge resulted in even more pronounced suppression of cutaneous inflammation demonstrating immunomodulatory properties of Vf. This indicates that cutaneous treatment with Vf in AD patients is effective by direct and immunomodulatory pathways (Gueniche *et al.*, 2008). Indeed, *in vitro* analyses demonstrated that signals of *V. filiformis* induced a DC phenotype dominated by IL-10 already suggesting regulatory and tolerogenic properties (Lutz and Schuler, 2002; Frick *et al.*, 2010). In fact, DCs activated by signals of *V. filiformis* effectively induced regulatory T cells.

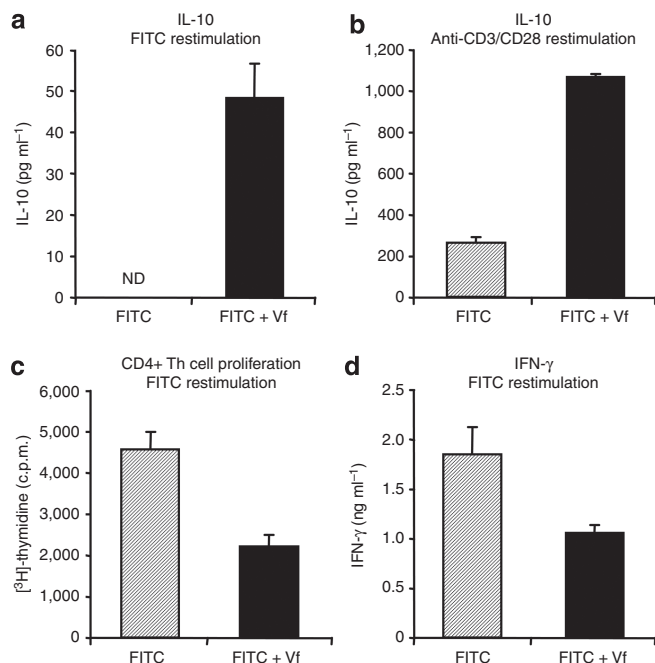


Figure 5. Cutaneous exposure to signals of *Vitreoscilla filiformis* (Vf) enhances T-cell IL-10 and inhibits T-cell proliferation *in vivo*. Induction of antigen-specific IL-10 production in draining lymph nodes was only detected in (a) mice previously exposed to Vf and (b) could be mapped to T helper (Th) cells as detected following anti-CD3/CD28 activation. (c) Vf-mediated immune modulation in previously Vf-exposed mice also reduced antigen-specific T-cell proliferation and (d) IFN- γ production as detected in lymph node cells 72 hours after *in vivo* challenge.

It was known that IL-10 production by DCs contributes to the induction of tolerance in various settings (Akbari *et al.*, 2001; Corinti *et al.*, 2001; Yu *et al.*, 2009). However, we demonstrate according to our knowledge the previously unreported finding that nonpathogenic Gram-negative bacteria induce this tolerogenic DC phenotype characterized by acquisition of a mature phenotype and a cytokine profile dominated by the production of IL-10, and Tr1 induction. The Gram-negative pathogen *Bordetella pertussis* has been shown to exploit a similar strategy to subvert host immunity and protective immune responses by induction of IL-10 and Tr1 cells (McGuirk *et al.*, 2002). In accordance with previously published results, we show that TLR adaptor molecule MyD88 is required for induction of both IL-10 and IL-12p70 production (Boonstra *et al.*, 2006). Surprisingly, however, DC maturation was independent of MyD88, indicating activation of other innate immune pathways (Medzhitov, 2009; Takeuchi and Akira, 2010). The dependence of IL-10 production on TLR2 confirms and extends data from previous reports demonstrating a dominant role for this pattern recognition receptor in the induction of IL-10 in DCs (Dillon *et al.*, 2006; Depaolo *et al.*, 2008). Lipoproteins have been isolated and identified from Gram-negative *E. coli* initially and lipopeptides derived thereof have been shown to activate TLR2 (Braun and Wu, 1994; Buwitt-Beckmann *et al.*, 2005). TLR2 activation by bacterial or synthetic ligands can result in either inflammatory or tolerogenic immune responses, but

detailed mechanisms are still to be deciphered (Oliveira-Nascimento *et al.*, 2012). Lipoproteins from *Staphylococcus aureus* binding to TLR2 have been shown to be crucial in induction of inflammation and clearance of bacteria (Schmalzer *et al.*, 2009). In contrast, host immunity is subverted by *Yersinia pestis* inducing Tr1 cells triggered by TLR2/6 binding of LcrV subsequently inducing IL-10 production (Depaolo *et al.*, 2008). Porin B from *Neisseria meningitidis* is also a TLR2 ligand inducing DC activation (Singleton *et al.*, 2005). As both *V. filiformis* and *N. meningitidis* are Gram-negative bacteria belonging to the family of Neisseriaceae, it is tempting to speculate that the TLR2-activating ligand of *V. filiformis* is a bacterial porin with predominant anti-inflammatory properties (Strohl, 2005). In contrast to Gram-negative pathogenic bacteria such as *Salmonella* spp. eliciting proinflammatory immune responses, innate immune sensing of nonpathogenic Gram-negative bacteria like *V. filiformis* is not dominated by TLR4 signaling over TLR2 signaling and induction of inflammation, but is characterized by a more dominant TLR2 signaling and the induction of tolerance. One may speculate that the functional dominance of tolerogenic signals may be a general principle of how nonpathogenic bacteria and their hosts organize their coexistence in the absence of inflammation. Previously, it has been shown that the exposure to Gram-negative gammaproteobacterium *Acinetobacter* prevents allergic disease in mouse models and correlates with IL-10 production from healthy but not from AD individuals (Debarry *et al.*, 2007; Conrad *et al.*, 2009; von Mutius and Vercelli, 2010; Hanski *et al.*, 2012). This already demonstrates that contact to Gram-negative nonpathogenic bacteria leads to active immune recognition resulting in tolerogenic cytokine production (Hanski *et al.*, 2012). Strikingly, atopic individuals harbor significantly lower amounts of *Acinetobacter* on the skin and show diminished IL-10 production (Hanski *et al.*, 2012). In light of these findings and our data, the pathways utilized by nonpathogenic Gram-negative bacteria to induce tolerance seem to be promising targets for therapeutic strategies.

DCs have been shown to play a central role in transmitting innate immune signals into various types of adaptive immune responses (Kapsenberg, 2003; Joffre *et al.*, 2009). "Tolerogenic DCs" can prime Th cells to become regulatory T cells. Among these, Tr1 cells are characterized by the production of low amounts of IFN- γ but high levels of IL-10 (Groux *et al.*, 1997; O'Garra and Vieira, 2004; Shevach, 2006). Moreover, it has been reported that immune modulation by Tr1 cells reduces antigen-specific IgE but not antigen-specific IgG1 and IgG2a levels, which is similar to our findings following exposure to Vf (Cottrez *et al.*, 2000), but in contrast to, e.g., low-zone tolerance that has been reported to tremendously reduce specific immunoglobulin levels (Steinbrink *et al.*, 1996).

We could demonstrate that DCs activated by signals of nonpathogenic *V. filiformis* are potent inducers of Tr1 cells producing these signature cytokines. Moreover, these Tr1 cells strongly inhibited Th cell responses demonstrating functionality. This is in accordance with previously published results demonstrating that Tr1 cells are equally potent to FoxP3^{pos} natural regulatory T cells in controlling effector T-cell

responses (Vieira *et al.*, 2004). However, in contrast to FoxP3^{POS} natural regulatory T cells, Tr1 cells are inducible, indicating a feasible therapeutic strategy.

Identifying nonpathogenic bacteria with tolerogenic potential, crucial active microbial components within these bacteria, and activation pathways mediating active tolerance—such as TLR2 signaling—points to very promising therapeutic strategies in the treatment of inflammatory and allergic diseases, especially of the surface organs such as AD.

MATERIALS AND METHODS

FITC induced antigen-specific contact hypersensitivity

NC/Nga mice (5 mice per group) were sensitized by administration of 0.25% FITC solution (dissolved in 1:1 acetone/dibutyl phthalate) on the shaved abdomen on days 0, 7, and 14. At 7 days after the last sensitization, mice were challenged by application of 0.25% FITC solution on both sides of the ears. To determine the direct immunosuppressive effects of Vf, one group of mice was treated with Vf (20% v/v) on the ear skin during the allergen challenge period. Ear thickness was measured with a micrometer (Oditest; Kroeplin, Germany) and data are expressed as change in ear thickness as compared with that before treatment. The immunomodulatory effects of Vf were investigated by coadministration of Vf (20% v/v) on the abdominal skin at days -1, 7, 14, and 21, and not during challenge. At 7 days after the last FITC contact, all mice were challenged by application of 0.25% FITC solution onto the ears in the absence of Vf. Draining lymph nodes were collected either 8 or 72 hours after challenge and whole lymph node cells were cultivated with FITC or anti-CD3/CD28 antibodies for another 3 days. Cell culture supernatants were subjected to ELISA.

Generation and activation of human monocyte-derived DCs

DCs were generated from adherent peripheral blood mononuclear cells as previously described (Guenova *et al.*, 2008). To induce DC maturation, day 6 immature DCs (CD11c⁺ CD14⁻ HLA-DR⁺ CD86⁺ CD83⁻) were cultured for an additional 24 or 48 hours in the presence of LPS R595 or Vf.

Generation and stimulation of murine BMDCs

Murine BMDCs were generated as described previously (Lutz *et al.*, 1999). At day 8, cells were collected, washed, and seeded in 1×10^6 ml per well in 24-well-plates. DCs were stimulated with LPS ($1 \mu\text{g ml}^{-1}$) or Vf (5% v/v) for 24 hours unless otherwise specified.

DC/T-cell coculture

Immature BMDCs were activated in the presence of ovalbumin ($50 \mu\text{g ml}^{-1}$) with LPS or Vf. Subsequently, DCs were washed extensively and cultivated together with naive CD4⁺CD62L⁺ OT-II Th cells in a ratio of 1:5 for 3 days. CD4⁺ T cells were then expanded using IL-2 (50 U ml^{-1}) for another 12 days. Resting T cells were washed and restimulated with plate-bound anti-CD3 ($2 \mu\text{g ml}^{-1}$) and anti-CD28 ($5 \mu\text{g ml}^{-1}$) in 96-well plates. Cell culture supernatants were harvested after 72 hours and subjected to ELISA.

Proliferation assays

Naive OT-II CD4⁺CD62L⁺ T cells (2×10^5) were activated with ovalbumin-pulsed and Vf-stimulated DCs (4×10^4) in 96-well flat-bottom plates in a total volume of 200 μl . After 5 days $0.25 \mu\text{Ci}$

[³H]-thymidine was added and cells were harvested after another 10 hours. Incorporated [³H]-thymidine was measured using a micro beta counter (Perkin Elmer, Wiesbaden, Germany).

To assess suppressive capacity of Vf-induced IL-10⁺ Tr1 cells, unprimed CD4⁺ Th cells were isolated using magnetic cell isolation as described. These CD4⁺ cells were labeled with $1 \mu\text{M}$ CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's protocol and 2×10^5 CFSE⁺ CD4⁺ T cells were activated with plate-bound anti-CD3 ($2 \mu\text{g ml}^{-1}$) and soluble anti-CD28 ($5 \mu\text{g ml}^{-1}$) in the presence of activated Vf-induced IL-10⁺ Tr1 cells at the indicated cell ratios. Controls were set up using CFSE⁺ CD4⁺ cells cultivated together with unlabeled Th cells to ensure balanced cell numbers. Proliferation was determined 72 hours after activation.

All mice were maintained under specific pathogen free conditions at the animal facilities of the University of Tübingen according to local and federal guidelines.

Additional methods concerning animals, reagents, antibodies, bacterial lysates, Th cell isolation, and description of FACS analysis can be found online.

Statistical analysis

All data are presented as means $\pm$ SD or SEM (where indicated) of one representative experiment. Experiments were repeated at least three times if not indicated otherwise and revealed comparable results. Statistical analysis was performed with Student's *t*-tests (two tailed). Values of $P < 0.05$ were considered as statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplementary Information

Non-pathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10 producing dendritic cells and regulatory Tr1 cells

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Supplementary Material and Methods

Animals

C57Bl/6 and OT-II mice were from Charles River, Sulzfeld, Germany and Jackson Laboratory, Bar Harbor, Maine, USA. TLR2^{-/-}, TLR4^{-/-} and MyD88^{-/-} mice were described elsewhere (Hoshino *et al.*, 1999; Kawai *et al.*, 1999; Spiller *et al.*, 2007). Nc/NgA mice were from Riken BioResource Center, Tsukuba, Japan. All mice were maintained under specific pathogen free conditions at the animal facilities of the University of Tübingen according to local and federal guidelines.

Reagents

DMEM, RPMI 1640, penicillin/streptomycin, L-glutamine, sodium-pyruvate, MEM-amino acids (50X) and HEPES were from Biochrom, Berlin, Germany. Fetal calf serum (FCS) was from PAA, Cölbe, Germany. Fluorescein-isothiocyanate (FITC) was from Sigma-Aldrich, Taufkirchen, Germany. LPS from *Salmonella minnesota* R595 (TLR4 grade, ultra pure) was from Alexis Biochemicals, Lausen, Switzerland. Recombinant human IL-2 was from Chiron, Munich, Germany. [³H] thymidine was from Amersham, GE Healthcare, Freiburg, Germany. Endotoxin-free ovalbumin was purchased from Profos, Regensburg, Germany. Streptavidin-horseradish peroxidase and TMB-substrate solution were from BD Biosciences, Heidelberg, Germany.

Antibodies

For flow cytometry analysis of murine DC the following fluorochrome labelled antibodies were used. CD11c (N418) was from Bio Legend, San Diego, CA, USA. CD80 (16-10A1), CD86 (GL-1), MHC class II (M5/114.15.2) were from Becton-Dickinson (BD), Heidelberg, Germany. CD83 (michel-17) was from eBioscience, San Diego, CA, USA. Fluorochrome matched isotype controls were from Biolegend, BD or eBioscience respectively. For human DC-analysis anti-CD14 (TüK4) and anti-CD11c (BU15) were from Caltag/Invitrogen Corporation, San Diego, CA, USA, anti-CD83 (HB15e) was from BD. Isotype controls were from Caltag or BD, respectively.

Murine cytokines were detected by ELISA using commercially available ELISA kits for determination of IFN- γ and IL-4 (BD Biosciences). IL-10 and IL-12p70 were quantified by ELISA using the following matched antibody pairs (all from BD): JES5-2A5, SCX-1 for IL-10 and clones 9A5, C17.8 for IL-12p70. Human IL-10 was detected using the IL-10 specific BD OptEIA™ kit.

Endotoxin-free anti-CD3 and anti-CD28 antibodies were from eBioscience.

FACS analysis

BMDCs and MoDC were washed twice with FACS-buffer (PBS + 2% FCS) and 2×10^5 cells were incubated with the respective antibodies for 30 minutes on ice. Acquisition was performed on a FACS Calibur or a LSR II flow cytometer (both from Becton Dickinson) using CellQuest or BD DIVA software, respectively. Samples were analyzed using FCS Express V3 Software (De Novo Software, San Diego, CA, USA).

CD4⁺ T cell isolation

Lymphnodes and spleens of OT-II TCR transgenic mice (carrying a transgenic TCR specific for OVA) were isolated and lymphocytes were purified using a CD4⁺CD62L⁺ isolation kit from Miltenyi Biotech, Bergisch Gladbach, Germany.

Generation of bacterial lysate from non-pathogenic Gram-negative *V. filiformis*

Bacterial lysates of three well characterized strains of *Vitreoscilla filiformis* (ATCC 15551, ATCC 43190 and 43191) were used (Strohl *et al.*, 1986). *V. filiformis* lysates (Vf) were either generated as described (Gueniche *et al.*, 2008) or bacteria were cultured in Beggiatoa broth (ATCC 138) supplemented with catalase at 30 °C for five days. The bacterial lysates were then heat treated at 121 °C for 20 minutes. Whole protein content of all lysates was determined using the BCA assay (Thermo Scientific Pierce, Rockford, IL USA) and equivalently concentrated lysates were used for experiments. DC and T cells exposed to different concentrations of Vf lysates failed to show reduced cell viability compared to controls as determined by trypan blue staining. If not otherwise indicated a lysate of *V. filiformis* strain 15551 was used.

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Supplementary Figures

Figure S1

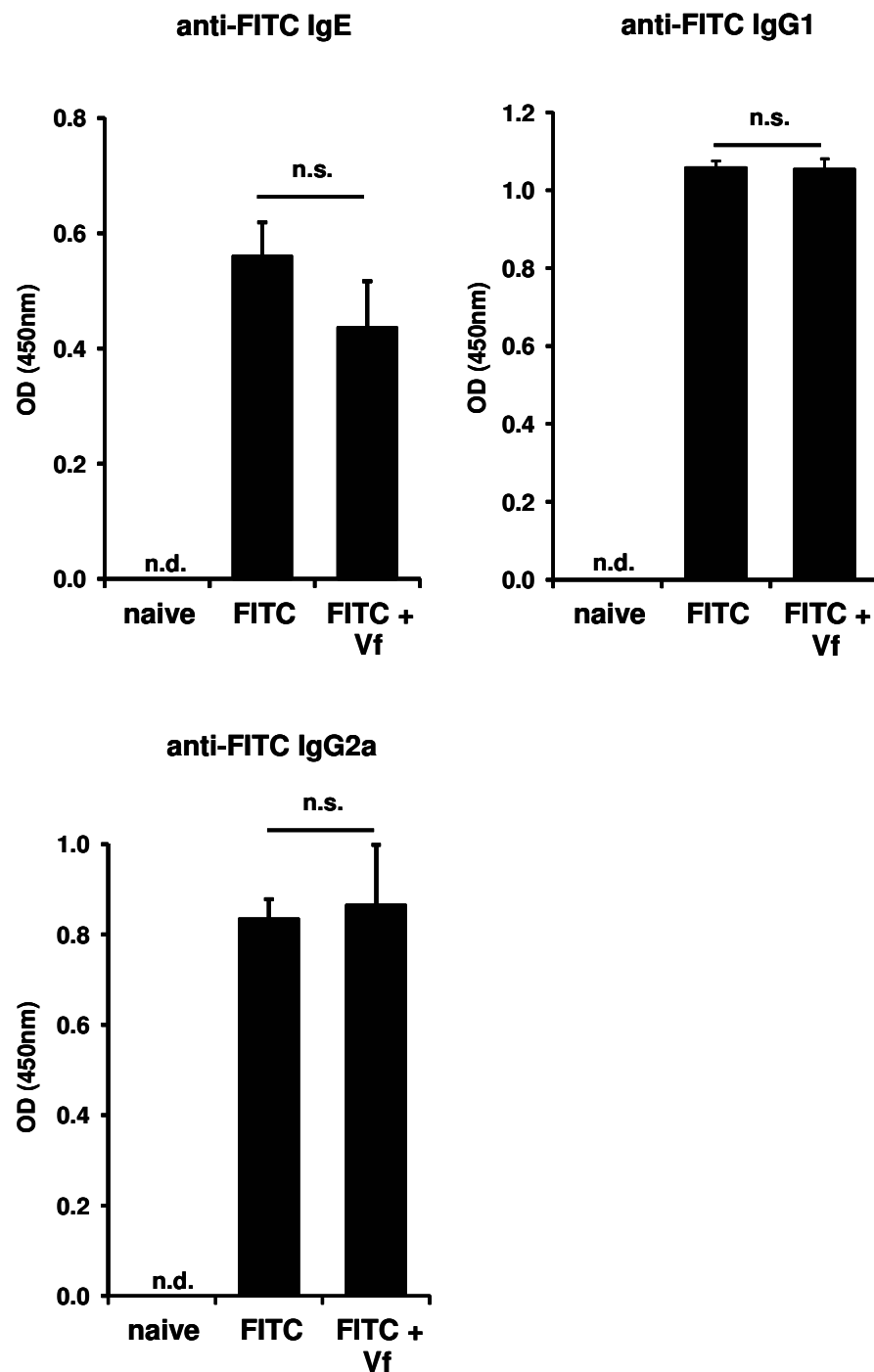
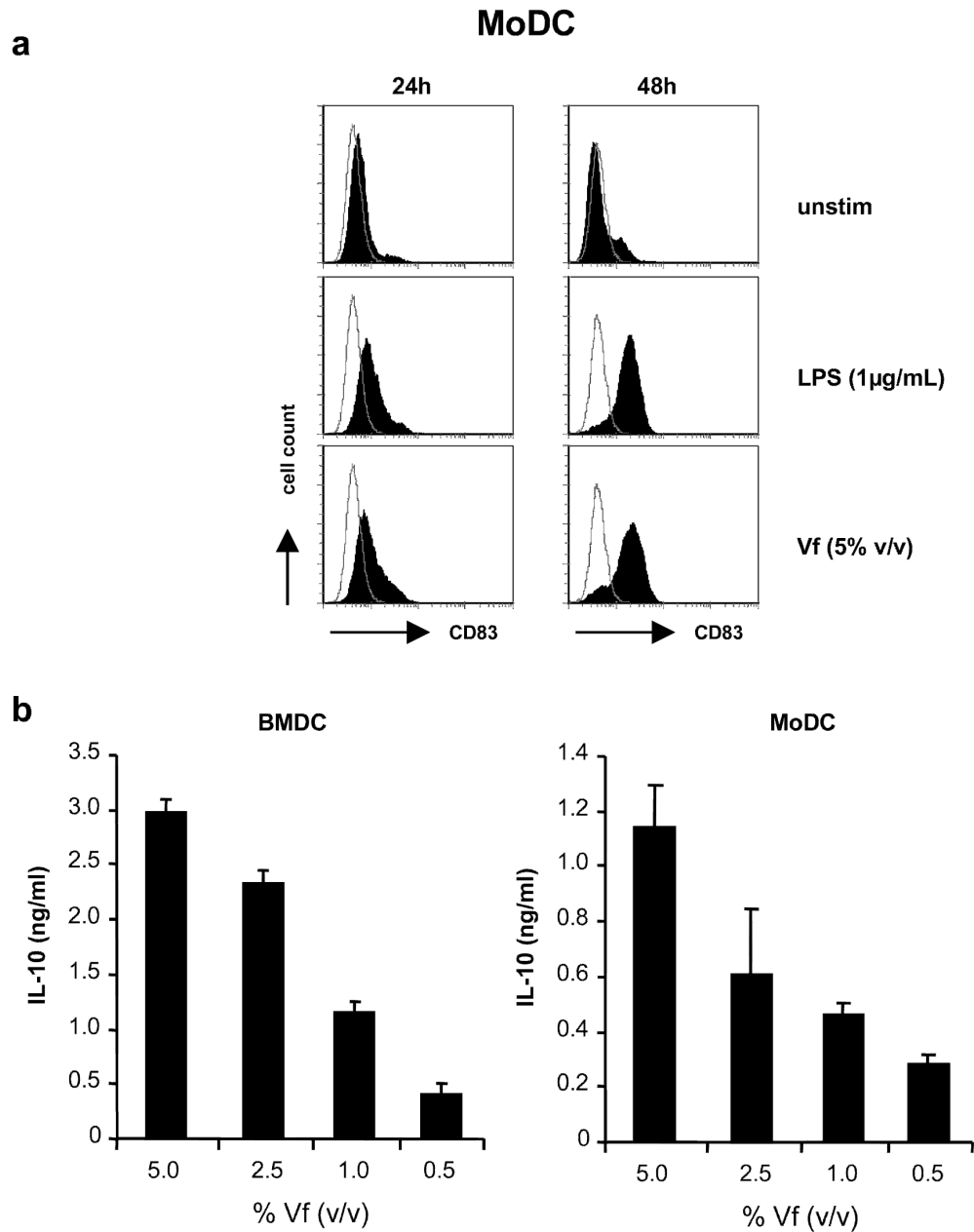


Figure S1. Antigen-specific immunoglobulin levels

FITC-specific immunoglobulin level of the subclasses IgE, IgG1 and IgG2a do not significantly differ between mice exposed to or not to Vf. As expected naïve mice do not display antigen-specific immunoglobulins. n.d., not detectable, n.s. not significant.

Figure S2**Figure S2. Vf induces maturation of human MoDC and dose dependent production of IL-10 in murine and human DC.**

(a) Human MoDC were exposed to either Vf or LPS leading to full maturation as detected by enhanced expression of CD83 (filled area) after 24 and 48 hours compared to unstimulated cells. Grey lines represent isotype controls. (b) Dose-dependent production of IL-10 in response to different concentrations of Vf by murine and human DC.

Figure S3

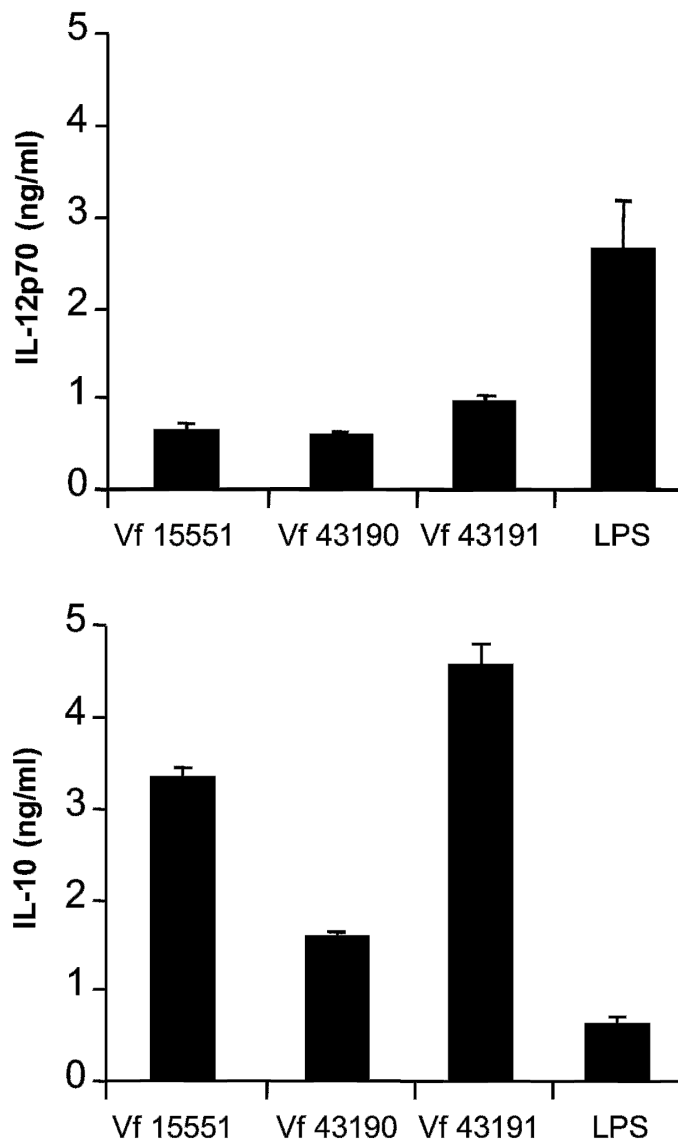


Figure S3. The IL-10 dominated cytokine profile in DC is a feature of the genus *Vitreoscilla filiformis* and independent of the bacterial strain.

BMDC were activated with Vf (5% v/v) derived of *V. filiformis* strain 15551, 43190 and 43191. After 24 hours DC produced high levels of IL-10 but only low amounts of IL-12p70 irrespective of the Vf used for activation. As before, LPS stimulation lead to high IL-12p70 levels but only low IL-10 production.

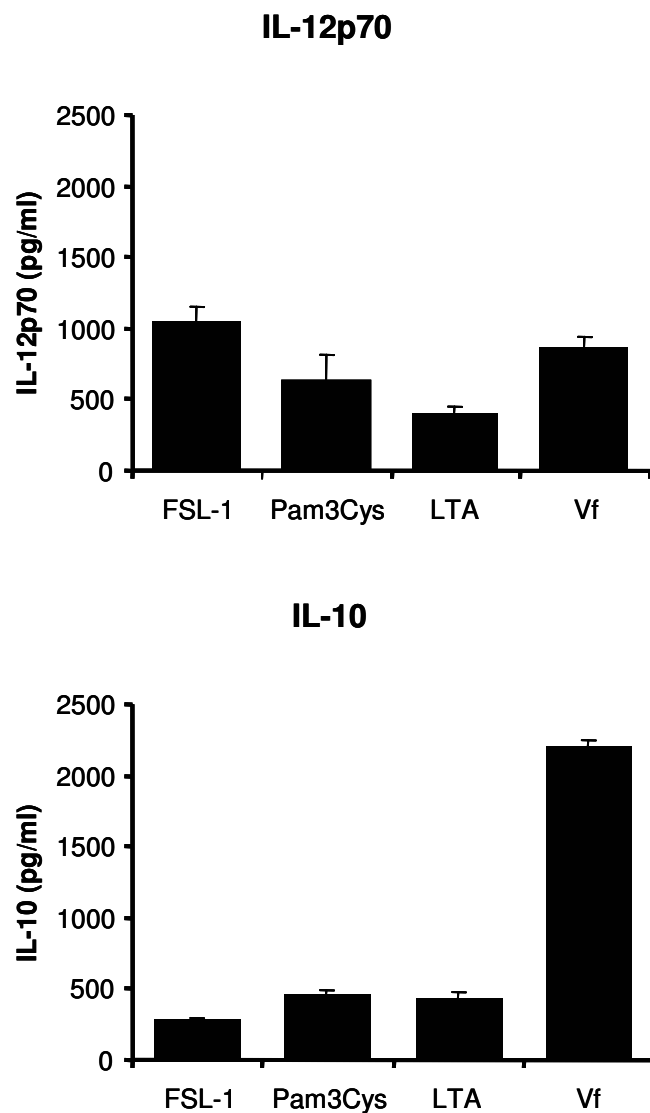
Figure S4

Figure S4. Vf is superior to numerous TLR2 ligands in induction of IL-10 producing DC.

DC were activated with 10µg/ml of FSL-1 (TLR2/6 ligand), Pam3Cys (TLR2/1 ligand), *S. aureus* derived LTA (TLR2/6 ligand) or 5%(v/v) Vf. Induction of an IL-10 dominated DC cytokine profile was preferentially found when DC were exposed to Vf.