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Virus like particles of GII.4 norovirus bind Toll Like Receptors 2 and 5

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ABSTRACT

Norovirus (NoV) is now recognized as a major cause of gastroenteritis outbreaks, worldwide. Norovirus replication mechanisms are still poorly understood, mainly because a reliable cell culture system is still lacking. The present study aims at understanding some aspects of the immune response against norovirus, and particularly the capacity of virus like particles (VLPs) from an Italian strain, belonging to the GII.4 genotype predominating worldwide, to interact with target cells via Toll Like Receptors (TLRs). The capacity of GII.4 NoV VLPs to interact and cause the activation of TLR2, 4 and 5 was studied in recombinant HEK cells. The results obtained show the ability of GII.4 NoV VLPs to induce activation of TLR2 and 5. The results on TLRs activation confirm that GII.4 NoV VLPs interact with TLR2 and 5, that may represent putative receptors and play a role in NoV infection of intestinal cells.

1. Introduction

Norovirus (NoV) has been recognized as a pathogen causing gastroenteritis worldwide, involved in large foodborne and waterborne outbreaks. NoV is a small non-enveloped RNA virus, belonging to the *Caliciviridae*. The genome is a single-stranded RNA of approximately 7.3 kb, containing three open reading frames (ORFs) [1]. ORF1 encodes non-structural proteins, ORF2 the viral capsid protein (VP1), while the functions of ORF3 are still unknown. NoV strains have been classified into five genogroups (GI - GV), two of which (GI and GII) include at least 25 distinct genotypes and the majority of strains responsible for human infections [2–4].

A sixth and a seventh canine genogroups have also recognized [5]. These viruses are highly heterogeneous complicating the understanding of the complex immune interactions that regulate susceptibility and disease [6]. Due to the absence of reliable cell culture systems, most important information on NoV has been obtained through the expression of the viral capsid proteins VP1, that self-assemble into VLPs in eukaryotic systems [1,6–10]. The VLP interacts with both antibodies and cell receptors, including the histo-blood group antigens (HBGAs) [9,11–15]. In order to investigate ligand specificity of newly identified NoVs and host-susceptibility factors favoring infection, recombinant VLPs have been widely used studying their binding to panels of human saliva samples, chemically synthesized oligosaccharides, human milk and epithelial cells of animal gastrointestinal tissues [16–18]. However, because there is a lack of both animal models and culture systems for human NoV genogroups, details of the mechanism for NoV infection are still unclear, thus hampering the development of efficient treatments [19].

Virus infection causes a variety of responses in infected cells, including changes in gene expression, interferon response and regulation of cell surface molecules. Cytokines and chemokines are involved in host response to virus infection and play a central role in the pathogenesis of disease [20–22]. In a previous paper [23], we showed that GII.4 NoV VLPs can induce activation and maturation of circulating antigen presenting cells (APC) derived from five independent donors. The innate and early adaptive immune response induced by GII.4 NoV VLPs was evaluated on *ex vivo* stimulated peripheral blood mononuclear cells (PBMCs) from volunteers by means of activation/maturation phenotype analysis and cytokine production analysis [23]. Our observations suggested that, despite an initial status characterized by IL-6 production, a prolonged stimulation may induce viable T cells to produce IFN-γ [23].

The complexity of viral proteins makes them likely to interact with different Toll-like receptors (TLRs) [24]. TLRs are a family of transmembrane receptors differentially expressed in cells of diverse tissues, including innate immune cells, which recognize microbial Pathogen Associated Molecular Patterns (PAMPs) and activate signaling

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pathways launching immune and inflammatory responses. TLRs play important role in innate and adaptive immunity including induction of Th1 and Th2 immune responses [25,26]. Elevated expression of several TLRs was observed in PBMCs of children with acute rotavirus diarrhea [27]. Moreover, TLRs mediate the cytokines production in response to a variety of viruses and viral ligands [28,29]. Little is known about the differential function and response of TLRs and cytokines to NoV infection in intestinal cells, whereas a better understanding of the human immune response, including innate immunity and its key role in directing the adaptive immune response, is instrumental in the design of new tools for the control of NoV infection.

In the present study, recombinant VLPs, produced by expressing in baculovirus the ORF2 gene from a GII.4 norovirus strain detected in Italy in 2000 [23], were used to study possible interactions with TLR2, 4 and 5 in recombinant cell lines. Results showed that GII.4 NoV VLPs may induce activation of TLR5 and 2.

2. Material and methods

2.1. GII.4 NoV VLPs production

The cloning of the capsid region of NoV GII.4 strain (Hu/GII.4/00/IT) into the pFastBac vector was reported previously [23]. The resulting recombinant baculovirus BacHu/GII.4/00/IT was transfected into Sf9 insect cells to prepare the high-titer BAC NoV virus stock used to produce VLPs. When a diffuse cythopatic effect was observed, infected Sf9 cell monolayers were harvested and VLPs were purified by ultracentrifugation through a 30% (wt/vol) sucrose cushion, followed by a CsCl (1.362 g/cm^3) density gradient. VLPs were resuspended in Phosphate Buffer Saline (PBS). Proper folding of the purified NoV VLPs was visualized by electron microscopy. VLP protein concentration was determined by a Bradford protein assay (Biorad), as described previously [23].

2.2. TLR-binding assay using HEK-Blue TLR cells

HEK-Blue human TLR2 (hTLR2) cells, HEK-Blue human TLR4 (hTLR4) cells, HEK-Blue Null2 cells (parental cell line of HEK-Blue hTLR2 cells) and HEK-Blue Null1 cells (parental cell line of HEK-Blue hTLR4 cells) were purchased from InvivoGen (San Diego, California), cultured and used for assays, according to the manufacturer's instructions. The NFkB–induced embryonic alkaline phosphatase secreted into the supernatant of HEK-Blue hTLR2 cells was tested following the binding between TLR2 and the TLR2 agonist, by spectrophotometry. The TLR2 ligand Pam3CSK4 (1 μg/ml; InvivoGen, San Diego, California) and LPS (1 μg/ml, Sigma) were used as positive control for TLR2 and TLR4, respectively. Three serial dilutions of VLPs at 10, 1 and 0.3 μg/ml in PBS were added to the cells and cultured for 24 h at 37 °C, 5% CO^2 following the manufacturer's instructions. Supernatants were collected for measuring NF-kB-induced secreted embryonic alkaline phosphatase resulting from TLR2 and TLR4 binding by tested stimuli and incubated with QUANTI-BLUE medium (InvivoGen, San Diego, California) for 3 h at 37 °C. Optical Density (OD) was measured at 650 nm. Each cell line was tested in two independent experiments with or without 10 μg/ml polymyxin B (PXB) (Sigma) prior to assay for phosphates activity (data not shown).

Surface TLR5 binding was studied using HEK293 cells stably expressing TLR5 and the NF-κB-luciferase reporter cassette (HEK293-TLR5; a kind gift of Ugo D'Oro). The NFkB–induced embryonic Luciferase secreted into the supernatant of HEK-Blue hTLR5 cells was tested following the binding between TLR5 and the TLR5 agonist, by luminometer. HEK293-TLR5 were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 4.5 g/ml glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (complete medium), 10 μg/ml Blasticidin and 250 μg/ml hygromycin. For luciferase assay HEK293-TLR5 cells (2.7*10^5 cell/ml) were seeded into 96-well bottom plates in 90 μl of complete medium in absence of selection antibiotics. After overnight incubation, cells were stimulated in duplicate with different concentration of GII.4 NoV VLP starting from 10 μg/ml, 1 μg/ml, 0.3 μg/ml diluted in PBS, for 24 h or purified flagellin (a kind gift of Dr. Maria Luisa Ricci) (10 μg/ml) as positive control. Then the medium was discarded and cells were lysed with 20 μl of Passive Lysis Buffer (Promega) for 20 min at room temperature. Luciferase levels were measured by addition of 100 μl/well Luciferase Assay Substrate (Promega) using a Lumat LB9501 luminometer (E&G Berthold, Bad Wildbad, Germany) unit Raw light units (RLU) The cell line was tested in two independent experiments with or without 10 μg/ml polymyxin B (PXB) (Sigma) prior to assay for phosphates activity (data not shown).

2.3. Statistical analysis

Values of data in each stimulation experiment were expressed as mean ± standard deviations of duplicate or triplicate wells. Statistical analyses were performed with Student's unpaired *t*-test with $^{**}p < 0.01$.

3. Results

3.1. Binding capacity of GII.4 NoV VLPs to TLRs

We determined the TLR2-stimulating activity of GII.4 NoV VLP with a TLR2-transfected HEK293 responder cell line in comparative studies with Pam3CSK4 peptides. To examine the physical binding of GII.4 NoV VLP, HEK-Blue hTLR2 cells were used with HEK-Blue Null2 cells as negative control. GII.4 NoV VLPs bound to TLR2 expressed on HEK-Blue hTLR2 cells, as Pam3CSK4 positive control, and did not bind on HEK-Blue Null2 cells (Fig. 1A). Moreover, GII.4 NoV VLPs stimulate TLR2 expressing recombinant HEK cells in a dose dependent manner. The results concerning the binding to TLR4 are showed in Fig. 1B. No binding to TLR4 was detected with GII.4 NoV VLPs compared to positive LPS control after 24 h stimulation.

The stimulation of HEK293-TLR5 cells, HEK293 cells stably expressing TLR5 and the NF-κB-luciferase reporter cassette, resulted in

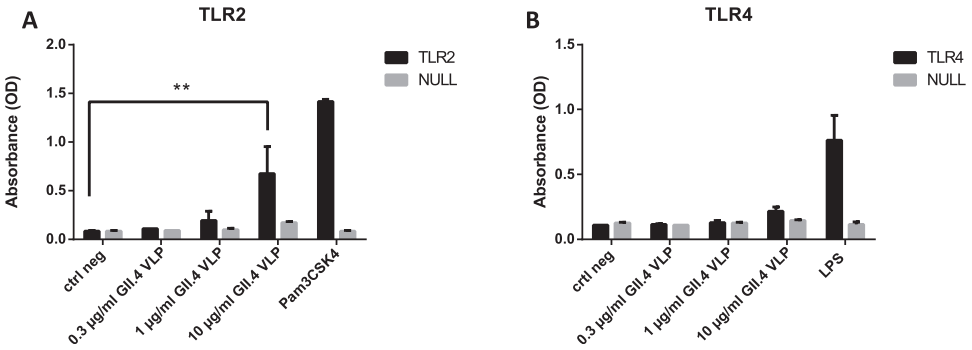

Fig. 1. Binding of GII.4 NoV VLPs to TLRs. HEK293 cells expressing human TLR2 (A) or TLR4 (B) were transfected with an NF-kB-driven firefly phosphates reporter plasmid and were stimulated for 24 h with three different doses of GII.4 NoV VLPs, with Pam3CSK4 (1 μg/ml; TLR2 ligand) and LPS (1 μg/ml; TLR4 ligand) respectively as positive control, or medium alone as negative control. HEK-Blue Null cells were used as parental cell line of HEK-Blue hTLR2 and TLR4 cells. The results are shown as the mean + SD of duplicate wells. Each cell line was tested in independent experiments. $^{**}p < 0.01$.

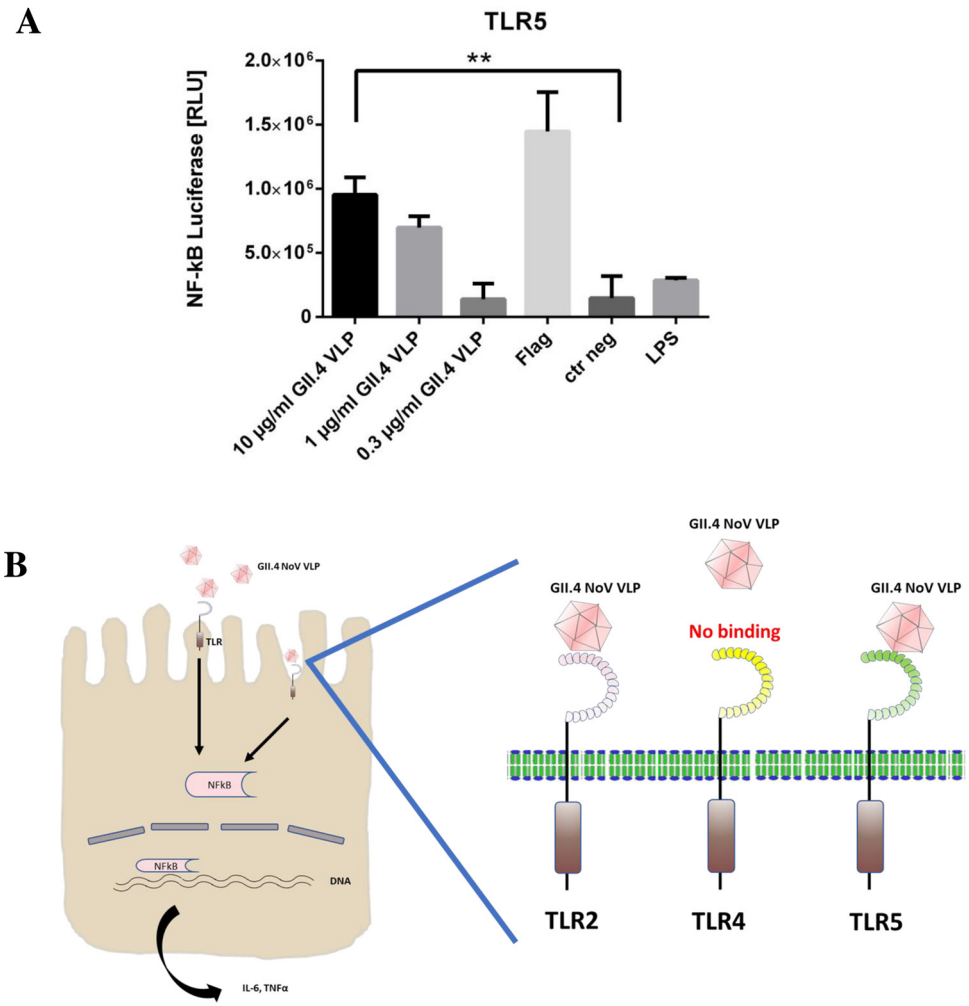

Fig. 2. GII.4 NoV VLPs bind TLR5. A) HEK293 cells were transiently transfected with NF-kB-Luciferase. Cultures were stimulated for 24 h with GII.4 NoV VLPs (10, 1 or 0.3 µg/ml), flagellin (10 µg/ml), LPS (1 µg/ml) or left unstimulated (negative control). Data are mean ± SD of triplicate experiments. RLU = Relative light units, $^{**}p < 0.01$. B) Simplified scheme of proposed TLR signaling by GII.4 NoV VLP: binding with TLR2 and TLR5 and possible NF-kB activity after interaction.

NF-kB activation. To investigate the physical binding of GII.4 NoV VLPs to HEK293-TLR5 recombinant cells, NF-kB activation was measured using flagellin and LPS as positive and negative controls, respectively. The activation of NF-kB was significantly increased in HEK293-TLR5 cells treated for 24 h with GII.4 NoV VLPs when compared to untreated negative control (Fig. 2) or LPS-treated cells. Additionally, GII.4 NoV VLPs stimulate TLR5 expressing recombinant HEK cells in a dose dependent manner.

Residual endotoxin activity, due to LPS possibly presents in GII.4 NoV VLP preparation, was blocked by pre-incubation with PXB at a concentration of 10 µg/ml. The absence of interference with activation in the presence of PXB was verified as previously described [23] (data not shown).

4. Discussion

VLPs are considered antigenically and morphologically indistinguishable from naïve virus [10,30], thus representing a suitable tool to study virus binding to cell ligands, the consequences of interactions with immune cells and the humoral immune response against NoV [31].

Binding of the surface exposed viral protein(s) to cellular receptors represents the first event leading to viral infection and the primary interaction with immune cells possibly leading to immune responses and immuno-pathogenesis. In this scenario, the analysis of VLPs interactions with their cellular receptor(s) represents an efficient model of investigation to decipher both the identity of receptors involved and the consequences of the interaction itself in terms of viral susceptibility and immune responses. In particular, the identification of viral cell receptors on host's cells is important not only to better understand the molecular events following viral infection, but also to develop novel anti-viral strategies.

Several papers reported that NoV VLPs bind to histo-blood group antigens (HBGAs), a diverse family of carbohydrates expressed on human cells including epithelial cells forming mucosal surfaces, which possibly represent the major NoV receptors [9,30,32–34]. Human noroviruses show large heterogeneity in recognition of HBGAs, but it seems possible that epidemic GII.4 strains predominate because they bind histo blood groups of individuals with A, B, and O secretors [35], who are representative of 80% of the world population [13,15]. Individuals who are homozygous for the G428A mutation in FUT2 are nonsecretors and are resistant to symptomatic infection by norovirus GI.1 [36] and most GII.4 strains [37]. However, other receptors in addition to HBGAs may be involved in viral entry and in interactions with immune cells [38,39].

In a previous paper, we analysed the cytokine release in PBMCs of adult donors and the results suggested a bimodal response with an initial phase characterized by a GII.4 NoV VLP-dependent IL-6 production and a later phase characterized by IFN-γ secretion [23]. Thus, the bimodal cytokine secretion may represent the consequence of different immune events that are triggered by NoV VLPs. In this paper, we

hypothesized that NoV VLPs may interact with epithelial cells and/or innate immune cells through receptors that these cells share, such as TLRs. Different studies have shown that TLR agonists may have a positive effect in anti-viral immunity [29,40–43]. For example, a bacterial ligand of TLR2/6, FSl-1, was shown to induce significant resistance to experimental Herpes simplex virus (HSV)-2 infection [28].

Intestinal epithelial cells are in continuous contact with enteric microbes as the first line of host microbial interaction inside the gut. Given that TLR2 and TLR4 responses are almost negligible in many intestinal epithelial cell lines [44,45] and most epithelial cell lines are strongly responsive to flagellin via TLR5 [46,47], TLR5 appears to have an important role in a communication between the intestinal epithelium and enteric microbes. The aberrant activation of TLR5 by flagellin is associated with developing intestinal inflammation [48–50] and we previously found that NoV activates a pro-inflammatory status. Then we focussed on the possible interaction of NoV VLPs with TLR5. Various viruses have been shown to trigger innate immunity via TLRs, suggesting that these receptors are likely to be important in the outcome of many viral infections, but as human NoV does not grow in cell culture, it is difficult to study its possible interactions with TLRs. Some studies regarding murine norovirus (MNV-1, which grows in cell culture) suggest that TLR3, which binds to dsRNA, is not involved in the antiviral response against MNV-1 infection, at odds with the MDA5 protein [51]. In other studies, authors have hypothesized that agonists of TLR7, which binds ssRNA, would be capable of inhibiting norovirus replication since the innate immune response plays an essential role in the elimination of norovirus infections [52].

In this study, results suggest that NoV VLPs interact with TLRs: GII.4 NoV VLPs bind TLR2 and TLR5 expressing recombinant HEK cells in a dose dependent manner, so that a physical interaction between VLP and TLR2 or TLR5 (Figs. 1 and 2) may be hypothesized. Therefore, TLR5 may represent an additional possible receptor mediating GII.4 NoV VLPs-host cell interactions to induce immune-mediated responses.

Moreover, we show that NoV VLPs bind to TLR2, but not to TLR4. These results both exclude an endotoxin contamination of our NoV VLPs preparation and are in line with the previously observed inflammatory status induced by NoV VLPs in PBMC [23]. In this light, it is interesting that the Hepatitis B Virus (HBV) surface antigen (HBsAg) binding TLR2 plays a pivotal role in HBV-related HCC pathogenesis, suggesting that the TLR2 pathway may be exploited by human viruses to infect and to favour inflammation [53]. We exclude the presence of baculovirus in the VLPs used in the experiments. In fact, the VLPs were purified by ultracentrifugation using a CsCl density gradient, and were recovered from a sharp band in the CsCl gradient by picking the tube sidewall, using a needle, that makes any cross-contamination of gradient bands impossible. Moreover, the purified GII.4 VLPs preparations were analysed by electron microscopy and baculoviruses were not visualized (data not shown).

This study represents a first description of the possible binding of GII.4 NoV VLPs to TLRs. The results obtained are in line with several previously published studies underlining the consequences of direct binding of viruses with TLRs in terms of immune response [39]. Even if interactions with other TLRs, and in particular TLR7, due to the single RNA genome of NoV, cannot be excluded, this preliminary work suggests a possible pivotal role of TLR5 and TLR2 in the control human NoV infections that deserves further studies. A deeper understanding of the signalling pathways involved in NoV interactions with host cells could lead to a better comprehension of the disease development and the possible identification of novel strategies for therapeutic interventions [54].

Author contributions

P.E. designed the project and conducted the experiments. C.T. contributed to a final draft. P.E. and S.M. analysed the results. P.E. wrote the main manuscript. F.M.R., R.N. critically revised the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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