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)-1β, 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.

INTRODUCTION

The epidemiology of Clostridium difficile infection has changed radically in the last 10 years (Peòpin et al., 2004) due to the emergence of a hypervirulent strain in North America and Europe, C. difficile NAP1/BI/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 MDDCs 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 MDDCs, 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 the expression of the surface marker CD14 (Ausiello et al., 2006).

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

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<th>Strain</th>
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<td>001-3</td>
<td>001</td>
<td>ECDC*</td>
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<tr>
<td>C253</td>
<td>012</td>
<td>Cerquetti et al. (2000)</td>
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<tr>
<td>630</td>
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<td>Wüst et al. (1982)</td>
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<tr>
<td>R20291</td>
<td>027</td>
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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-1β, 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-1β 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-1β, 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-1β 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-1β, 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 μg ml⁻¹) was followed by an increase in release of IL-1β 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. 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 &
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 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
high levels of IL-1
Indeed, SLP-treated monocytes induced the production of influence the cytokine profile in monocytes and MDDCs. strains included in this study showed the ability to strongly
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
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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.


distributed 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.

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REFERENCES


