The *Candida* Th17 response is dependent on mannand β-glucan-induced prostaglandin E2

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Abstract

The fungus *Candida albicans* is a potent inducer of the Th17 response. Prostaglandin E2 (PGE2) is a strong pro-inflammatory mediator, which has proven to be essential for the Th17 response. In this study, we have investigated the role of PGE2 in the Th17 response induced by *C. albicans* in humans. PBMC were stimulated with *C. albicans* in the absence or presence of a non-steroidal anti-inflammatory drug (NSAID). In separate experiments, PGE2 or the prostaglandin receptors agonists butaprost or misoprostol were added to the cells. PBMC were also stimulated with fungal components and small interfering RNA for mannose receptor (MR) was performed. PGE2 and cytokines were measured by ELISA or luminex, and the source of IL-17 production was determined using FACS analysis. Blocking *Candida*-induced PGE2 production by an NSAID resulted in decreased IL-17 and IL-22 production and inhibited expression of RAR-related orphan receptor gamma T mRNA. Furthermore, when PGE2 production was blocked, IL-6, IL-23 and IL-10 were decreased, while tumor necrosis factor α increased. Stimulation with PGE2 or E prostanoid (EP)2/EP4 agonists restored IL-17 production. *Candida albicans* mannan was the only fungal component that was able to directly induce PGE2 and silencing of the MR resulted in a reduction of *Candida*-induced PGE2. β-Glucan engagement of dectin-1 synergistically increased Toll-like receptor 2 (TLR2)-induced PGE2 production. These data provide evidence that PGE2 pathway is important for the Th17 response induced by *C. albicans* and that PGE2 is induced by the fungal components mannan and β-glucan that are recognized by the MR and the dectin-1/TLR2 pathway, respectively.

Keywords: IL-17, MR, NSAIDs, PBMC

Introduction

Prostaglandin E2 (PGE2) is the most widely produced prostanoid in the body in response to pro-inflammatory cytokines (1) and plays an important role in the regulation of inflammatory responses. It is primarily involved in the induction of the classical signs of inflammation, namely, erythema, increased vascular permeability, and edema, pain and fever (2–4). Furthermore, PGE2 alters the capacity of antigen-presenting cells (APCs) and T cells to produce certain cytokines and may therefore influence the functional phenotype of T cells during priming (5). PGE2 favors a Th2 response by inhibiting the production of the Th1 cytokine IFN-γ and by up-regulating the production of the Th2-associated cytokines IL-4 and IL-5 (6).

The recently discovered Th subset called Th17 cells plays an important role in the protection against extracellular bacteria and fungi (7). Th17 cells are characterized by the production of IL-17A (IL-17), IL-17F, IL-21 and IL-22 (7). IL-17 is capable of initiating and maintaining inflammation and plays an important role in autoimmune diseases like rheumatoid arthritis, multiple sclerosis and psoriasis (8). The early differentiation of Th17 cells is initially regulated by IL-1β signaling (9), while IL-23 plays an important role in the amplification and late stage of Th17 development (10). Interestingly, PGE2 induces IL-23 production (11–13) and together with IL-23 synergistically favors human Th17 expansion (1, 14). PGE2 has also been reported to be necessary for the production of the Th17 effector cytokine IL-17 (15). Furthermore, it has been shown that human memory T cells induce a robust Th17 response in reaction to the fungus *Candida albicans* (16, 17).

In the present study, we investigated the mechanisms through which *C. albicans* induces PGE2 production...
and whether this influences the Candida-induced Th17 response. It is shown that C. albicans is a potent inducer of PGE2 and that the Candida-induced Th17 response is dependent on the induction of prostaglandins. Candida albicans mannann was the only fungal component that was able to directly induce PGE2 production, while β-glucan exerted synergistic effects on Toll-like receptor 2 (TLR2)-induced prostaglandins.

Materials and methods

Volunteers

Blood was collected from healthy volunteers who were free of infectious or inflammatory disease after informed consent was given. Blood was collected by venipuncture into 10 ml EDTA syringes (Monoject, s-Hertogenbosch, The Netherlands).

Reagents

The following study materials were used: anti-CD3- and anti-CD28-coated (αCD3×αCD28) beads prepared from a T cell activation/expansion kit (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) and Pam3Cys (EMC Microcollections, Tuebingen, Germany). Chitin was kindly provided by Prof. Neil A. R. Gow (School of Medical Sciences, Aberdeen, UK) and prepared according to previous protocols (18). Candida mannan and particulated β-glucan were kindly provided by Prof. David Williams and were prepared as previously described (19, 20). Curdlan was purchased from WAKO (Richmond, VA, USA) and diclofenac from Novartis (Sanquin, Amsterdam, The Netherlands). Isolated RNA was being reverse transcribed into complementary DNA using oligo(dT) primers and MMLV reverse transcriptase. PCR was performed using a 7300 real-time PCR system (Applied Biosystems, Lennik, Belgium). Primer sequences for human RAR-related orphan receptor gamma (ROγR) T—sense: 5’-TGA-GAA-GGA-CAG-GGA-GGC-AA-3’ and anti-sense: 5’-CCA-CAG-ATT-TTGCAA-GGG-ATC-A-3’. B2M was used as a reference gene, for which the primers were as follows: 5-ATG-ATG-ATG-CCT-GCC-GTG-TG-3 (forward) and 5-CCA-AAT-GGC-GCA-TCT-TCA-AAC-3 (reverse). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of PCR reaction at 95°C for 15 min and 60°C for 1 min.

Microorganism

Candida albicans American Type Culture Collection MYA-3573 (UC 820) (21) was grown overnight in Sabouraud broth at 37°C; cells were harvested by centrifugation, washed twice, and re-suspended in culture medium. Candida albicans was heat killed for 1 h at 100°C.

In vitro cytokine production

Separation and stimulation of PBMC were performed as described previously (22). Briefly, the PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMC were washed twice in saline and suspended in culture. The cells were counted in a hemocytometer, and their number was adjusted to 5 × 10⁶ cells ml⁻¹. PBMC (5 × 10⁵) in a volume of 100 μl per well were incubated at 37°C in round-bottom 96-well plates (Greiner, Nurnberg, Germany) in the presence of 10% human pooled serum, with C. albicans or culture medium alone. After 1, 2 or 7 days of incubation, supernatants were collected and stored at −20°C until assayed.

Cytokine assays

The concentrations of IL-1β, IL-17, IL-22, IL-23 and tumor necrosis factor (TNF) α (R&D Systems, Abingdon, UK) and IL-6, IL-10 and IFN-γ (Sanquin, Amsterdam, The Netherlands) were measured in cell culture supernatants using ELISA according to the instructions of the manufacturer. IL-2, IL-4 and IL-12p70 were measured by using luminex technology according to instructions of the manufacturer (BioRad, Veenendaal, The Netherlands).

Quantitative PCR

Two million freshly isolated PBMC were incubated with the various stimuli. After 24 h of incubation at 37°C, total RNA was extracted in 800 μl of TRIzol reagent (Invitrogen, Breda, The Netherlands). Isolated RNA was being reverse transcribed into complementary DNA using oligo(dT) primers and MMLV reverse transcriptase. PCR was performed using a 7300 real-time PCR system (Applied Biosystems, Lennik, Belgium). Primer sequences for human RAR-related orphan receptor gamma (ROγR) T—sense: 5’-TGA-GAA-GGA-CAG-GGA-GGC-AA-3’ and anti-sense: 5’-CCA-CAG-ATT-TTGCAA-GGG-ATC-A-3’. B2M was used as a reference gene, for which the primers were as follows: 5-ATG-ATG-ATG-CCT-GCC-GTG-TG-3 (forward) and 5-CCA-AAT-GGC-GCA-TCT-TCA-AAC-3 (reverse). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of PCR reaction at 95°C for 15 min and 60°C for 1 min.

Fluorescence-activated cell sorting

One million freshly isolated PBMC were stimulated for 7 days with heat-killed C. albicans in the presence of 10% human pool serum and in the absence or presence of non-steroidal anti-inflammatory drugs (NSAIDs). After 7 days, the supernatant was removed and replaced with RPMI with Golgiplug (555029; BD Biosciences, Breda, The Netherlands), phorbol myristate acetate (50 ng ml⁻¹) (P8139, Sigma-Aldrich) and ionomycin (1 μg ml⁻¹) (10834, Sigma-Aldrich) for 4 h. After that, the cells were labeled with anti-CD4–APC (555349, BD Biosciences) according to instructions of the manufacturer. Accordingly, Fix & Perm (GAS0015-100 and GAS002S-100, Invitrogen) were used so the cells could be stained intracellularly with anti-IL-17–FITC (11-7179-82; eBioscience, Frankfurt, Germany). The cells were re-suspended in 200 μl 1% BSA and fluorescence was measured on a Cytomics FC500 FACS machine (Beckmann Coulter, Woerden, The Netherlands). Gates were drawn to determine the percentage of IL-17-producing CD4+ lymphocytes.

Small interfering RNA transfection

PBMC were transfected by electroporation with the Amaxa Human Monocyte Nucleofector kit (Lonza, Cologne, Germany) in accordance with the manufacturer’s instructions. In brief, PBMC (2 × 10⁵) were harvested and re-suspended in 100 μl of nucleofector solution. After addition of mannose receptor (MR) small interfering RNA (siRNA) (Dharmacon, Epson, UK; 011730) or control green fluorescent protein (GFP) siRNA (VSC-1001, Lonza) at final concentration of 125 nM, cells were electroporated with Amaxa program.
Y-001 and recovered for 24 h before further stimulation. Both MR expression on the cell surface and mRNA expression for MR were decreased after MR siRNA transfection compared with GFP siRNA transfection (data not shown).

Statistical analysis
Experiments were performed in duplicate, and supernatants were pooled. The differences between groups were analyzed using the Wilcoxon signed rank test. The level of significance between groups was set on P < 0.05. Data are presented as mean ± SEM, unless stated otherwise.

Results
Candida albicans-induced IL-17 and IL-22 is dependent on PGE2
To determine whether C. albicans could induce PGE2 production, PBMC from eight healthy volunteers were stimulated for 48 h with C. albicans blastoconidia. Stimulation of C. albicans resulted in PGE2 production by PBMC (Fig. 1a). In addition, when PBMC were stimulated with C. albicans in the presence of the prostaglandin inhibitor diclofenac, the PGE2 production was completely blocked (Fig. 1a). To investigate if the Th17 response induced by C. albicans is PGE2 dependent, PBMC were stimulated for 7 days with C. albicans or beads coated with αCD3αCD28 in the absence or presence of diclofenac. Both IL-17 and IL-22 were produced upon stimulation with C. albicans, and both cytokines were lower when PBMCs were cultured in the presence of diclofenac (Fig. 1b). Mitogenic T cell stimulation with αCD3αCD28 beads resulted in IL-17 production, but not IL-22 production. The IL-17-induced production by beads was not influenced by PGE2 inhibition (Fig. 1b). In addition, RORγT mRNA expression was decreased in the presence of an NSAID in the system (Fig. 1c). To indicate the source of IL-17 production, FACS analysis was performed on CD4 and intracellular IL-17. It appeared that IL-17 was exclusively produced by CD4+ cells. Furthermore, the percentage of IL-17-producing CD4+ cells decreased when cells were cultured in the presence of NSAID’s (Fig. 1d). To assess whether the decrease in IL-17 production caused by the NSAIDs was due to the absence of PGE2, we added exogenous PGE2 to assess whether this could restore IL-17 production. Adding PGE2 to the cell culture partially restored IL-17 production (Fig. 1e). Also butaprost, which is a selective EP2 receptor agonist, and misoprostol, a non-selective agonist with the highest affinity for the EP4 and EP3 receptor, restored the NSAID-inhibited IL-17 production (Fig. 1e). PBMC in the presence of PGE2, butaprost or misoprostol alone did not produce IL-17 (data not shown).

Candida albicans mannan is the only fungal component that directly induces PGE2 production
To elucidate which fungal components were responsible for the PGE2 production induced by C. albicans, PBMC were cultured with RPMI, mannan, β-glucan or curdlan or chitin and PGE2 production was measured in cell culture supernatants. Interestingly, C. albicans mannan was the only component able to induce PGE2 production (Fig. 2a). In line with this, when the transcription of the MR was inhibited using siRNA, PGE2 production decreased (Fig. 2b). β-glucan and curdlan alone were not able to induce PGE2. Also, the TLR2 ligand Pam3Cys alone was not able to induce PGE2 production. However, β-glucan and curdlan were able to synergize with the TLR2 ligand Pam3Cys and induce PGE2 production (Fig. 2c).

PGE2 skewes the cytokine profile favoring a Th17 response
To determine the effects of prostaglandins on the cytokine profile induced by C. albicans, we stimulated human PBMC with C. albicans in the presence of absence of the prostaglandin inhibitor diclofenac. Strikingly, IL-6 and IL-23 decreased when PBMC were pre-incubated with diclofenac. In addition, IL-10 decreased when PGE2 was blocked, while TNF-α increased (Fig. 3). IL-1β was not affected by inhibition of PGE2 production.

Discussion
In the present study, we provide evidence for an important role of PGE2 in the Candida-induced Th17 response. PGE2 contributes to the Candida-induced Th17 response by inducing the Th17-polarizing cytokines IL-6 and IL-23. Furthermore, the fungal components mannan and β-glucan were shown to be responsible for the induction of PGE2 production.

In line with previous studies, we show that C. albicans is capable of inducing a strong PGE2 production in human PBMC (23). A notable difference is that most studies used viable C. albicans. It has been reported that live C. albicans can produce PGE2 (24, 25); this is why we used heat-killed C. albicans to investigate the induction and functional role of PGE2 produced by the host. Boniface et al. (15) found that PGE2 is necessary for the production of IL-17 in the presence of APCs and that PGE2 directly promotes differentiation and pro-inflammatory functions of Th17 cells, while differentially regulating IFN-γ production. In line with this, we found that blocking PGE2 production resulted in reduced RORγT mRNA expression, IL-17 and IL-22 production and increased TNF-α production. This indicates that PGE2 favors a Th17 response. In line with these findings, the addition of PGE2 partially restored the IL-17 production. Furthermore, PGE2 signals through the EP2 and EP4 receptors and both receptors are important for mediating Th17 responses (15, 26, 27). Our observations suggest that PGE2 induced by Candida also signals through the EP2 and EP4 receptor, since stimulation of these receptors in the absence of PGE2 partially, and sometimes completely, restored the Candida-induced Th17 response. These data further support the concept that the Th17 response induced by C. albicans is dependent on the PGE2 signaling pathway.

However, although in certain conditions the PGE2 release is completely blocked, the production of IL-17 and IL-22 is not completely inhibited. This indicates that other mechanisms contribute to the fungal-induced Th17 responses. Importantly, blocking PGE2 did not affect IL-1β, and there was still IL-6 and IL-23 production. Since IL-1β is essential for the early development of the Th17 response, the IL-1 pathway is most likely responsible for the IL-17 production.
Fig. 1. *Candida albicans*-induced IL-17 and IL-22 is dependent on PGE2. (A) Human PBMC were stimulated for 48 h, in the presence of human serum, with RPMI or *C. albicans* (1 x 10^6 ml^-1) and in the absence or presence of NSAIDs (n = 11). (B) Human PBMC were cultured with human serum for 7 days with RPMI, *C. albicans* or αCD3αCD28 beads (2.5 x 10^6 ml^-1) in the presence or absence of NSAIDs. Production of IL-17 (n = 20) and IL-22 (n = 7) in the supernatants was measured by ELISA. (C) Quantitative PCR of RORγT mRNA expression in human PBMC that were stimulated for 24 h, in the presence of human serum, with RPMI or *C. albicans* and in the absence or presence of NSAIDs (n = 5). (D) Human PBMC were stimulated for 7 days with *C. albicans*, in the presence of human serum and in the absence or presence of NSAIDs. Expression of CD4 and intracellular IL-17 were determined with FACS analysis. Figure is representative for four healthy volunteers from two different experiments. (E) Human PBMC were stimulated for 7 days, in the presence of human serum, with *C. albicans* (gray bars), in the absence or presence of NSAIDs and in the absence or presence of PGE2 (10 mM), butaprost (10 μM) or misoprostol (35 μM) (n = 3). Cytokines and PGE2 were measured by ELISA. (A–C) Data are pooled and expressed as mean ± SEM.
Th17 response has been linked to mucosal candidiasis (28, 29). The residual production of IL-17 is thus most likely to be sufficient to maintain adequate host defense against mucosal candidiasis when NSAIDs are used.

In a subsequent set of experiments, we tried to elucidate which components of *C. albicans* were responsible for the induction of PGE2. Mannans were the only fungal components that were able to directly induce PGE2 production. We have previously reported that mannan from *C. albicans* was also the only component capable of inducing IL-17 production (17). Furthermore, blocking of the MR with siRNA leads to inhibition of PGE2 production, suggesting that the MR receptor plays an important role in the induction of PGE2 production by PBMC. This is supported by the previous observation that over-expression of the MR in HeLa cells that are challenged with mannan results in a significant induction of cyclooxygenase-2 expression (30). Another C-type lectin, the β-glucan receptor dectin-1, also plays an important role in anti-*Candida* host defense, and prostaglandin production can be enhanced by over-expressing dectin-1 in a mouse macrophage cell line (31). However, β-glucans alone did not stimulate PGE2 production (31). In line with the known and previously reported synergism of dectin-1 and TLR2 (32, 33), β-glucans strongly increased the production of PGE2 induced by TLR2. Furthermore, dectin-1 and TLR2 have also been linked to the induction of the T<sub>17</sub> response (17). In contrast, we previously found that β-glucans or curdlan did not augment the mannan-induced IL-17 response (17). Therefore, β-glucans contribute through the dectin-1/TLR2 pathway to the *Candida*-specific T<sub>17</sub> response by inducing the production of PGE2 by the host.

To understand how PGE2 polarizes the immune response toward a T<sub>17</sub> profile, we investigated the changes in cytokine profiles when PGE2 production was inhibited. PGE2 is known to have a potent IL-6-inducing effect on monocytes (34). We observed that blocking of PGE2 by diclofenac reduced the production of IL-6 induced by *Candida*. IL-6 has been reported to be essential for the induction of T<sub>17</sub> memory cells in humans (35). This suggests that skewing of PGE2 toward a T<sub>17</sub> response is dependent on IL-6. When PGE2 production was blocked, the induction of IL-23 by *Candida* was also lower. The role of IL-23 in the T<sub>17</sub> response has been well established and it is now generally accepted that IL-23 is needed to maintain the T<sub>17</sub> response (10, 36). Furthermore, it has been suggested in an experimental inflammatory bowel disease model that the pro-inflammatory effects of PGE2 are due to the induction of dendritic cell-derived IL-23 that subsequently supports the T<sub>17</sub> response (9). Interestingly, we found no effect on IL-1β when we blocked PGE2 production. Since IL-1β is one of the most important cytokines in the induction of an early T<sub>17</sub> response (9), it is therefore notable that PGE2 does not induce the T<sub>17</sub> response directly through IL-1β. We found an increase of TNF-α when PGE2 was inhibited, which is in line with the literature which has reported that PGE2 inhibits TNF-α (37). However, the exact role of TNF-α in the T<sub>17</sub> response is not clear and still has to be elucidated (38). Finally, we found that IL-10 production was also lower in the presence of a PGE2 inhibitor. Veldhoen et al. (39) already speculated that IL-10 production, which suppresses IL-12, might aid the development of IL-17-producing T cells and subsequently reported that anti-IL-10 caused a reduction in the proportion of T cells that were able to produce IL-17. Therefore, the loss of the suppressive effects of IL-10 could be a possible explanation for the observed lower IL-17

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**Fig. 2.** *Candida albicans* mannan is the only fungal component that directly induces PGE2 production. (A) Human PBMC were stimulated for 48 h in presence of human serum with RPMI, mannan (10 μg ml<sup>-1</sup>), β-glucan (10 μg ml<sup>-1</sup>), chitin (20 μg ml<sup>-1</sup>) and curdlan (10 μg ml<sup>-1</sup>). (n = 4) (B) PGE2 production in cells from two healthy volunteers transfected with control siRNA (GFP) or MR siRNA and stimulated with RPMI or *C. albicans*. (C) Human PBMC were stimulated for 48 h in presence of human serum with RPMI, mannan, β-glucan or curdlan and in the absence or presence of Pam3Cys (n = 6). Production of PGE2 in the supernatants was measured using ELISA. Data are pooled and expressed as mean ± SEM.
production when PGE2 production was blocked. This would ultimately result in a stronger Th1 response that is able to subvert the Th17 response.

In conclusion, prostaglandins were shown to play an important role in the C. albicans-induced Th17 response. PGE2 production induced by C. albicans skewes the cytokine profile toward a Th17 response by decreasing IL-6, IL-23, IL-17 and IL-22. Interestingly, C. albicans mannan, which is the main inducer of the Th17 response in C. albicans, was also the only fungal component that was able to directly induce PGE2. Furthermore, dectin-1, the other major pathway important for the induction of a Th17 response, was shown to act synergistically on the TLR2-induced PGE2 production. These data provide evidence that the Th17 response induced by the MR and dectin-1/TLR2 is enhanced by prostaglandins, which in turn skew the cytokine profile favoring Th17 polarization.

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