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Immunomodulators Released During Rotavirus Infection of Polarized Caco-2 Cells

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Abstract

Rotavirus preferentially replicates in enterocytes and "danger signals" released by these cells are likely to modulate viral immunity. As a model of these events, we studied selected immunomodulators released during rotavirus infection of polarized Caco-2 cells grown in transwell cultures (TW). At early time points post-infection the virus was detected mainly in the apical side of the TWs, but this tendency was progressively lost concomitantly with disruption of the cell monolayer and cell death. Rotavirus-infected cells released IL-8, PGE_2, small quantities of TGF-β1, and the constitutive and inducible heat shock proteins HSC70 and HSP70, but not IL-1β, IL-6, IL-10, IL-12p70, or TNF-α. This set of immunomodulators is known to induce a non-inflammatory (non-Th-1) immune response, and may be determining, in part, the relatively low T-cell immune response observed in blood samples after RV infection.

Introduction

Rotavirus (RV) is the single most important etiological agent of severe gastroenteritis in children (1). Notwithstanding, our knowledge of RV immunity in humans is incomplete and does not permit us to rationally design or improve existing vaccines (1). RV replicates preferentially in enterocytes, and "danger signals" released by these cells, both endogenous (cytokines and other cellular products not released by uninfected cells [29]) and exogenous (the virus itself and individual viral components) are likely to be key immunomodulators (IM). Since enterocytes are difficult to grow *in vitro* (47), intestinal epithelial cell (IEC) lines derived from tumors (mostly Caco-2 and HT-29 cells) infected with RV (RV-IEC) have been used as a model to study these IM. RV-IEC secrete IL-8, GROα, and RANTES, but not IFN-α, TNF-α, IFN-γ, IL-1α/β, or IL-6 (9,46). Moreover, in RV-IEC the mRNA of IL-8, GROα, IP-10, RANTES, MCP-1, and GM-CSF, but not IL-1α/β and TNF-α, are induced (43). Increases of IFN-α mRNA but not IFN-β, were detected in the RV-IEC (43). A microarray analysis of RV-IEC confirmed the increase of RANTES mRNA, and in addition, showed noteworthy increases in the mRNA levels of prostaglandin-endoperoxide synthase 2, and several stress-response and interferon-inducible genes (13). Other authors confirmed the induction of prostaglandin endoperoxide synthase mRNA and found that, as expected, PGE_2 is secreted by infected IEC (44). Osteopontin (42) and nitric oxide (40) are two more recently described IM secreted by RV-IEC.

Of note, the majority of these studies have been done with nonpolarized IEC. Studies with polarized IEC are important because toll-like receptors (TLR), involved in the recognition of pathogens, are differentially expressed in the apical and basolateral surfaces of IEC and enterocytes, and stimulation of either of these surfaces may induce differential secretion of IM (25). Moreover, studies to comprehensively evaluate IM released by interaction of a pathogen with IEC require that the *in vitro* model be adapted to include cells from the immune system (dendritic cells [DC] [39] or PBMC [18], for example), and this has not been done for the RV model.

Heat shock proteins (HSPs) are "endogenous danger signals" released during tissue injury (20,29). Purified HSPs, synthetic fragments derived from them (20), or HSP that chaperone pathogen molecules like LPS (52), have been shown to directly activate antigen-presenting cells. In addition, HSPs can chaperone viral peptides to antigen-presenting cells for cross-priming of specific $CD8^+$ T cells *in vitro* (20). Although the occurrence of this last function *in vivo* is controversial (20), HSP have been shown to bind viral peptides *in vivo* (30), and HSP loaded *in vitro* with viral peptides can induce protective antiviral immune responses (26,28,38). Typically, HSPs are thought to be released passively after cell lysis; however, they can also be actively released before cell death (4).

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In this study, we investigated selected IM released by polarized Caco-2 cells following RV infection in transwell cultures (TW). As a first step in our studies, we compared the interaction of RV with cells grown in TW with membranes that have pores of 3 μm and 0.4 μm. Both types of TW have been used in the past to study RV-infected cells (10,11,45,48). The first type of TW is necessary for studies of IM when DCs are incorporated in the model (39), while the latter type of TW seems to be better suited for studying the polarized released of IM, since in the former Caco-2 cells can partially traverse the pores (11). We found that IM released in both systems are in general comparable, and that RV induced the release of IL-8, PGE_2, and small quantities of TGF-β1, HSP70, and HSC70 (a constitutive non-inducible HSP).

Materials and Methods

Cells and cell culture

Caco-2 cells (a gift from C. Sapin INSERM U 538, Université Pierre et Marie Curie, Paris, France) were cultured in Dulbecco modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (FCS; Invitrogen-Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 0.1 mM non-essential amino acids (Invitrogen-Gibco), and used between passages 68 and 77. Stocks of cells used were found to be free of *Mycoplasma* (*Mycoplasma* detection kit for conventional PCR VenorGem; Sigma, St. Louis, MO), and experiments were performed in the presence of 0.5 μg/mL ciprofloxacin to maintain them in this state. Cells were seeded at 10,000 cells/cm^2 on TW (tissue culture treated polycarbonate membrane with 3.0 μm or 0.4 μm pores and a 24-mm insert) 6-well plates (Costar; Corning Inc., Corning, NY). Monolayer integrity was monitored by measuring the transepithelial electrical resistance (TER) using a Millicel ERS apparatus (Millipore, Bedford, MA). The TER was calculated by subtracting the background resistance from the experimental resistance (measured in TW with medium only), and then multiplying this value by the area of the filter. Culture medium (both from the apical and basolateral chambers) was replaced every 2 d until day 10 of culture, and from this day on medium was replaced every day. The cells were used at day 21 post-seeding, when they were fully differentiated (23).

Cell death was assessed by measurement of lactate dehydrogenase (LDH) in the culture medium using an LDH kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. An LDH standard curve was used for some experiments to express results as units of LDH/mg of protein in the samples. Similar conclusions were obtained expressing results with this system or as ELISA optical densities, and results are presented in the latter format.

Fluorescence microscopy was performed using a Zeiss LSM512 microscope (Carl Zeiss, Jend, Germany) with a Plan-Neo Fluor 40×/1.30 oil objective and a 405-nm wavelength.

CpG treatment

Fully differentiated Caco-2 cells were treated apically or basolaterally with 10 μg/mL of CpG 2395 (Coley Pharmaceutical Group Inc., Wellesley, MA) for 24 h (25), and then the supernatants from both sides of the chamber were collected to quantify IL-8 as described above.

RV infections

Rhesus RV (RRV; obtained from Harry Greenberg, Stanford University, Palo Alto, CA) was grown and tittered on MA104 cells (33). RRV was used after cesium purification or used as an unpurified supernatant of infected MA104 cells. The medium used to dialyze the purified virus was used as a negative control for experiments with purified virus. When unpurified RRV was used, the supernatant of mock-infected MA104 was used as a negative control. RRV was activated for 30 min by treatment with 2 μg/mL of trypsin. At day 20 post-seeding the cells were deprived of FCS and infected at day 21 (23). Cells grown in TW were inoculated apically with RV for 45 min. Then the inoculum was removed, the monolayers were washed twice, and new DMEM without FCS was added. Apical and basolateral supernatants were collected at 6, 12, 16, 24, or 48 h post-infection (h.p.i.), centrifuged at 250×g for 10 min to remove cellular debris, and frozen at −20°C. In pilot experiments infections with a multiplicity of infection (MOI) of 5 or 10 FFU/cell yielded similar results, and all experiments shown are with an MOI of 5. Tissue culture-adapted human RV strains Wa and ITO were obtained from Harry Greenberg and Sue Crawford, Baylor College of Medicine, Houston, TX, respectively, and grown as described above for RRV.

ELISAs for the measurement of cytokines and HSP70

Cytokines and HSP70 were measured with commercial kits, following the manufacturer's instructions. IL-1β, IL-6, IL-10, IL-12p70, and TNF-α were detected using a cytometric bead array (CBA) human inflammation kit (BD Biosciences, San Jose, CA; limit of detection from 1.9–7.2 pg/mL). IL-8 was measured with the CBA or with an ELISA Duoset kit (R&D Systems, Minneapolis, MN; detection limit of 31.2 pg/mL). Total (active plus latent activated according to the manufacturer's instructions) and active transforming growth factor-β1 (TGF-β1) was quantified by ELISA with a Duoset kit (R&D Systems; detection limit of 31.2 pg/mL). To indirectly measure PGE_2 an EIA kit (Cayman Chemical, Ann Arbor, MI; detection limit 0.39 pg/mL) that quantifies the prostaglandin E metabolite 13,14-dihydro-15-keto prostaglandin E_2 (PGE_2M) was used. The PGE_2M, and not the PGE_2 itself, was measured because PGE_2 is detected only transiently after RV infection (44). HSP70 was measured with the EKS700 kit (StressGen Biotechnologies, Victoria, BC, Canada; detection limit of 0.78 ng/mL).

Western blot for the measurement of HSC70

Samples tested were resuspended in reducing (DTT 0.1 M) Laemmli buffer and evaluated by Western blot. Proteins were separated by SDS-PAGE (10% gels) and transferred to polyvinylidene fluoride membranes (Biorad, Hercules, CA). The membranes were blocked with Tris-HCl (pH 7.5) containing 5% skim milk and 0.05% Tween 20 for 1 h and then incubated with appropriately tittered primary antibodies for 1 h (mouse monoclonal antibody [Mab] anti-HSC70, clone B-6; Santa Cruz Biotechnology, Santa Cruz, CA, and anti-VP6, clone 1026; a generous gift from E. Kohli, Université de Dijon, Dijon, France). After washing, the blots were incubated for 50 min with ImmunoPure peroxidase conjugated goat anti-mouse IgG (Pierce Biotechnology, Inc., Rockford, IL).

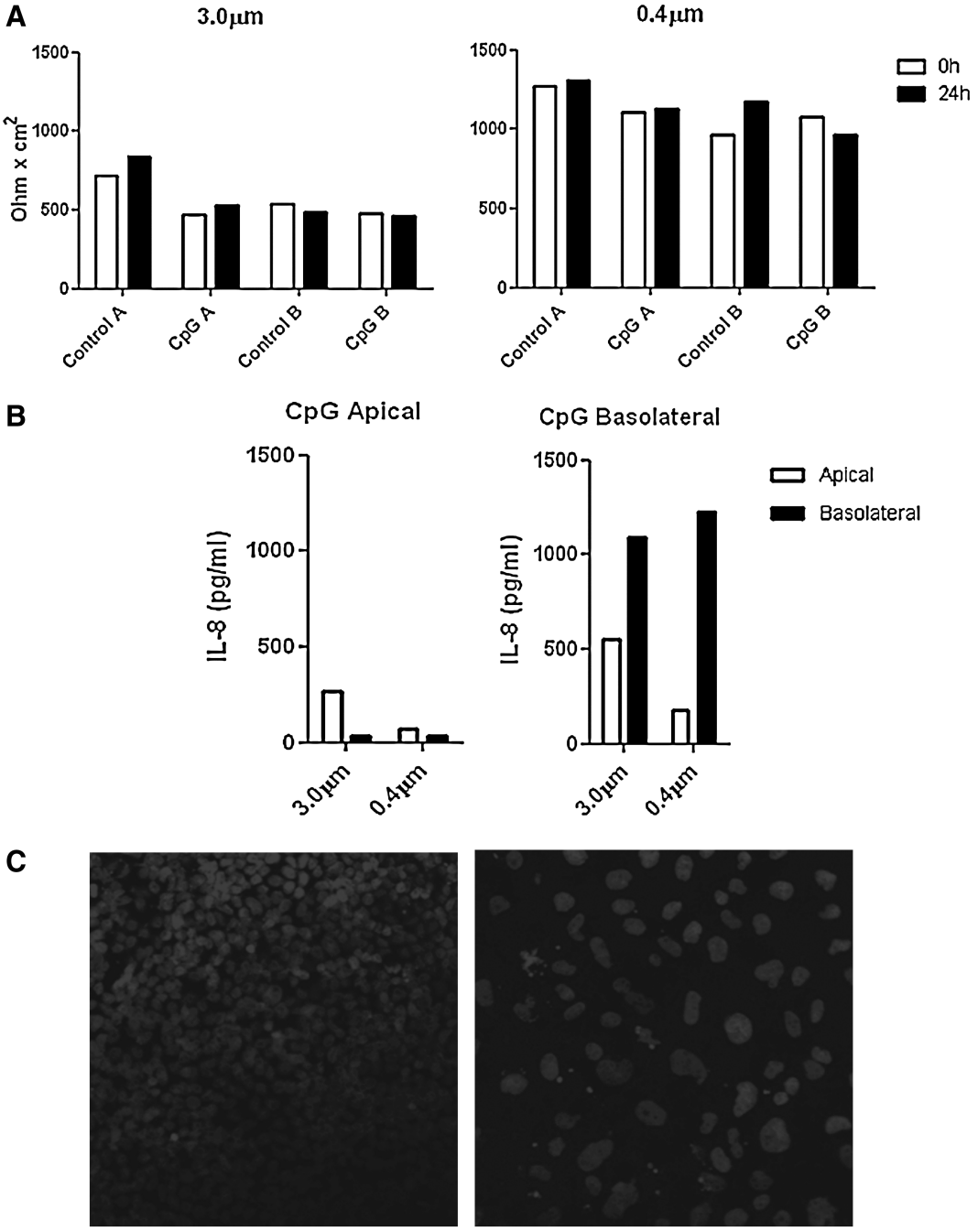

FIG. 1. Basolateral treatment of Caco-2 cells with CpG preferentially induces the basolateral secretion of IL-8. Cells grown in 3-μm or 0.4-μm pore size TW were treated apically or basolaterally with CpG 10 μg/mL and the TER and IL-8 in the supernatants were measured 24 h after stimulation. (**A**) Monolayer integrity was monitored by measurement of the TER. (**B**) IL-8 secretion was measured in the apical and basolateral supernantants by ELISA. Shown is one experiment representative of three performed. (**C**) Caco-2 cells cultured in 3.0-μm pore size TW grow in the opposite side of the filter. A Caco-2 cell monolayer grown in 3-μm TW after 21 d of culture were stained with DAPI and analyzed by Fuorescence microscopy (left panel, apical side of the filter; right panel, basolateral side of the filter).

The blots were developed using the chemiluminescent Supersignal West Dura Extended Duration substrate (Pierce Biotechnology) and CL-XPosure films (Pierce Biotechnology). As a positive control for the detection of HSC70, a recombinant bovine HSC70 (SPP-751) from Stressgen Biotechnologies was used.

Statistical analysis

Statistical analysis was performed with SPSS software version 12.0 (SPSS Inc., Chicago, IL) and GraphPad Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA), using a non-parametric test. Differences between paired

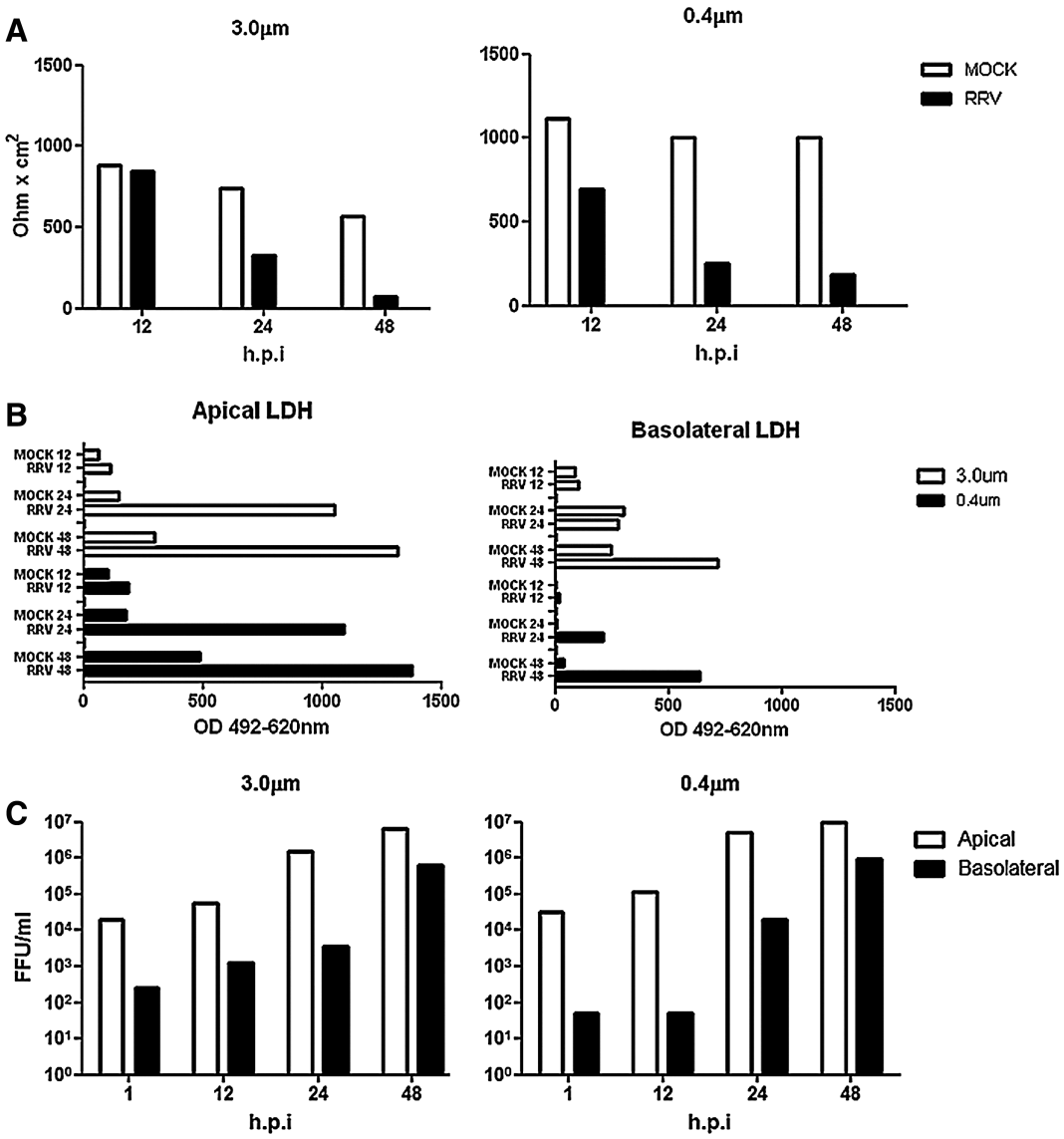

FIG. 2. Time course of RRV infection in 3.0-μm or 0.4-μm TW systems. (**A**) Monolayer integrity of mock or RV treated cells was monitored by measurement of the TER. (**B**) Cell death was evaluated using LDH. (**C**) Infectious particles released in the apical or basolateral compartments were quantified at different time points after infection by titration of supernatants on MA-104 cells. Data are representative of two comparative independent experiments with the two types of TW, and from 3–11 experiments with 3.0-μm TW.

results were determined with the Wilcoxon test. Significance was set at $p < 0.05$. Data are shown as medians unless otherwise noted.

Results

Basolateral CpG treatment preferentially induces the basolateral secretion of IL-8 in Caco-2 cells grown in both 3-μm and 0.4-μm TW

Polarized Caco-2 cells display many of the functional and morphologic characteristics of human mature enterocytes, in which RV replicates (23). Lee *et al.* recently showed that in these cells TLR9 activation through apical and basolateral poles have distinct transcriptional responses (25). To compare this functional differentiation of Caco-2 cells grown in 3-μm and 0.4-μm TW, cells were stimulated apically or basolaterally with CpG (a ligand of TLR9) and IL-8 was measured 24 h after stimulation in the apical and basolateral compartments (25). Although the TER was lower in the 3-μm than in the 0.4-μm TW, it was not changed by CpG treatment, suggesting that the integrity of the monolayers was conserved during the experiments (Fig. 1A). In addition, CpG treatment did not induce cell death, as determined by the measurement of basolateral and apical LDH before and after treatment in both types of TW (data not shown). In agreement with a previous report (25), low amounts of IL-8 were detected in the apical and basolateral compartments when CpG was administered to Caco-2 cells apically (Fig. 1B). In contrast, CpG administered basolaterally induced IL-8 secretion in the basolateral, but not the apical, compartment (Fig. 1B).

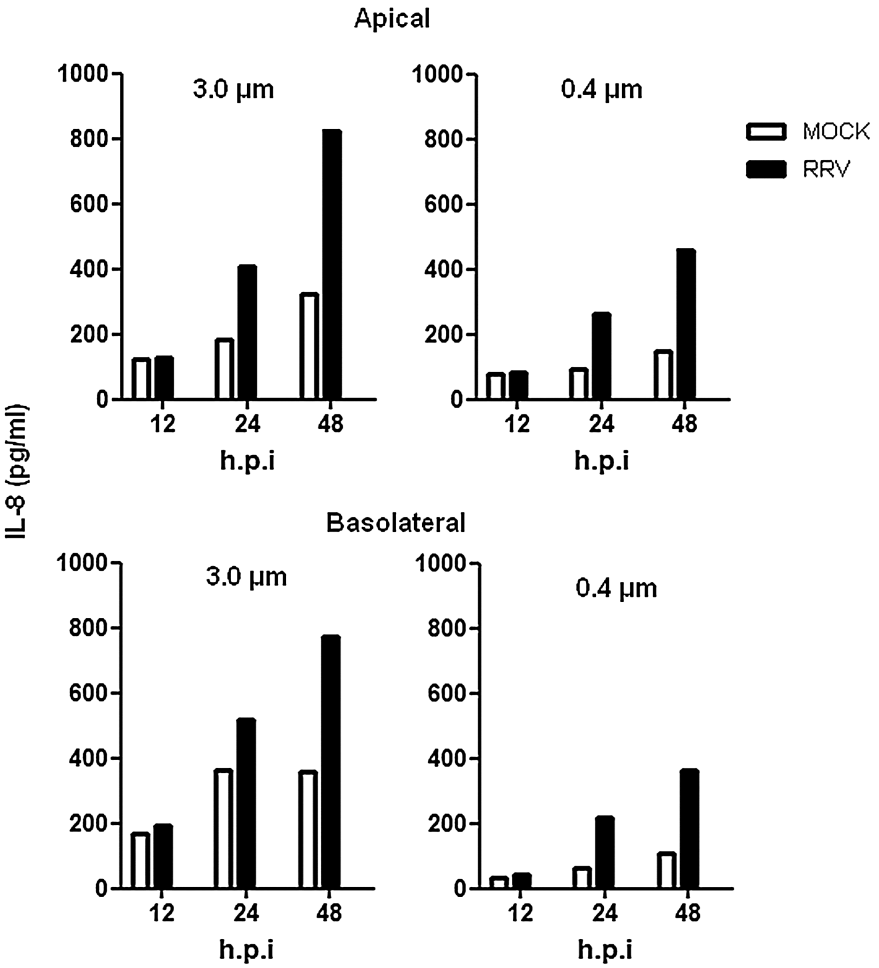

FIG. 3. IL-8 detection in polarized Caco-2 cells using 3.0-μm and 0.4-μm TW. Apical and basolateral supernatants from Caco-2 infected with RRV or treated with a control preparation (mock) were collected at different times post-infection. IL-8 was measured by ELISA. Data shown are the mean of two independent experiments.

The results observed were comparable with cells grown in 0.4-μm or 3-μm TW, but relatively more polarization of the IL-8 secretion was observed in the 0.4-μm TW (Fig. 1B).

It has been reported that Caco-2 cells cultured in a 3.0-μm TW system can grow on the opposite side of the TW from which they are seeded (11). Since this fact could influence the interpretation of our experiments, we determined the extent to which this occurs with our culture conditions. For this purpose, the nuclei of cells grown in 3-μm TW for 21 d were stained with DAPI. We found that approximately 11.4% of Caco-2 cells grow on the basolateral side of the filter, and their nuclei were larger than those of cells grown on the apical surface (Fig. 1C). In conclusion, although a small fraction of cells can traverse the 3-μm TW, these cultures still permit evaluation of the functional polarization of Caco-2 cells.

Polarized apical release of RV is similar in 3.0-μm and 0.4-μm TW

It has been reported that RV is released preferentially to the apical compartment of polarized Caco-2 cells (23). To determine if this polarized release varies depending on the type of TW used, cells were treated with a control preparation (supernatant of mock-infected cells), or infected with RRV apically, at a MOI of 5, and apical and basolateral supernatants were collected 12, 24, and 48 h.p.i. In both types of TW a progressive increase in the disruption of the cell monolayer (decrease in the TER; Fig. 2A) and cell death (evaluated by the release of LDH; Fig. 2B) were observed. Polarized apical release of RRV was detected at 12 and 24 h.p.i. in both TW. At 48 h.p.i., concomitantly with disruption of the monolayer and transmembrane leakage, infectious virus tended to be equally distributed apically and basolaterally (Fig. 2C). Of note, at 1 h.p.i. low numbers of RRV were found in the basolateral side of both TW, but more markedly in the 3-μm TW (Fig. 2C). This result suggests that the monolayers are intrinsically permeable to low levels of virus, and that some of the virus administered apically can also stimulate the cells by the basolateral pole (Fig. 2C). However, experiments adding RV to the basolateral chamber suggest that it is unlikely that these low quantities of virus can induce the release of IM in our model (data not shown).

RV induces comparable secretion of IL-8 in 3-μm and 0.4-μm TW

To determine if the release of IL-8 induced by RV infection varied depending on the type of TW used to culture the cells, this cytokine was measured in the supernatants of the experiments described above. Similar quantities of IL-8 were detected on both sides of the TW at 48 h.p.i. in both the 3.0-μm and the 0.4-μm TW (Fig. 3). Levels of IL-8 were greater in the 3-μm than in the 0.4-μm TW, but release of

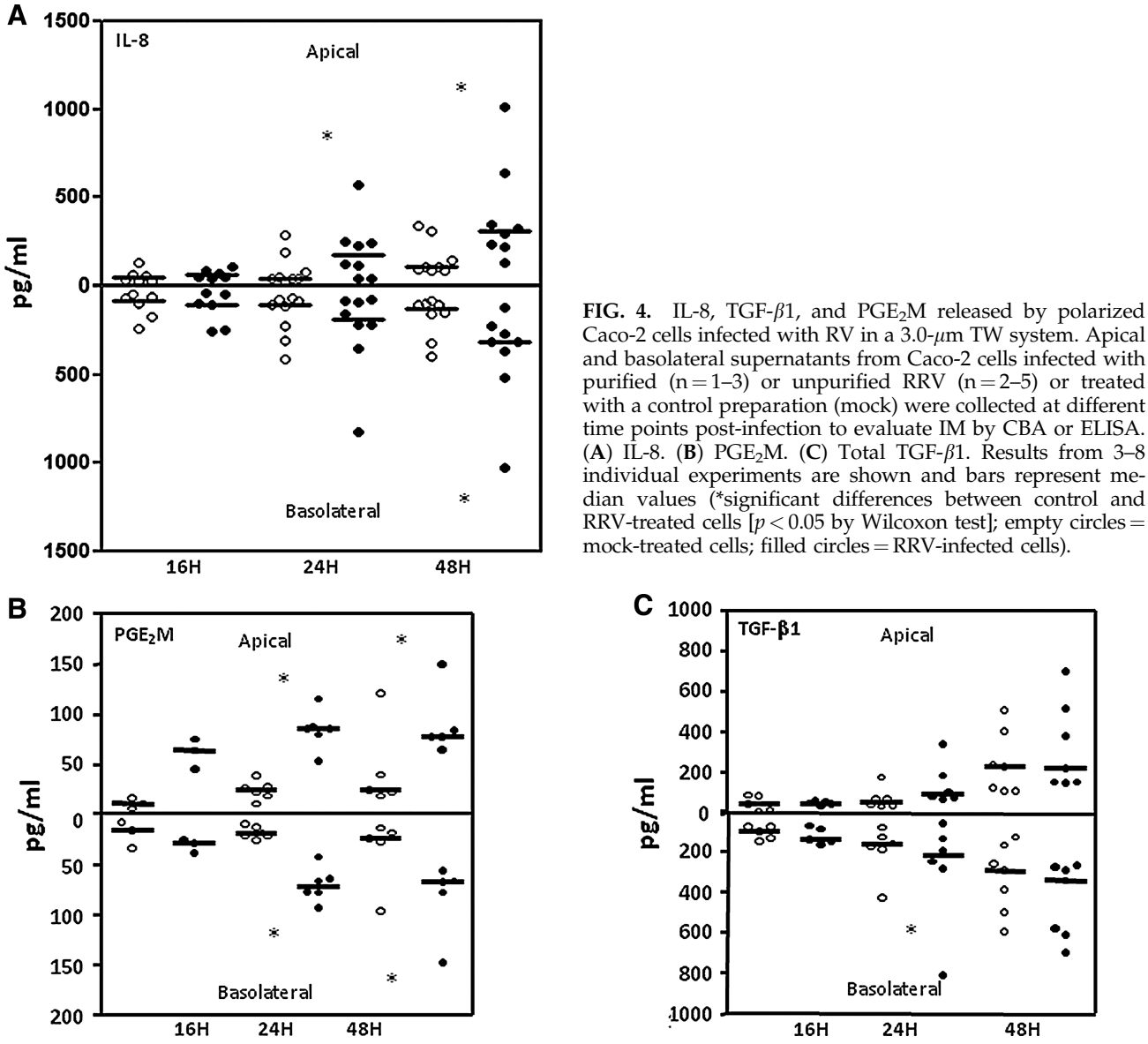

FIG. 4. IL-8, TGF-β1, and PGE_2M released by polarized Caco-2 cells infected with RV in a 3.0-μm TW system. Apical and basolateral supernatants from Caco-2 cells infected with purified (n = 1–3) or unpurified RRV (n = 2–5) or treated with a control preparation (mock) were collected at different time points post-infection to evaluate IM by CBA or ELISA. (**A**) IL-8. (**B**) PGE_2M. (**C**) Total TGF-β1. Results from 3–8 individual experiments are shown and bars represent median values (*significant differences between control and RRV-treated cells [$p < 0.05$ by Wilcoxon test]; empty circles = mock-treated cells; filled circles = RRV-infected cells).

IL-8 by mock treated cells was also higher in the 3-μm TW (Fig. 3). Thus, both types of TW are comparable to evaluate RV-induced IL-8 release.

IM released by polarized Caco-2 cells in 3-μm TW

To evaluate the endogenous IM released during RV infection, we quantified several cytokines (IL-12, IL-6, IL-10, IL-1β, TNF-α, and IL-8) by CBA, and other potentially important IM by ELISA (PGE_2M, TGF-β1, and HSP70) and Western blot (HSC70), in the apical and basolateral supernatants of cells grown in 3-μm TW. Cells infected with purified (n = 3) or unpurified RRV gave similar results and are presented together in Figs. 4 and 5. A statistically significant increase of IL-8 was detected in the apical side of RV-infected cells at 24 h.p.i., and on both sides at 48 h.p.i. (Fig. 4A). Similar quantities of IL-8 were detected in both compartments (Fig. 4A), probably due to loss of integrity of the cell monolayer at these time points (Fig. 2A and data not shown). None of the other five cytokines measured by CBA was released after RV infection (n = 4–6; Fig. 4A and data not shown). These results suggest that active secretion and not cellular death or lysis is responsible for the detection of IL-8 in RRV-infected cells.

The PGE_2M was detected in both compartments of RRV-infected cells at 16 h.p.i. (Fig. 4B). A small, but statistically significant, increase of total (active plus latent) TGF-β1 was observed in RV-infected cells at 24 h.p.i. in the basolateral compartment (Fig. 4C). RV did not induce release of active TGF-β1 (n = 3; data not shown). At 24 h.p.i. HSP70 was significantly increased apically, and at 48 h.p.i. in both the apical and basolateral compartments of the infected cells, concomitantly with important disruption of the monolayer and cell death (Fig. 5A). At 24 and 48 h.p.i. HSC70 was detected by Western blot in the apical chamber of the 3-μm TW (Fig. 5B). HSC70 was detected at lower levels and inconsis-

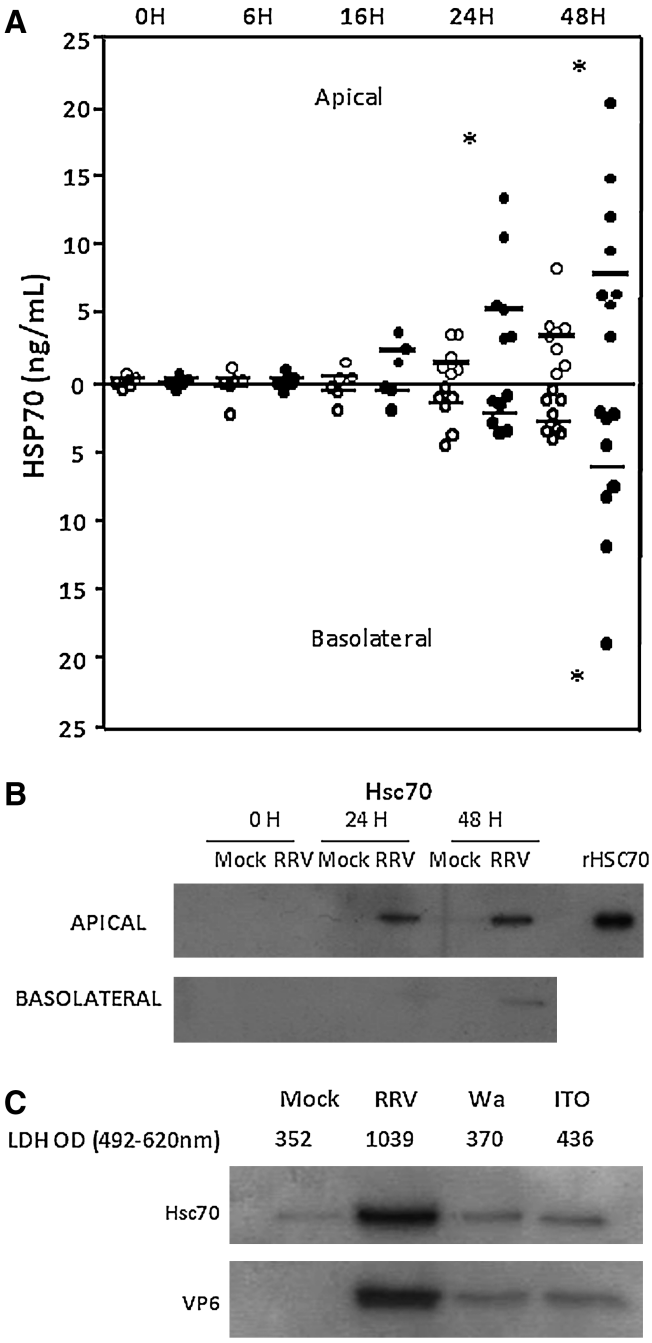

FIG. 5. Heat shock proteins are released during RV infection. (**A**) Apical and basolateral supernatants from Caco-2 cells infected with purified (n = 0–3) or unpurified RRV (n = 3–5) or treated with a control preparation (mock) were tested by ELISA for HSP70 (*significant differences between control and RRV-treated cells [$p < 0.05$ by Wilcoxon test]). (**B**) HSC70 was detected by Western blot in the apical and basolateral supernatants. A representative blot of three independent experiments with 3-μm TW is shown. A recombinant bovine HSC70 (0.1 μg) was used as a positive control. (**C**) HSC70 was detected by Western blot in supernatants of polarized Caco-2 cells grown in a T175 culture flask 24 h.p.i. with RRV, Wa, or ITO. Values of LDH in the corresponding supernatants are shown.

tently (in one of three experiments) in the basolateral chamber at these time points (Fig. 5B and data not shown). Comparable results were obtained using the 0.4-μm TW (n = 2; data not shown).

Finally, to determine if the release of HSPs induced by RV was associated with loss of cell viability, we measured HSC70 and LDH in supernatants of cells infected with RVs of lower cytopathogenicity (human strains Wa and ITO) than RRV (Fig. 5C). Higher levels of HSC70 and LDH were detected in supernatants of RRV-infected cells, compared to cells infected with human RV strains, suggesting that the release of HSPs goes hand in hand with that of LDH (Fig. 5C).

Discussion

We have shown that Caco-2 cells are functionally polarized in 3.0-μm and 0.4-μm TW, and that RV infection and release of IL-8 is similar in both TW. Our results are the first to evaluate IM released by RV-infected IEC in which functional polarization has been documented. We found that RV infection increases the release of IL-8, PGE_2M, HSP70, and HSC70, and small quantities of TGF-β1.

Caco-2 cells cultured for 21 d are functionally differentiated in both 3-μm and 0.4-μm TW, since they predominantly respond to basolateral CpG, and after stimulation with CpG, IL-8 was detected mainly in the basolateral compartment (Fig. 1B). In the monolayers of 0.4-μm TW the TER (Fig. 2A), the degree of functional polarization (Fig. 1B), and the monolayer integrity at the beginning of the infection (Fig. 2C) tended to be higher. However, the kinetics of disruption of the cell monolayer (Fig. 2A), cell death (Fig. 2B), polarized apical release of RV (Fig. 3B), and release of IL-8 (Fig. 3) after infection were comparable in both groups of TW. Taken together, these results suggest that both culture systems may be used for the study of IM released by RV-infected IEC, and that future studies using 3-μm TW and DC (39) may be useful as an *in vitro* simplified model of human intestinal immune response to RV infection.

RV was released mainly into the apical compartment at 12 h.p.i. (Fig. 2C), when only small changes in the integrity of the cell monolayer and cell lysis were observed (Fig. 2A and B). By 24 and 48 h.p.i. the difference between infectious RV in the apical and basolateral chambers decreased, accompanied by a loss of the TER and cell death (Fig. 2A and B). These results are in agreement with observations of other investigators showing that RV is released almost exclusively from the apical pole of polarized Caco-2 cells before cell death (23). However, in these previous studies loss of integrity of the cell monolayer was seen at 48 h.p.i. (23), and not beginning at 16 or 24 h.p.i. (Fig. 2A and data not shown). Other investigators have shown similar kinetics of cell death and loss of TER as those shown here (15,36,48), and it has been reported that up to 40% of polarized Caco-2 cells are apoptotic 24 h.p.i. (14). Thus the kinetics of release of infectious virus by polarized IEC before cell death varies, probably depending on cell culture conditions and the virus strain used. In experiments with Wa (a human RV strain that is less lytic than RRV), decreases in TER and LDH release were observed at later time points post-infection (data not shown) than with RRV (Fig. 2). However, the virus and IL-8 release in these experiments was comparable (data not shown) to those with

RRV (Figs. 2C and 3, respectively), suggesting that the IM released may be similar.

As previously reported (9,46), RV induced the secretion of IL-8 (Fig. 3A). However, concomitant disruption of the monolayer did not permit us to determine if polarized release of this cytokine occurs in RV-infected cells. Moreover, since a subset of cells grows on both sides of the 3-μm TW (Fig. 1C), and small amounts of virus are present in the basolateral chamber at 1 h.p.i. (Fig. 2C), it is difficult to know if RV stimulates polarized receptors to induce the IL-8. Using HT29 cells, other authors have shown that IL-8 production after RV infection is comparable in different states of cellular differentiation, and that this response is stronger than in Caco-2 cells (46). Thus our results and those of previous investigators suggest that IL-8 responses to RV in polarized and non-polarized cells are similar. The role of IL-8 in RV infection remains to be determined, given that the concentration of this cytokine is inconsistently elevated in acute serum samples from RV-infected children, and similar concentrations of IL-8 from plasma samples are detected in children with and without RV gastroenteritis ([22] and data not shown). However, these results do not exclude the possibility that locally, in the intestine, this cytokine may be elevated during RV infection.

In agreement with previous reports (44), RV induced the release of the PGE_2M (Fig. 3B). PGE_2 seems to play a role in RV immunity because it is detected in plasma and stool samples from RV-infected children (55). Of note, PGE_2 produced by infected enterocytes may induce the anti-RV immune response to have non-inflammatory characteristics (34), and induce regulatory T cells (3).

Small amounts of TGF-β1 were released preferentially to the basolateral pole of infected cells at 24 h.p.i. (Fig. 3C). The TGF-β1 released was mostly latent (Fig. 4C, and release of active TGF-β1 was not detected), and thus is non-covalently linked to latency-associated peptide, and latent TGF-β1 binding protein-1 (21,54). However, it is very likely that this TGF-β1 can be rapidly activated *in vivo* by integrins by protease-dependent or protease-independent mechanisms (21,54). It has been reported that TGF-β1 is not increased in the serum or intestinal contents of neonatal gnobiotic pigs infected with RV (2). However, these results do not exclude the possibility that, as suggested by our results, small increases of TGF-β1 can occur in the intestines of infected individuals. Since TGF-β1 is a key cytokine modulating intestinal tolerance and intestinal B-cell responses (24,37), further examination of the role of this cytokine in RV immunity is needed.

At present it is unknown how the intestinal immune system differentiates pathogenic from non-pathogenic microorganisms (35). Studies with IEC suggest that the interaction of the microorganism with enterocytes is critical, since pathogens, but not commensal microorganisms, are capable of inducing in these cells an inflammatory response that includes the release of TNF-α, IL-1β, and IL-8 (24,35). Like some commensal microorganisms (24,32), RV infection induced the release of relatively low levels of IL-8 (Fig. 4). Moreover, IL-12, IL-6, IL-1β, IL-10, or TNF-α were not found in supernatants of RV-infected cells (data not shown). Thus, as previously suggested (43), the pattern of cytokines released by RV-infected polarized IEC resembles that induced by commensal bacteria. In conclusion, the lack of TNF-α and IL-1β, plus the presence of PGE_2 and TGF-β1 (Fig. 4), suggest that the supernatant of RV-infected cells tends to induce a non-inflammatory (non-Th-1) response. This finding is in accordance with our previous reports that Th-1 cells circulate at very low levels in RV-infected children (19,41).

HSC70 is involved in RV entry (17), and HSP70 has been shown to be rapidly and transiently induced after RV infection (8). These HSPs may also play a role in RV immunity because they are released from infected polarized Caco-2 cells (Fig. 5). HSPs can be released by cells exposed to different types of stressful conditions, including viral infection (4,31). HSC70, HSP70, and LDH (Figs. 2B and 5) are detected simultaneously, suggesting that most HSPs may be passively released after cell death. However, it has been recently described that these proteins can be actively released in association with exosomes (5,12,16,50), and this also seems to be the case in our model (unpublished data). Soluble HSPs (49), or HSPs contained in exosomes (51), have been shown to have important IM properties in other models, and we are currently testing their potential role in RV immunity. Of note, HSPs can chaperone antigenic peptides to antigen-presenting cells (6,7), and for this and other reasons they have been used as adjuvants (27,38,53). In this context, identification of RV peptides associated with HSPs released by infected cells may aid in the identification of RV-immunogenic epitopes.

In summary, we have identified potentially important IM released during RV infection of Caco-2 polarized cells that seem to induce a non-inflammatory response. The role of the released cytokines and HSPs in RV immunity remains to be established.

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Disclosure Statement

No competing financial interests exist.

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