Unmethylated CpG motifs in *Toxoplasma gondii* DNA induce TLR9- and IFN-β-dependent expression of α-defensin-5 in intestinal epithelial cells

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SUMMARY

The gut epithelial barrier is a strategic place to prevent, or at least to limit, parasite dissemination upon oral infection with *Toxoplasma gondii*. Innate immunity to this pathogen results from delicate interactions involving different components of the infecting agent and the host. We herein aimed to examine the molecular mechanism by which protozoan DNA boosts the production of α-defensin-5 (DEFA-5), the main antimicrobial peptide at the target site of infection. The present study shows that DEFA-5 is rapidly upregulated in intestinal epithelial cells following intracellular Toll-like receptor 9 (TLR9) activation by unmethylated CpG motifs in DNA from *T. gondii* (CpG-DNA). Concomitantly, CpG-DNA purified from the pathogen markedly increased TLR9 mRNA expression levels in the Caco-2 cell line. We further verified that DEFA-5 production was dependent on interferon-β released from these cells upon treatment with CpG-DNA prepared from tachyzoites. Our results suggest that, in protozoan DNA-stimulated intestinal epithelial cells, the TLR9/interferon-β/DEFA-5 pathway may initiate an innate anti-*T. gondii* response without the need of parasite invasion. These findings highlight the key role of the gut epithelium in Toxoplasma recognition and amplification of local host defence against this microbe, thereby contributing to gain insight into immunoprotective mechanisms and to improve therapeutic strategies.

Key words: *Toxoplasma gondii*, parasite DNA, unmethylated CpG, Toll-like receptor 9, α-defensin-5, interferon-β, intestinal epithelial cells.

INTRODUCTION

The intestinal epithelium functions as a selective barrier that plays an active role in maintaining mucosal homoeostasis and also contributes to host resistance to several infections, including human toxoplasmosis raised by the apicomplexan parasite *Toxoplasma gondii* (Schulthess *et al*. 2008; Peterson and Artis, 2014). Among multiple innate immune mechanisms involved in the early protective response to *T. gondii*, those mediated by members of the defensin family of antimicrobial peptides have lately received much attention (Foureau *et al*. 2010; Tanaka *et al*. 2010; Mowat, 2011). Defensins are small cationic moieties that exert their activity by damaging the microbial membrane and by acting as chemokines (Jarczak *et al*. 2013). Two main subfamilies, α- and β-defensins, differ in their disulphide connectivities and spacing between cysteine residues (Raj and Dentino, 2002). In the intestine, β-defensins are predominantly expressed in the colonocytes, whereas α-defensins are a component of secretory granules released by Paneth cells (Frye *et al*. 2000). Oral toxoplasma infection has been shown to induce intestinal production of α-defensin-5 (DEFA-5), via Toll-like receptor 9 (TLR9) activation, leading to reduced parasite viability and infectivity (Foureau *et al*. 2010; Tanaka *et al*. 2010). This innate immune response mechanism is mediated by TLR9-dependent type I interferon (IFN) production (Foureau *et al*. 2010; Beiting, 2014). Moreover, Rumio *et al*. (2004) have found that Paneth cells from mice treated *in vivo* with CpG-containing oligonucleotides display high expression of antimicrobial peptides. Since microbial unmethylated CpG DNA, the classic ligand for TLR9 signalling, has the ability to stimulate gut epithelium (Watson and McKay, 2006), our current goal was to determine whether the interaction of CpG sequences in *T. gondii* genome with their cognate receptor is responsible for triggering IFN-β-upregulated DEFA-5 expression in intestinal epithelial cells.

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MATERIALS AND METHODS

Cells

The Caco-2 human cell line was obtained from the American Type Culture Collection and cultured using minimal essential medium (Life Technologies, Carlsbad, CA, USA) supplemented with 20% fetal

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calf serum as described (Corral *et al*. 2007). This cell system was chosen for the study due to its ability to spontaneously differentiate and produce a monolayer of intestinal epithelial cells, constituting the cell model most commonly used to reproduce the immune and barrier functions of the human small intestine (Sambuy *et al*. 2005).

Parasites

Toxoplasma gondii parasites of the virulent RH (type I) strain were isolated from the peritoneal exudates of intraperitoneally infected BALB/c mice. Three days after the inoculation, fluids were collected in cold phosphate-buffered saline, pH 7·2, containing 1 mM $CaCl_2$ (PBS-Ca). Highly purified tachyzoites, the rapidly multiplying form of the parasite, were obtained by two consecutive discontinuous sucrose gradients as reported previously (Garberi *et al*. 1990). Briefly, sucrose step gradients were prepared by carefully layering equal volumes of 40–50–60% sucrose in PBS-Ca solution in 40 mL centrifuge tubes. The parasite suspension (2 mL) was loaded on top of the gradient and centrifuged at 10 000 × ***g*** for 30 min at 4 °C. Thereafter, gradients were fractionated into 0·5 mL aliquots and repeatedly washed in PBS-Ca buffer at 3000 × ***g*** for 10 min. Tachyzoite-enriched fractions were pooled, washed as above and finally stored at −70 °C until used.

Infection of intestinal epithelial cells

Tachyzoites of the RH strain were maintained *in vitro* by infection of human foreskin fibroblasts. Parasites were collected and purified using a 3 μM polycarbonate membrane filter (Millipore, Bedford, MA, USA) (Sonaimuthu *et al*. 2014). Cells (1×10^5/ml) were grown at 70% confluence in six-well plates until exposed to *T. gondii*. Infection of Caco-2 cells was performed at a 1:2 cell-to-parasite ratio and incubated for 24 h.

DNA purification and methylation

Tachyzoite DNA was extracted from 10^9 parasites by using Wizard Genomics Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Nucleic acid concentrations and purity were determined by nanodrop spectrophotometry method. The ratio of O.D. at 260 and 280 nm was used to assess the purity of tachyzoite DNA. A ratio of 2·0 was considered as 100% pure DNA. Those samples yielding a 1·9–2·0 ratio (⩾95% purity) were chosen for further analysis. Measurement of the G + C content of DNA preparation was accomplished by enzymatic digestion and high-performance liquid chromatography as indicated by Mesbah and Whitman (1989). Some aliquots of genomic *T. gondii* DNA were methylated as previously reported (Shoda *et al*. 2001) using CpG methyltransferase (3 U M.SssI μg^{-1} DNA; New England Biolabs, Ipswich, MA). DNA methylation was complete after 24 h, as determined by measuring the resistance of treated DNA samples to cleavage by *Hpa*II restriction endonuclease (New England Biolabs). Another aliquot of parasite DNA was digested for 2 h at 37 °C with 5 μg mL^{-1} DNase 1 (Life Technologies) as described elsewhere (Lazarovici *et al*. 2013) and used as control preparation. All DNA specimens were purified, quantified, and subjected to agarose gel electrophoresis with ethidium bromide staining.

Limulus amebocyte lysate assay

Cell culture reagents and all DNA preparations were intensively tested for the absence of contaminating lipopolysaccharide (LPS) using the *Limulus* amebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, MD, USA). The sensitivity of the assay is 0·06 EU mL^{-1} (6 pg mL^{-1}) endotoxin. All cell culture reagents had undetectable levels of endotoxin (<6·0 pg in 10 μg mL^{-1} DNA specimen). In addition, all media were supplemented with 10 μg mL^{-1} polymyxin B sulphate (Sigma-Aldrich, St. Louis, MO, USA) which has proved to efficiently inhibit LPS contamination (Cardoso *et al*. 2007).

Quantitative real-time PCR analysis

After 5 h treatment with protozoan nucleic acid, RNA was isolated from Caco-2 cells using RNAzol RT (Molecular Research Center, Cincinnati, OH, USA). cDNA synthesis and quantitative real-time PCR (qRT-PCR) were carried out as previously described (Kingma *et al*. 2011). The primer sequences used for qRT-PCR are: forward sequence, 5′-GCTGCGTCTCCGTGACAATTA-3′; reverse sequence, 5′-AGCTGACATCCAGCCTCCG-3′. The number of cycles obtained for TLR9 gene was normalized by subtracting the number of cycles obtained for the internal control (GAPDH gene).

Toxoplasma gondii DNA transfection of intestinal epithelial cells

Caco-2 cells were exposed for 5 or 24 h to free DNA (0·5–10·0 μg mL^{-1}) purified from tachyzoites. Alternatively, the cells were transfected with unmethylated or methylated *T. gondii* DNA complexed with 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP; Sigma-Aldrich) at 10·0, 5·0, 2·5, 1·0 and 0·5 μg mL^{-1} DNA for 24 h. All experiments were conducted in serum-free media.

Immunoblotting

Disrupted and solubilized cell extracts (20 μg) were subjected to SDS–PAGE as described elsewhere

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(Foureau *et al*. 2010), and then transferred to Immobilon-P filters (Millipore, Billerica, MA, USA). The membranes were probed with rabbit anti-human DEFA-5 polyclonal antibody (Alpha Diagnostics, San Antonio, TX, USA) and mouse monoclonal antibody (mAb) against β-tubulin (Sigma-Aldrich) for 18 h at 4 °C. The signal was visualized by further incubation with the corresponding horseradish peroxidase-conjugated secondary Ab (Thermo Scientific Pierce, Rockford, IL) and a chemiluminescent peroxide substrate (ECL; Amersham, GE Healthcare Biosciences, Pittsburgh, PA, USA).

Measurement of IFN-β levels

The production of IFN-β in 24 h supernatants from *T. gondii* DNA-stimulated Caco-2 cells was analysed by ELISA (R&D Systems, Minneapolis, MN, USA) following the instructions of the supplier. The assay sensitivity is 50 pg mL^{-1}.

TLR9 inhibition and IFN-β blocking assays

For inhibition of TLR-9, a G-type TLR9 inhibitory oligonucleotide (forward 5′-CTCCTATTGGGGT TTCCTAT-3′) (Alexis, Enzo Life Sciences, Farmingdale, NY, USA) was used (Takeda *et al*. 2011). For blocking IFN-β activity, cells were treated with specific neutralizing antibody (10 μg mL^{-1}, R&D Systems) or isotype control antibody (BD Biosciences, San Jose, CA, USA) (Colonne *et al*. 2011).

Flow cytometry

Flow cytometry assays were performed in the presence of 1·0 μg mL^{-1} of Brefeldin A (Sigma-Aldrich) for 6 h to inhibit release of defensin into the culture medium. The cells (10^5) were stained intracellularly with fluorochrome-labelled DEFA-5 mouse monoclonal Antibody (8c8, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and analysed as reported previously (Andrade *et al*. 2013).

Immunocytochemical analysis

Cells were washed and processed using the Novolink system (Leica Biosystems, Newcastle, UK) according to the manufacturer's suggested procedure. The reaction was developed using a 3–3′-diaminobenzidine tetrahydrochloride solution activated with H_2O_2 for 10 min. Cell nuclei were counterstained with Mayer's haematoxylin. Preparations were dehydrated to xylene, mounted with BioMount (Bio-Optica, Milan, Italy) and analysed by microscopy.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism (San Diego, CA, USA) 5·0 software. Results are presented as the mean ± standard error of the mean (S.E.M.) of triplicate determinations and are representative of at least three independent experiments. Significant differences among groups were made using the one-way analysis of variance test followed by Tukey's test. A difference between groups of $P < 0{\cdot}05$ was considered significant.

RESULTS

Toxoplasma DNA contains unmethylated CpG motifs

Preliminary characterization studies determined that DNA obtained from tachyzoites was LPS-free (<6·0 pg in 10 μg per ml DNA specimen) and had a G + C content of 54·1%, similar to that (52·2%) reported for this apicomplexan pathogen (Khan *et al*. 2007). To determine the extent to which protozoan DNA is unmethylated, *T. gondii* DNA was methylated with CpG methylase and examined for *Hpa*II sensitivity. DNA purified from tachyzoites was sensitive to *Hpa*II digestion (Fig. 1A), suggesting the existence of unmethylated CG dinucleotides in the parasite genome. Confirming this observation, Toxoplasma DNA proved resistant to *Hpa*II cleavage following M.SssI methylation (Fig. 1B).

Unmethylated Toxoplasma gondii DNA elicits defensin response in intestinal epithelial cells

To assess whether induction of DEFA-5 expression may be directly stimulated by unmethylated CpG motifs in Toxoplasma genome, we treated Caco-2 cells *in vitro* with purified tachyzoite DNA at increasing concentration. *Toxoplasma gondii*-derived nucleic acid induced a dose-dependent boost in the expression of DEFA-5 in starved cells transfected for 24 h with DOTAP-complexed parasite DNA at 2·5 and 5·0 μg mL^{-1}, although some reactivity can be seen at 1·0 μg mL^{-1} (Fig. 2A). Caco-2 cells also produced DEFA-5 when free Toxoplasma DNA was added to the culture medium though 5-fold lower than that achieved using DOTAP-mediated transfection. Both enzymatic digestion of DNA and CpG methylation completely abrogated antimicrobial peptide induction, indicating the specificity of unmethylated *T. gondii* DNA effect on enhanced defensin response. Purified human DNA neither displayed ability for upregulating DEFA-5 levels in the Caco-2 cell line (data not shown). Consistent with these observations, immunocytochemical studies revealed augmented DEFA-5 expression in intestinal epithelial cells cultured for 24 h in the presence of unmethylated, but not

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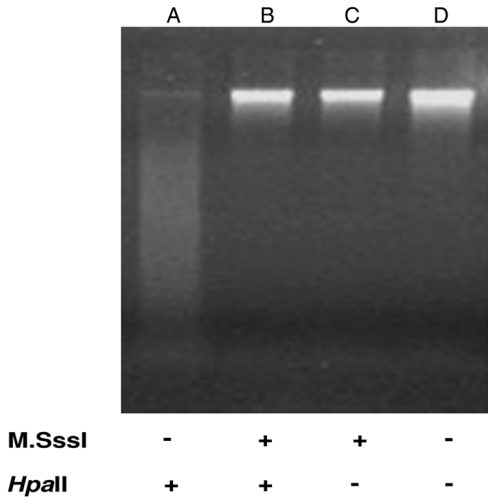

Fig. 1. Sensitivity of unmethylated and methylated *T. gondii* DNA to enzymatic digestion with *Hpa*II. DNA purified from tachyzoites was methylated or not with M. SssI methylase and tested for sensitivity to *Hpa*II. DNA was visualized following electrophoresis on ethidium bromide-agarose gel. (A) and (D), unmethylated DNA; (B) and (C), methylated DNA. Specimens in lanes (A) and (B) were subjected to *Hpa*II digestion, whereas DNA in lanes (C) and (D) was not treated with *Hpa*II.

methylated, protozoan DNA at 5 μg mL^{-1} (Fig. 2B). Accordingly, cytofluorometric analysis showed that, in response to unmethylated *T. gondii* DNA, the number of DEFA-5-containing Caco-2 cells was substantially elevated, compared with methylated microbial DNA-treated controls (Fig. 2C).

Purified tachyzoite DNA stimulates TLR9 mRNA expression

Because TLR9 is involved in pathogen DNA sensing, the effect of *T. gondii* DNA on the mRNA levels of this pattern recognition receptor was studied. Parasite DNA stimulation of Caco-2 cells with pure unmethylated tachyzoite DNA (5 μg mL^{-1}) resulted in a 6-fold increment in the expression of TLR9 (Fig. 3). Oppositely, TLR9 transcripts did not augment after treatment with methylated protozoan DNA. These results suggest that unmethylated CpG motifs in *T. gondii* genome are key inducers of TLR9 expression in intestinal epithelial cells.

Toxoplasma gondii DNA drives TLR9- and IFN-β-mediated DEFA-5 production in Caco-2 cells

Upon unmethylated tachyzoite DNA (5 μg mL^{-1}) transfection of Caco-2 cells, we detected a robust induction of the antimicrobial peptide DEFA-5 that was markedly reversed by blocking TLR9 with a specific inhibitory oligonucleotide in a concentration-dependent fashion (Fig. 4A). Addition of the anti-TLR9 oligonucleotide (30 nM) did not modify the lack of DEFA-5 response to methylated parasite DNA (Fig. 4B). Remarkably, the TLR9 inhibitory oligonucleotide was also able to impair ignition of DEFA-5 expression turned on after 24 h infection of intestinal epithelial cells with live *T. gondii* tachyzoites of the RH strain (Fig. 4C).

We next investigated whether TLR9-linked DEFA-5 expression was further associated with intestinal IFN-β secretion. Unlike methylated nucleic acid, unmethylated *T. gondii*-derived DNA significantly ($P < 0{\cdot}05$) induced IFN-β production in Caco-2 cells (Fig 5A). Specificity of the stimulatory pathway involved was confirmed by cell transfection with a TLR9 inhibitory oligonucleotide, which drastically impaired protozoan DNA-triggered IFN-β release. More important, intestinal DEFA-5 upregulation was strongly dependent on the TLR9-mediated IFN-β response to unmethylated CpG motifs in Toxoplasma genome (Fig. 5B). Addition of cytokine-specific blocking mAb abrogated the effect of unmethylated tachyzoite DNA and TLR9 activation on the induction of α-defensin response, suggesting that this type I IFN plays a central role in eliciting high levels of DEFA-5 in the intestinal epithelium.

DISCUSSION

Oral infection with *T. gondii* has been described to strongly promote α-defensin secretion in the gut (Foureau *et al*. 2010). As human defensin response is known to be upregulated by microbial DNA and immunostimulatory nucleotides (Platz *et al*. 2004; Han *et al*. 2009), we asked whether unmethylated CpG motifs present in Toxoplasma genome could boost α-defensin release from Caco-2 cells, an intestinal epithelial cell line susceptible to infection by this apicomplexan pathogen (Monroy, 2008). We verified DEFA-5 overexpression in *T. gondii*-infected cells and also upon microbial DNA transfection of uninfected intestinal epithelial cells, suggesting that parasite invasion is not a pre-requisite for α-defensin induction. In this sense, a previous report has documented broad release of DEFA-5-containing secretory granules from Paneth cells throughout the small intestine, even when infected at very low parasite dose and independently of the gut microflora (Foureau *et al*. 2010). Therefore, enhanced protozoan DNA uptake and internalization into the intestinal epithelium should be considered a novel alternative mechanism by which *T. gondii* elicits a rapid innate response that could be greatly amplified by immune mediators. We demonstrated that DNA purified from RH strain tachyzoites has a high G + C content and these nucleotides are not methylated. Our findings suggest that α-defensin induction is due to the effect of unmethylated CpG sequences in *T. gondii* nucleic acid on Caco-2 cells, as both DNA digestion

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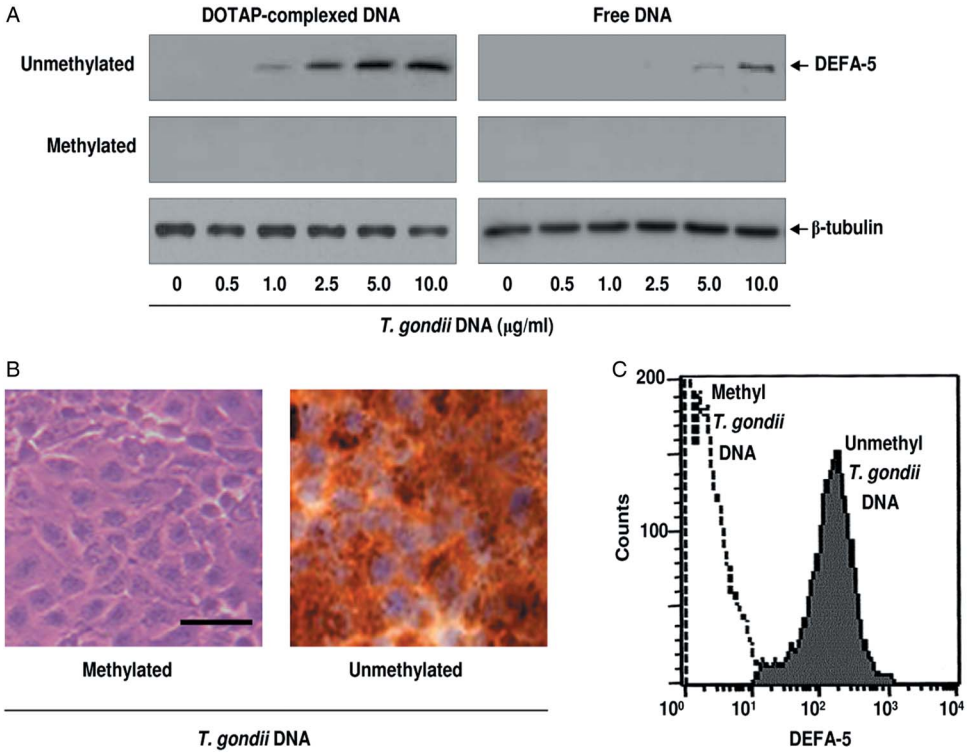

Fig. 2. DEFA-5 expression in intestinal epithelial cells *in vitro* stimulated with *T. gondii* DNA. (A) Dose effect of free and DOTAP-complexed tachyzoite DNA (unmethylated and methylated, see Materials and Methods section) on DEFA-5 expression in the Caco-2 cell line. The cells were DNA-transfected at the indicated concentrations for 24 h, and the levels of DEFA-5 and β-tubulin (loading marker) proteins were analysed by immunoblotting. (B) DEFA-5 overexpression in Caco-2 cells stimulated with unmethylated protozoan DNA. DAB staining of cell monolayers labelled using polymer immunohistochemical detection system (see Materials and Methods section). Bar: 100 μM. (C) Intracellular DEFA-5 production in intestinal epithelial cells in response to unmethylated and methylated Toxoplasma DNA. The expression of the antimicrobial peptide was determined by flow cytometry upon treatment with Brefeldin A and direct immunofluorescence staining with mAb (see Materials and Methods section). The histogram was obtained after analysing 10^5 cells. DEFA-5-related fluorescence intensity is shown on a four-decade log scale. The solid line (dark grey filled curve) represents Caco-2 cells stimulated with unmethylated DNA, whereas the dashed line (open curve) represents cells transfected with methylated nucleic acid. In every case, microphotographs and plots show one of at least three independent experiments.

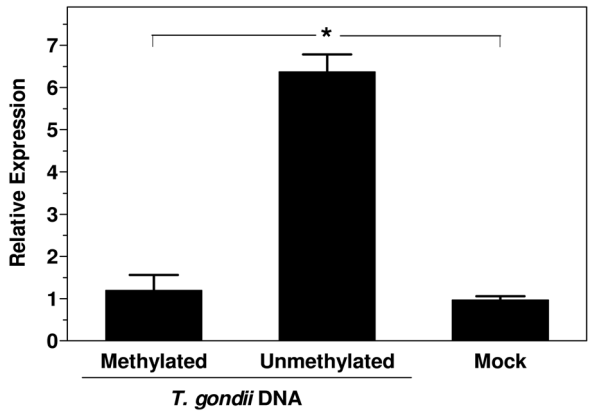

Fig. 3. Effect of *T. gondii* DNA on TLR9 mRNA expression levels in intestinal epithelial cells. Caco-2 cells were treated with pure unmethylated or methylated tachyzoite DNA (5 μg mL^{-1}) for 5 h. Mock-treated cells were used as controls. RNA was isolated, analysed by qRT-PCR with specific probes and normalized to GAPDH gene. Each bar represents the mean value ± S.E.M. Similar results were obtained in two additional experiments. *$P < 0{\cdot}05$.

and CpG methylation abolished DEFA-5 upregulation. In agreement with this, tachyzoite-derived CpG residues have been found to be mostly unmethylated and hundreds of these immunostimulatory motifs have been identified in the parasite genome (Andrade *et al*. 2013).

Since Toxoplasma DNA can activate phagocytic cells via TLR9 (Andrade *et al*. 2013), we next examined the participation of this receptor in parasite DNA-elicited DEFA-5 response by human intestinal cells. Akin to previous data from other infection models (Knuefermann *et al*. 2008; Juarez *et al*. 2010), exposure of Caco-2 cells to unmethylated DNA from *T. gondii* resulted in a significant elevation of TLR9 mRNA levels, suggesting that the intestinal epithelial inflammatory response to pathogen-derived nucleic acid is potentiated by increased TLR9 expression. More important, we identified a central role for the interaction of CpG-containing Toxoplasma DNA with TLR9 in triggering the antimicrobial peptide production in human gut epithelium. As a result of the

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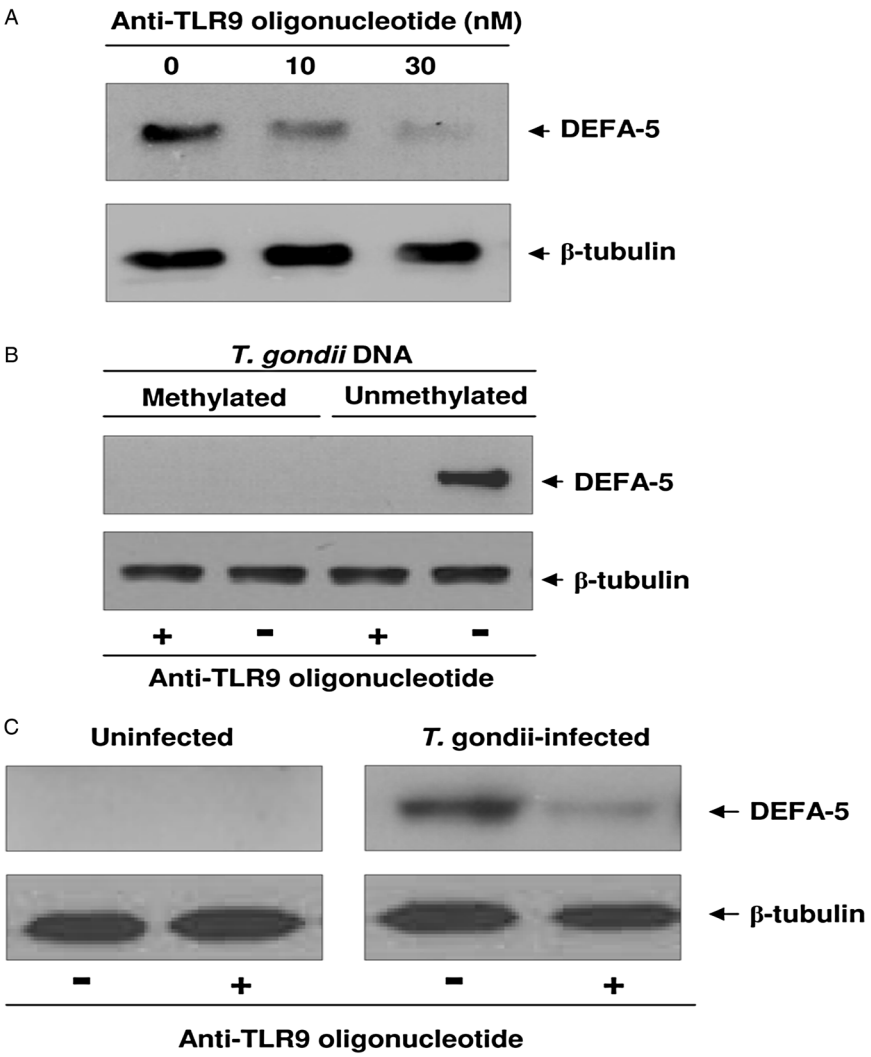

Fig. 4. Influence of TLR9 signalling on *T. gondii* infection and parasite DNA-mediated antimicrobial peptide production in intestinal epithelial cells. (A) Dose effect of specific inhibitory (anti-TLR9) oligonucleotide on DEFA-5 expression levels. Caco-2 cells were transfected with unmethylated tachyzoite DNA (5 μg mL^{-1}) in the absence or in the presence of TLR9 blocking oligonucleotide at increasing concentrations. DEFA-5 and β-tubulin proteins were assessed by immunoblotting as described under Materials and Methods section. (B) DEFA-5 response to methylated and unmethylated parasite DNA preparations following treatment with anti-TLR9 oligonucleotide (30 nM). (C) Specific oligonucleotide-mediated inhibition of TLR9-dependent DEFA-5 induction in Caco-2 cells exposed for 24 h to live *T. gondii* RH strain tachyzoites. Uninfected cells were included as controls. Representative results of Western immunoblot analysis are presented.

intracellular dynamics of parasite proliferation, tachyzoite DNA is released from the pathogen leading to TLR9 engagement (Melo *et al*. 2013). TLR9 co-localizes with intracellular tachyzoites in the endolysosomal compartment and functions as a key sensor of *T. gondii* through the recognition of unmethylated CpG sequences in Toxoplasma DNA (Andrade *et al*. 2013). Further, protozoan DNA fragments liberated by local inflammatory macrophages might enter intestinal epithelial cells by diffusion and act as a functional ligand for TLR9 to induce α-defensin secretion and degranulation (Rumio *et al*. 2004). Interestingly, extracellular Toxoplasma CpG dinucleotide sensing by TLR9 located on the surface of intestinal epithelial cells cannot be ruled out either (Ewaschuk *et al*. 2007). It is now accepted that TLR9 activation is essential for evoking the innate response to oral toxoplasmosis as well as for developing Th1-type anti-*T. gondii* immunoprotection in the small intestine (Minns *et al*. 2006). Human cells produce increased levels of proinflammatory cytokines in response to parasite-derived RNA and DNA, but not to *T. gondii* profilin (Andrade *et al*. 2013). Hence, in consideration of the lack of functional *TLR11* and *TLR12* genes in humans, it is tempting to speculate that the endosomal nucleic-acid sensing TLRs (i.e. TLR3, TLR7 and TLR9) and their downstream signalling pathways are important players in the early recognition of Toxoplasma DNA and RNA,

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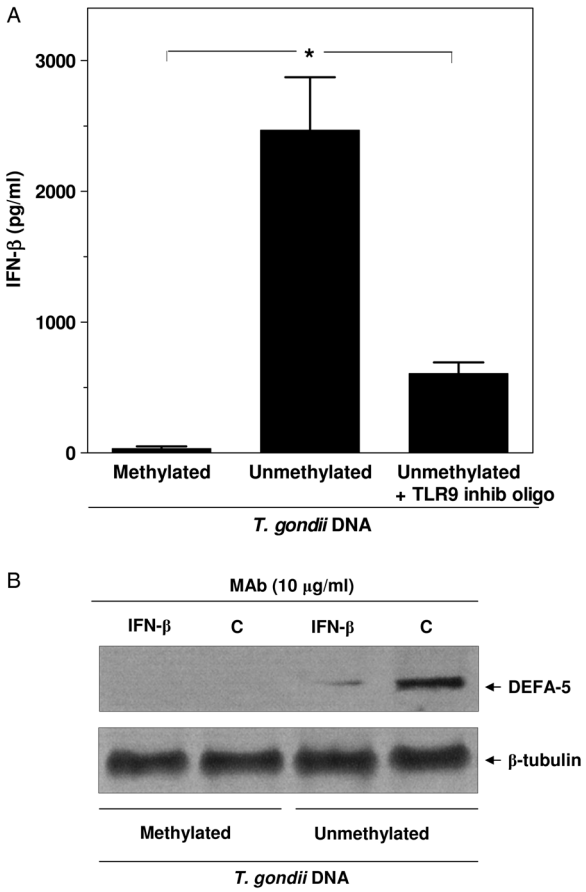

Fig. 5. Effect of *T. gondii*-derived DNA and TLR9 activation on IFN-β-associated DEFA-5 production in intestinal epithelial cells. (A) Role of TLR9 in inducing IFN-β release from Caco-2 cells. The enterocytes were transfected with unmethylated or methylated tachyzoite DNA in the absence or in the presence of anti-TLR9 oligonucleotide as indicated. IFN-β concentration (mean ± S.E.M.) in 24-h culture supernatants was quantified by ELISA. The results are representative of three independent experiments performed in triplicate. $*P <$ 0·05. (B) Analysis of IFN-β-dependent DEFA-5 response. Cells were stimulated with Toxoplasma DNA in the absence or in the presence of specific neutralizing mAb (IFN-β mAb) or isotype control antibody (C mAb) at 10 μg mL^{-1} as indicated. Protein expression of DEFA-5 and β-tubulin was visualized by immunoblotting. Results show a representative experiment of three performed.

as well as primary determinants of resistance to infection and the clinical outcome of human toxoplasmosis (Minns *et al*. 2006; Andrade *et al*. 2013).

Experimental oral toxoplasmosis leads to enhanced DEFA-5 synthesis and release from Paneth cells via a TLR9-dependent production of type I IFNs, which stimulate their own expression in an autocrine or paracrine manner (Minns *et al*. 2006; Foureau *et al*. 2010). IFN-α/β has been described to promote protozoan parasite killing and also to improve IFN-γ liberation from NK cells (Orellana *et al*. 1991; Diefenbach *et al*. 1998; Han *et al*. 2014), a crucial feature for the clearance of *T. gondii* (Ronet *et al*. 2005). Recently, Han *et al*. (2014) observed that IFN-β produced in response to heat-killed Toxoplasma organisms was greater than that achieved with live parasites. DNA isolated from different pathogens, including *T. gondii*, has been reported to efficiently drive type I IFN response (Wattrang *et al*. 2005; Kingma *et al*. 2011; Parker *et al*. 2011; Melo *et al*. 2013). Our results indicate that DEFA-5 release by intestinal epithelial cells is mediated at least in part by the TLR9-dependent IFN-β induction elicited by unmethylated CpG-containing Toxoplasma DNA. These observations shed light on the role of the human intestinal epithelium in pathogen recognition and in the first line of defence against this obligate intracellular parasite.

The precise contribution of DEFA-5 to immunoprotection against oral infection with *T. gondii* remains to be fully elucidated. Nonetheless, results from a study by Tanaka *et al*. (2010) revealed that DEFA-5 is an innate immune molecule that displays a beneficial role in controlling human toxoplasmosis. Exposure of tachyzoites to this antimicrobial peptide induces parasite aggregation, leading to Toxoplasma destruction and reduced intracellular infection. Besides, DEFA-5 has been shown to modulate nuclear factor κB-dependent expression of adhesion molecules, cytokines and chemokines in Caco-2 cells, and to act chemotactic to a variety of immune cells, including naïve and mature T cells, mast cells and macrophages (Grigat *et al*. 2007; Lu and de Leeuw, 2013). As demonstrated for enteric salmonellosis (Salzman *et al*. 2003), use of a transgenic mouse model showing human DEFA-5 expression specific to Paneth cells could be helpful in providing support for a critical *in vivo* role of epithelial-derived defensins in mammalian host defence against *T. gondii* infection.

The effective response to infection is now understood to involve productive interactions between *T. gondii*, the epithelial immune function and the host intestinal microbiota (Cohen and Denkers, 2015). Paneth cells are entangled in the innate defence and in preventing bacterial translocation that can occur during infection (Vaishnava *et al*. 2008). In addition to their ability to eliminate pathogens, Paneth cell-derived α-defensins are important regulators of the gut microbial community thereby ensuring a beneficial homoeostasis at the intestinal barrier (Salzman *et al*. 2010). Specific α-defensin deficiency has been identified as an integral element in the pathogenesis of inflammatory bowel disease (Wehkamp *et al*. 2005). Alterations in DEFA-5 expression can have a significant impact on the bacterial composition, rather than on total bacterial numbers, of the small intestine microbiome, which may result in skewing of the mucosal immunity (Salzman *et al*. 2010). Thus, the innate DEFA-5 response to oral Toxoplasma challenge

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could further contribute to modulate the parasite-triggered gut inflammatory activity responsible for Paneth cell death, dysbiosis and intestinal immunopathology (Raetz *et al*. 2013). On the other hand, DEFA-5 at very high concentration or over a prolonged period of time might provoke excessive inflammation favouring pathogen growth (Pagnini *et al*. 2011; Lu and de Leeuw, 2013).

During oral infection, the gut epithelial barrier is a strategic site to prevent, or at least to limit, disseminated toxoplasmosis. In the current study, we identified tachyzoite DNA carrying unmethylated CpG motifs as one of the parasite molecules deeply implicated in modulating the initial steps of immunity to *T. gondii* at the target site of oral infection. Our findings reveal that, in intestinal epithelial cells, protozoan DNA sensing results in activation of the TLR9/IFN-β/DEFA-5 pathway that may contribute to generate and amplify effector mechanisms to fight off the infection. A clear knowledge of the parasite components and the host receptors and mediators involved in the innate response is crucial for gaining a better insight into immunoprotection against human toxoplasmosis. This will in-turn broaden our ability to develop strategies for safe and more effective therapies.

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