Immunomodulatory activities of surface-layer proteins obtained from epidemic and hypervirulent *Clostridium difficile* strains

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Surface-layer proteins (SLPs) have been detected in all *Clostridium difficile* strains and play a role in adhesion, although an involvement in the inflammatory process may also be supposed, as they cover the bacterial surface and are immunodominant antigens. The aim of this study was to evaluate the immunomodulatory properties of SLPs obtained from hypervirulent and epidemic (H/E) or non-H/E *C. difficile* strains, to try to determine whether they contribute to hypervirulence. SLPs were purified from H/E PCR ribotype 027 and 001 and non-H/E PCR ribotype 012 *C. difficile* strains, and the ability to modulate these properties was studied in human *ex vivo* models of monocytes and monocyte-derived dendritic cells (MDDCs). The results indicated that SLPs were able to induce immunomodulatory cytokines [interleukin (IL)-1 β , IL-6 and IL-10] in monocytes. SLPs induced maturation of MDDCs, which acquired enhanced antigen-presenting activity, a crucial function of the mature stage. SLP-primed MDDCs expressed high levels of IL-10, an important regulatory cytokine. No significant differences were found in the activation induced in monocytes and MDDCs by SLP preparations from H/E and non-H/E strains. Overall, these findings show an important role for SLPs in modulation of the immune response to *C. difficile*. However, SLPs from H/E strains did not show a specific immunomodulatory pattern compared with SLPs from non-H/E strains, suggesting that SLPs are not involved in the increased severity of infection peculiar to H/E strains.

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INTRODUCTION

The epidemiology of *Clostridium difficile* infection has changed radically in the last 10 years (Pépin *et al.*, 2004) due to the emergence of a hypervirulent strain in North America and Europe, *C. difficile* NAP1/B1/027 (PCR ribotype 027) (Killgore *et al.*, 2008). The emergence of this hypervirulent and epidemic (H/E) strain has altered the face of the disease, with increased numbers of nosocomial outbreaks and concomitant morbidity (Hookman & Barkin, 2009).

Apart from the toxins, a small number of putative virulence determinants of the bacterium have been identified, including adhesins, flagella and heat-shock proteins (Hennequin *et al.*, 2001; Tasteyre *et al.*, 2001; Waligora *et al.*, 2001). One structure that has been implicated in adhesion of *C. difficile* to enteric cells is the bacterial surface (S) layer, a proteinaceous two-dimensional

paracrystalline array that completely surrounds the vegetative cell. The S layer of *C. difficile* is composed of two S-layer proteins (SLPs), the high-molecular-weight and low-molecular-weight proteins, which are derived from post-translational cleavage (Waligora *et al.*, 2001; Karjalainen *et al.*, 2001). SLPs have been detected in all *C. difficile* strains examined so far, although with sequence variability between different PCR ribotypes (Karjalainen *et al.*, 2001; Kato *et al.*, 2005; P. Spigaglia, F. Barbanti & P. Mastrantonio, unpublished data), suggesting that they are basic components of the bacterium. SLPs are immunodominant antigens (Mukherjee *et al.*, 2002), and thus a role in the pathological process and specifically in the inflammatory process has been hypothesized. In a previous study, we demonstrated that *C. difficile* SLPs may perturb the fine balance of inflammatory and regulatory cytokines in human monocytes and monocyte-derived dendritic cells (MDDCs). In particular, we showed that SLPs from *C. difficile* strain C253 were able to induce the release of elevated levels of inflammatory cytokines by monocytes and induce maturation of human MDDCs that become producers of both interleukin (IL)-12p70 and IL-10, acquire antigen-presenting cell (APC) activity and drive a mixed T-helper 1

Abbreviations: APC, antigen-presenting cell; BrdU, bromodeoxyuridine; DC, dendritic cell; FCS, fetal calf serum; H/E, hypervirulent and epidemic; IL, interleukin; MDDC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; SLP, surface-layer protein; Th, T helper.

(Th1)/Th2 polarization (Ausiello *et al.*, 2006). Moreover, the ability of SLP-matured MDCCs to drive a polarized T-cell effector profile suggests a possible use of these surface proteins as adjuvants.

Monocytes and dendritic cells (DCs) play important roles in the immune response as cells deeply involved in innate and adaptive immune defences (Taylor *et al.*, 2005; Sozzani *et al.*, 2000). Besides their role in phagocytosis and APC processes, they are potent immune regulatory cells that exert this ability by secreting a vast spectrum of modulating substances, including chemokines and cytokines (Sozzani *et al.*, 2000).

The aim of this study was to evaluate the capacity of SLPs purified from H/E *C. difficile* strains of PCR ribotypes 027 and 001, compared with non-H/E *C. difficile* strains of PCR ribotype 012, to modulate the functions of human monocytes and MDCCs, in particular their role in the induction of immunomodulatory cytokines, and to evaluate whether differences in the SLP structure are sufficient to trigger different inflammatory processes, in order to highlight possible mechanisms to explain the dissimilar levels of severity of infection caused by H/E and non-H/E strains.

METHODS

Reagents. Human recombinant granulocyte-macrophage colony-stimulating factor and recombinant IL-4 were from R&D Systems. FITC-conjugated anti-CD1a, phycoerythrin (PE)-conjugated anti-CD14, PE-conjugated anti-CD80 and PE-conjugated anti-CD83 mAbs were all from BD Biosciences.

Bacteria and growth conditions. Five *C. difficile* strains belonging to three different PCR ribotypes were used in this study (Table 1). Strains were cultured under anaerobic conditions on *Brucella* agar plates containing 0.5 mg vitamin K₁ l⁻¹, 5 mg haemin l⁻¹ and 5% defibrinated sheep red blood cells, and in brain-heart infusion (BHI) broth (Oxoid) for SLP extraction.

Extraction and characterization of SLPs from *C. difficile* strains. Bacteria were grown anaerobically in BHI broth for 18 h at 35 °C. *C. difficile* SLPs were extracted from whole cells by treatment with 0.2 M glycine (pH 2.2) as described previously (Calabi *et al.*, 2001), purified by gel filtration using a PD-10 desalting

column (GE Healthcare) and suspended in 0.05 M Tris/HCl (pH 7.4). To check the purity of the SLP preparations, the extracts were separated by SDS-PAGE on a 12% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Two bands were clearly visible corresponding to the high-molecular-weight and low-molecular-weight SLPs, and the purity of the extraction was ≥95%, as checked by densitometry, confirming the data of Calabi *et al.* (2001). Protein quantification was performed using a Pierce BCA Protein Assay kit – Reducing Agent Compatible (Thermo Fisher Scientific).

Monocyte isolation, MDCC generation and culture conditions.

Human monocytes were purified from peripheral blood mononuclear cells (PBMCs) of healthy blood donors (courtesy of Dr Girelli, 'Centro Trasfusionale Policlinico Umberto I', University La Sapienza, Rome, Italy) by centrifugation on density gradients (Lympholyte-H; Cedarlane). Monocytes were purified from PBMCs by using a subsequent purification step performed by magnetic bead separation protocols (Miltenyi Biotec). Monocytes were >90% pure, as assessed by the expression of the surface marker CD14 (Ausiello *et al.*, 2006).

Monocytes were washed and suspended at a concentration of 1×10^6 cells ml⁻¹ in RPMI 1640 (Gibco) supplemented with 5% LPS-free fetal calf serum (FCS) and antibiotics (50 IU penicillin ml⁻¹ and 50 µg streptomycin ml⁻¹; Hyclone Laboratories) in the presence of a pre-determined optimal dose (40 µg ml⁻¹) of different SLP preparations in 14 ml tubes (Falcon; Becton Dickinson) at 37 °C in a 5% CO₂ atmosphere. Supernatants were collected after 24 h and used to measure the levels of IL-1β, IL-6 and IL-10.

MDCC generation was performed as described previously (Ausiello *et al.*, 2006) by culturing human monocytes in RPMI 1640, supplemented with heat-inactivated 10% LPS-screened FCS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 25 mM HEPES, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (all from Hyclone Laboratories) and 0.05 mM 2-mercaptoethanol (Sigma Chemicals), hereafter defined as complete medium, in the presence of 25 ng granulocyte-macrophage colony-stimulating factor ml⁻¹ and 25 ng IL-4 ml⁻¹. After 6 days, immature MDCCs were washed and analysed by cytofluorometric analysis for CD1a and CD14 expression. MDCCs were used in the experiments if they had >80% CD1a and <10% CD14.

MDCCs (10^6 cells ml⁻¹) were resuspended in complete medium and treated with different SLP preparations. After 48 h, treated MDCCs were harvested for immunophenotypic analysis and the supernatants for cytokine measurement by ELISA.

Isolation of T lymphocytes and MDCC/T-cell allogenic mixed leukocyte reaction. CD3 T cells were purified from PBMCs of healthy blood donors by negative sorting using magnetic beads (Pan T Cell Isolation kit; Miltenyi Biotec). The purity of cell preparations was assessed by cytofluorometric staining.

MDCCs (10^6 cells ml⁻¹) were resuspended in complete medium and treated with different SLP preparations for 48 h, washed extensively and cultured in a mixed leukocyte reaction with T cells (3×10^5) at different ratios in 48-well culture plates for 6 days. Cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation evaluated by direct immunofluorescence with an FITC-conjugated anti-BrdU mAb (BD Biosciences). Briefly, BrdU (10 mg ml⁻¹; BD Biosciences) was added to an MDCC and T-cell mixed leukocyte reaction at a final concentration of 3 µg ml⁻¹ on days 3 and 5 of culture. Cells were collected on day 6, fixed in 0.5% paraformaldehyde, permeabilized and stained for intracellular BrdU by direct immunofluorescence with an FITC-conjugated anti-BrdU mAb. Cells were examined by flow cytometry, and T-cell proliferation was evaluated.

Table 1. *C. difficile* strains used in this study

Strain	PCR ribotype	Source or reference
001-3	001	ECDC*
C253	012	Cerquetti <i>et al.</i> (2000)
630	012	Wüst <i>et al.</i> (1982)
R20291	027	ECDC*
AI13	027	Barbut <i>et al.</i> (2007)

*These strains were kindly supplied by Dr Ed Kuijper on behalf of the European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden.

Immunophenotypic analysis. Cells were washed and resuspended in PBS containing 3 % FCS and 0.09 % NaN₃, and incubated with a panel of fluorochrome-conjugated mAbs (BD Biosciences) specific for CD14, CD1a, CD80 and CD83 surface markers.

Isotype-matched antibodies were used as negative controls. Cells were analysed with a FACScan (BD Biosciences). Fluorescence data are reported as the percentage of positive cells when treatment induced the expression of the marker in cells that were negative; median fluorescence intensity was used when treatment increased the expression of the marker in cells that were already positive.

Cytokine measurement by ELISA. IL-1 β , IL-6 and IL-10 were measured in monocyte culture supernatants by ELISA (Quantikine; R&D Systems) with a sensitivity of 0.7 pg ml⁻¹ for IL-6, 1 pg ml⁻¹ for IL-1 β and 3.9 pg ml⁻¹ for IL-10. The production of IL-10 and heterodimeric IL-12p70, the bioactive form of IL-12, was assessed in MDDC culture supernatants by ELISA (Quantikine; R&D Systems) with a sensitivity of 5 pg ml⁻¹ for IL-12p70.

Statistical analysis. Statistical descriptive analyses were carried out using the SPSS statistical package. Differences between mean values were assessed using a two-tailed Student's *t*-test and were statistically significant for *P* < 0.05.

RESULTS

SLPs from H/E and non-H/E *C. difficile* strains induce IL-1 β , IL-6 and IL-10 in monocytes

In a previous study, we showed that SLPs purified from non-H/E *C. difficile* strain C253, PCR ribotype 012, induced IL-1 β and IL-6 pro-inflammatory cytokine production in human monocytes (Ausiello *et al.*, 2006). Here, we measured the capacity of a broader panel of H/E and non-H/E SLPs to induce IL-1 β , IL-6 and also IL-10, a regulatory cytokine, in human monocytes. Resting monocytes were obtained after positive selection from PBMCs of healthy individuals and stimulated for 24 h with SLPs. Treatment of the monocytes with a pre-determined optimal dose of the different SLP preparations (40 μ g ml⁻¹) was followed by an increase in release of IL-1 β and IL-6 pro-inflammatory cytokines and IL-10, an anti-inflammatory/regulatory cytokine (Fig. 1). SLPs derived from all the *C. difficile* strains induced a statistically significant higher level of all the cytokines tested compared with untreated cultures. No statistically significant differences in the capacity of SLPs from H/E and non-H/E strains to induce cytokine production were found (Fig. 1).

SLPs from H/E and non-H/E *C. difficile* strains induce maturation and functional activation of MDDCs

The ability to induce MDDC maturation implies an important role for the proteins under examination, influencing MDDC functions. The transition of MDDCs from phagocytic to APC functions is depicted by modification of the surface phenotype (Rescigno, 2002). The ability of MDDCs to induce the expression of well-established maturation markers when activated by *C.*

Fig. 1. SLP extracts from H/E and non-H/E *C. difficile* strains induce IL-1 β , IL-6 and IL-10 in human monocytes. Supernatants of resting monocytes either untreated (none) or treated with different SLP preparations derived from H/E strains R20291, A113 and 001-3 or from non-H/E strains C253 and 630, or with *Escherichia coli* (Ec) LPS (0.1 μ g ml⁻¹, positive control), were collected after 24 h, and IL-1 β , IL-6 and IL-10 levels (pg ml⁻¹) were measured by ELISA. Values were expressed as mean $\pm$ SEM of six independent experiments performed with monocytes from different healthy donors. *, *P* < 0.05 relative to unstimulated monocyte cultures.

difficile preparations was studied. Fig. 2 summarizes the comparative analysis of phenotypic maturation of MDDCs obtained from different healthy individuals. SLPs induced increased expression on MDDCs of the co-stimulatory molecule CD80 and the maturation marker CD83, which reached statistical significance compared with untreated MDDCs.

As the essential aspects of MDDC function *in vivo* are antigen presentation and T-cell activation (Lanzavecchia &

Fig. 2. SLP extracts from H/E and non-H/E *C. difficile* strains induce phenotypic maturation of MDDCs. MDDCs either untreated (none) or treated with SLPs derived from H/E strains R20291, AI13 and 001-3 or from non-H/E strains C253 and 630, or with *E. coli* (Ec) LPS ($0.1 \mu\text{g ml}^{-1}$, positive control) for 48 h, were analysed for surface markers associated with the mature phenotype. Fluorescence data are reported as median fluorescence intensity (MFI) when treatment increased the expression of the marker in cells that were already positive (CD80); otherwise the percentage of positive cells was used (CD83). Columns represent cumulative results (mean $\pm$ SEM) of six independent experiments performed with MDDCs obtained from different healthy donors. *, $P < 0.05$ relative to untreated DC cultures.

Sallusto, 2001), experiments were performed to evaluate the capacity of mature MDDCs to stimulate alloreactive T cells. Fig. 3 shows that MDDCs obtained after culturing with SLPs were able to promote a statistically significant increase of the basal degree of proliferation of allogenic T cells achievable with unstimulated MDDCs. Again, no major differences were observed among SLPs from H/E and non-H/E strains.

H/E and non-H/E SLP-matured MDDCs induce the production of regulatory cytokines

MDDCs are powerful producers of key pro- and anti-inflammatory and immunoregulatory cytokines, especially

Fig. 3. SLP extracts from H/E and non-H/E *C. difficile* strains enhance MDDC allostimulatory activity. MDDCs either untreated (○) or treated with SLPs derived from H/E strains R20291 (□), AI13 (●) and 001-3 (■) or from non-H/E strains C253 (▲) and 630 (△) for 48 h were co-cultured in different numbers with allogenic purified T cells (3×10^5) for 6 days. Proliferation was assessed by BrdU incorporation. Results are reported as the percentage (mean $\pm$ SEM) of positive cells from three independent experiments performed with MDDCs and T cells obtained from different healthy donors. *, $P < 0.05$ relative to SLP-treated MDDC cultures.

when activated by microbial antigens and adjuvants (Lanzavecchia & Sallusto, 2001; Ausiello *et al.*, 2002; Fedele *et al.*, 2005). Thus, we tested the ability of SLPs derived from H/E and non-H/E *C. difficile* strains to promote cytokine production by MDDCs, particularly IL-12p70 and IL-10, which are pro- and anti-inflammatory cytokines, respectively (Trinchieri, 2003; Moore *et al.*, 2001). Fig. 4 shows that MDDCs stimulated with SLPs were able to produce high levels of IL-10, which were statistically significant compared with untreated cells, and low or undetectable levels of IL-12p70. The only exception was SLPs derived from *C. difficile* strain C253, a non-H/E ribotype, which induced substantial levels of IL-12p70, confirming our previous results (Ausiello *et al.*, 2006).

DISCUSSION

C. difficile SLPs are the outermost surface components of the bacterium and have been shown to be involved in the mechanism of gut colonization and adhesion to the intestinal mucosa (Cerquetti *et al.*, 2000; Calabi *et al.*, 2002). It has been proposed that SLPs mediate binding to both the intestinal epithelial cells and some components of their extracellular matrix fibres, contributing to further tissue damage (Cerquetti *et al.*, 2000; Calabi *et al.*, 2002).

On the basis of direct and indirect evidence, SLPs have been proposed to have a role in modulation of the host

Fig. 4. SLP extracts from H/E and non-H/E *C. difficile* strains induce IL-12p70 (■) and IL-10 (□) regulatory cytokines in MDDCs. MDDCs were either untreated (none) or treated with SLPs derived from H/E strains R20291, AI13 and 001-3 or from non-H/E strains C253 and 630, or with *E. coli* (Ec) LPS (0.1 µg ml⁻¹, positive control) for 48 h. Cytokine production was assessed by ELISA. Values are expressed as pg ml⁻¹ (mean ± SEM) from six independent experiments performed with MDDCs obtained from different healthy donors. *, $P < 0.05$ relative to untreated DC cultures.

immune response (Mukherjee *et al.*, 2002; O'Brien *et al.*, 2005). In particular, IgM anti-SLPs have been associated with a reduced risk of recurrent *C. difficile* infection in humans (Kyne *et al.*, 2001). Furthermore, a protective effect of anti-SLP serum has been observed recently in a lethal hamster challenge model (O'Brien *et al.*, 2005). This study aimed to compare the immunomodulatory activities of SLPs purified from H/E and non-H/E strains to understand whether differential modulation of immune-cell functions by SLPs obtained from strains with different spreading ability and capacity to cause severe disease participated in the hypervirulence mechanisms. Indeed, it has been shown that the nucleotide sequences of SLP-encoding genes from H/E and non-H/E strains seem rather conserved within the same PCR ribotype, whilst they present differences among the different PCR ribotypes (Eidhin *et al.*, 2006; Spigaglia *et al.*, 2011).

We found that SLPs from the H/E and non-H/E *C. difficile* strains included in this study showed the ability to strongly influence the cytokine profile in monocytes and MDDCs. Indeed, SLP-treated monocytes induced the production of high levels of IL-1β and IL-6, two potent pro-inflammatory cytokines, confirming our previous results with strain C253 SLPs (Ausiello *et al.*, 2006), but also IL-10, an anti-inflammatory and regulatory cytokine. MDDCs treated with SLPs from H/E strains acquired an anti-inflammatory phenotype witnessed by the expression of IL-10 but not IL-12p70. In the case of the non-H/E strains, this point requires further investigation as SLPs of strain 630 did not induce IL12p70, whilst strain C253 SLPs clearly induced IL-12p70 secretion, confirming results obtained in our previous work (Ausiello *et al.*, 2006).

Microbial infections induce the recruitment of circulating monocytes and DCs to gut-associated lymphoid tissues (Serbina *et al.*, 2008). Monocytes take part in the innate immune defence through direct antimicrobial activity and the release of inflammatory mediators that further sustain the recruitment and activation of immune effectors (Yona & Jung, 2010). DCs are professional APCs that possess the unique ability to bridge innate and adaptive immunity.

Upon maturation, DCs migrate to the secondary lymph nodes where they encounter and activate naïve T cells. DCs, through cytokine production, dictate the development of Th cells towards the Th1, Th2, Th17 or T-regulatory subsets (Steinman & Hemmi, 2006; Acosta-Rodriguez *et al.*, 2007).

Our findings suggested that *C. difficile* SLPs may foster mucosal inflammation through their action on recruited monocytes, which were induced to secrete high levels of IL-1β and IL-6. Although the inflammatory response is pivotal in counteracting microbial infections, an exacerbated inflammatory state is often detrimental and the cause of adverse pathology in several disease settings including intestinal diseases (Koboziev *et al.*, 2010). Here, we showed novel results concerning SLPs purified from H/E *C. difficile* strains: a clear induction of an anti-inflammatory profile characterized by IL-10 production in both monocytes and MDDCs and the failure to induce IL-12p70 in MDDCs. These findings may indicate that a regulatory mechanism has evolved to maintain gut immune homeostasis by generating a non-inflammatory environment.

Moreover, SLP-treated MDDCs were efficient APCs driving the expansion of T-cell effectors that may contribute to counteract bacterial aggressiveness.

Overall, our results suggest that SLPs are recognized by effectors of the innate and adaptive cell immune system and possess very similar immunomodulatory activities. SLPs of H/E strains trigger inflammatory processes similar to those obtained from PCR ribotypes usually associated with non-H/E sporadic cases. Therefore, SLP-mediated immunity does not seem to be linked to a specific ribotype and does not appear to contribute to the recently emerged hypervirulence phenomenon.

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