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The *Vibrio cholerae* cytolysin (VCC) promotes activation of mast cell (T helper 2) cytokine production

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Summary

Many strains of *Vibrio cholerae* produce a cytolysin (VCC) that forms oligomeric transmembrane pores responsible for vacuolization of several cell types in culture. Here we report that VCC is a TLR2 agonist able to stimulate mast cells to produce several cytokines (IL-4 included) which could contribute to the Th2 response seen in the natural infection. Moreover, VCC-induced cytokine production was dependent on increased cytosolic Ca²⁺ and on the presence of the two Src family kinases Lyn and Fyn, known to be required for FcεRI-dependent activation of mast cells. These findings strongly suggest that VCC is endowed with pro-inflammatory activity that promotes a Th2-type immune profile.

Introduction

Cholera has long been considered a classic paradigm of a non-inflammatory toxigenic diarrhoea until observations showing that *V. cholerae* infection induces a Th2-type of immune profile and the recruitment of inflammatory cells in the intestinal mucosa (Mathan *et al.*, 1995; Qadri *et al.*, 2000). More recently, a significant accumulation of mucosal mast cells in the crypt and villus lamina propria of adult cholera patients has been reported (Qadri *et al.*, 2004).

Enteropathogenic *V. cholerae* can elaborate basically two different categories of exotoxins. The first is represented by classic cholera toxin (CT), zonula occludens toxin and accessory cholera toxin. A membrane damaging toxin, referred to haemolysin or *V. cholerae* cytolysin (VCC) represents the second type of *Vibrio* exotoxin. VCC consists of two major toxin groups, namely, *V. cholerae* O1 (VCC1) and *V. cholerae* non-O1 (VCC2) and is produced by all strains.

It is a water soluble toxin secreted as a 79 kDa inactive pro-hemolysin by *V. cholerae* (Alm *et al.*, 1988; Yamamoto *et al.*, 1990). A proteolytic cleavage by different proteases that remove a N-terminal segment (Nagamune *et al.*, 1997), generates the mature toxin of 63 kDa. In cholesterol- and ceramides-rich membranes (Zitzer *et al.*, 1999) VCC forms heptameric channels, with a moderate anion preference, responsible for vacuolization and eventual lysis of several cell types in culture (Coelho *et al.*, 2000; Figueroa-Arredondo *et al.*, 2001; Moschioni *et al.*, 2002; Pantano and Montecucco, 2006). VCC is proposed as virulence factor causing diarrhoea (Hichinose *et al.*, 1987), although the ability of non-hemolytic strains of *V. cholerae* to cause fluid accumulation, suggests that it is not the sole etiologic factor involved (Singh *et al.*, 2001).

CT has been found to affect several steps in the induction of a mucosal immune response, which alone or in combination might explain its strong adjuvant action after oral immunisation. It has been claimed that CT primarily induces Th2 type immune responses characterised by CD4⁺ T cells producing IL-4, IL-5, IL-6 and IL-10 and by the production of IgA, IgG1 and IgE antibodies (reviewed in Holmgren *et al.*, 2003).

The immune modulatory property of CT is primarily directed on APC cells which are induced to mature and efficiently present to T lymphocytes, whereas no strong evidence are reported on a possible role in activating mast cells: indeed, just one report described the ability of CT in inducing peritoneal mast cells to release IL-6 (Leal-Berumen *et al.*, 1996).

Considering the recent demonstration of the abundance of mast cells in cholera affected patients and on the basis that no other bacterial factors have been identified as immune modulator so far, we decided to address the possibility that VCC could be such a factor, by evaluating its ability in activating mucosal mast cells. Here we show that VCC stimulates mast cells to produce IL-4, IL-5 and IL-6, all cytokine which directs Th2 development (Mowen and Glimcher, 2004); this effect, depends on a cytosolic calcium increase and on the two Src kinases Fyn and Lyn.

Altogether our results support the idea that VCC, other than CT, may play a major role in the induction of the Th2 response seen upon *Vibrio cholerae* infection, by acting on mast cells.

Results

Recent reports indicate increased levels of inflammatory mediators in patients infected by *Vibrio cholerae* that occur in parallel with recruitment of innate immune cells, including mast cells and neutrophils, in the intestinal mucosa (Qadri *et al.*, 2004). Although cholera toxin may contribute to such inflammatory milieu, (Leal-Berumen *et al.*, 1996, Rocha *et al.*, 2003), other bacterial factors are likely to be involved, particularly in the case of *Vibrio* strains which do not produce the enterotoxin. In order to assess whether VCC is one of these factors, we examined its effect on mouse bone marrow-derived mast cells (BMMC) (Gilead *et al.*, 1990). As shown in table 1, treatment of BMMC with 500 pM VCC for 4 h led to the production and secretion of substantial amounts of IL-4, IL-5, IL-6 and TNF. VCC may induce cell lysis at concentration < 100 pM (Moschioni *et al.*, 2002); however, we found no effect on cell viability under the present experimental conditions (data not shown).

The above findings suggested that VCC treatment caused mast cell activation. IgE-dependent activation of both human and rodent mast cells is characterized by an influx of extracellular Ca²⁺, which is essential for subsequent release of both pre-formed (granule-derived) mediators and newly generated eicosanoids and cytokines (Bradding and Conley, 2002). Moreover, other toxins, such as the cytotoxin VacA of *Helicobacter pylori*, stimulate mast cells to produce and secrete cytokines following the induction of cytosolic Ca²⁺ increase (Molinari *et al.*, 1998; de

Bernard *et al.*, 2005). Thus, we tested whether VCC induces changes in the intracellular Ca^{2+} concentration of BMMC. Figure 1 shows that 500 pM VCC induces a rapid increase in cytosolic Ca^{2+} concentration in Fura2-AM-loaded BMMC. The bi-modal response suggested that VCC might induce Ca^{2+} release from intracellular stores followed by a more sustained ion influx possibly through store-operated Ca^{2+} channels (SOCs). In mast cells, the release of Ca^{2+} from intracellular stores is primarily controlled by endoplasmic reticulum (ER) inositol 1,4,5-trisphosphate receptors (IP3Rs) which deplete intracellular stores and activate Ca^{2+} influx through plasma membrane channels (capacitative entry) (reviewed in Rivera and Gilfillan, 2006). To test whether VCC triggered this signalling pathway, VCC-treated cells were incubated with the Ca^{2+} chelator EGTA or with the PLC γ inhibitor U73122, which prevents IP3 synthesis. As shown in Figure 2A, the presence of EGTA in the extracellular medium impaired the late phase increase of cytosolic Ca^{2+} , but did not abolish the initial release of Ca^{2+} from intracellular stores. In contrast, U73122 inhibited the initial release of Ca^{2+} from intracellular stores (Figure 2B). These results support the hypothesis that VCC-induced Ca^{2+} mobilization involves both intracellular stores and capacitative currents.

To test whether the intracellular Ca^{2+} increase evoked by VCC was involved in cytokine synthesis and secretion, mast cells were treated with the intracellular Ca^{2+} chelator BAPTA-AM (10 μM) before stimulation with VCC. As shown in figure 3, the chelation of intracellular Ca^{2+} almost completely abolished the VCC-induced cytokine secretion. Same results were obtained when the experiment was carried on in low- Ca^{2+} medium, thus permitting to exclude that the effects observed with BAPTA-AM would be a consequence of the toxicity of the chelator by-products. Similarly, a marked decrease was also observed after the inhibition of IP3 synthesis with U73122 (Figure 3).

Cells were harvested after 30 min and processed for determination of cytokine mRNA for IL-4, IL-5, TNF and IL-6. Compared with cells treated only with VCC, the expression of cytokine mRNA decreased dramatically after BAPTA-AM treatment or in low Ca^{2+} conditions, and was also impaired by treatment with the PLC γ inhibitor U73122 (data not shown).

The flow of ions such as Cl^- is likely to play an important role in mast cell activation through their effects on membrane potential and therefore Ca^{2+} influx. Store-operated Ca^{2+} channels (SOCs), that carry Ca^{2+} into cells, conduct larger currents at negative membrane potentials (Hoth and Penner 1992). In order to determine whether Cl^- flux was required for VCC-induced BMMC activation and cytokine release, we monitored cells pre-treated with DIDS (100 μM) before exposing to VCC. As illustrated in figure 4, cytokine release was markedly reduced; concomitantly mRNA production was impaired as well (data not shown). Given that Ca^{2+} mobilization occurred from intracellular stores in an IP3-dependent manner, this suggested that the mast cell activation by VCC was, at least in part, a consequence of a membrane receptor engagement. Thus, once the receptor is activated, phospholipase C would generate IP3 and the Ca^{2+} released from intracellular stores would trigger extracellular Ca^{2+} entry, sustained by chloride influx.

To study the issue of VCC-mediated Ca^{2+} fluxes as dependent on receptor engagement, we took the approach of determining if known early mast cell signalling events were required for the VCC-induced cytokine responses. Drugs that increase the intracellular Ca^{2+} concentrations by transiting Ca^{2+} across the plasma membrane, like ionomycin or the Ca^{2+} ionophore A23187, can induce expression of cytokine genes in a manner independent from early signalling events (Plaut *et al.*, 1989). Thus, a requirement for the Src family protein tyrosine kinases (PTK) Fyn or Lyn would provide a strong argument for a receptor-mediated event. These kinases have been demonstrated to be key regulators of phosphatidylinositides and Ca^{2+} responses in BMMC activated through the high affinity IgE receptor (Fc ϵ RI) (reviewed in Blank and Rivera, 2004). As shown in Figure 5, the absence of Lyn in BMMC caused a marked impairment of

VCC-mediated cytokine production, at variance from previous reports showing that a IgE-dependent stimulation of Lyn-deficient BMMC resulted in an unaltered or enhanced cytokine response relative to wild-type cells (Nishizumi and Yamamoto, 1997; Kawakami *et al.*, 2000; Hernandez-Hansen *et al.*, 2005). Additionally, the absence of Fyn showed an even more dramatic impairment of VCC-mediated cytokine release from these cells. A reduction of cytokine mRNAs in both mutant cells, with respect to wild type cells (data not shown), was consistent with that of the released protein. Impaired cytokine release from Fyn-deficient BMMC was also previously observed after stimulation via FcεRI (Gomez *et al.*, 2005). While Fyn and Lyn were suggested to have a predominant positive and negative regulatory role, respectively, in mast cell effector responses, (Parravicini *et al.*, 2002; Odom *et al.*, 2004), a recent report has demonstrated a cooperative role between Fyn and Lyn in regulating the activation of sphingosine kinases (Olivera *et al.*, 2006). Thus the results shown in Figure 5 identify another point of cooperation for these two kinases in VCC-mediated cytokine responses. The requirement for both Fyn and Lyn in VCC-mediated BMMC cytokine production supports the requirement of early signalling events that impact on both phosphatidylinositol metabolism and Ca^{2+} responses and suggests a receptor-mediated activation by VCC.

The presence of Toll-like receptors (TLRs) on mast cells (reviewed in Marshall, 2004) and the recent demonstration of a link between Src kinases and TLRs (Chang *et al.*, 2004; Chun and Prince, 2006; Kannan *et al.*, 2006), prompted us to investigate the possibility that VCC activated mast cells by binding to a TLR. In order to address this point we used human embryonic kidney (HEK) 293 cells transfected with plasmids encoding distinct human TLRs. HEK293 cell lines lack expression of endogenous TLRs, although their TLR signalling machinery is fully functional. The common pathway leading to NF-κB activation requires the phosphorylation and degradation of the cytosolic inhibitor of NF-κB, IκB-α. The engagement of a specific TLR, expressed on HEK293, was monitored by evaluating the phosphorylation of IκB-α after exposure to VCC. As shown in Figure 6 phosphorylation of IκB-α was observed only in cells expressing TLR2, whereas no activation was observed in cells expressing either TLR3 or TLR4. These results indicate that VCC functions as an agonist of TLR2, but not of the TLR3 or TLR4.

Discussion

In our study we addressed the possibility that VCC had a role in promoting the inflammation associated with the *V. cholerae* infection. Considering that mast cells are particularly abundant in the intestinal mucosa of infected patients (Qadri *et al.*, 2004), we evaluated the effect of the hemolysin on these cells in terms of cytokine production. VCC efficiently induced BMMC to produce IL-4, IL-5 and IL-6, all cytokines that could contribute to the Th2 response as observed in the natural infection (Marinaro *et al.*, 1995; Qadri *et al.*, 2000). VCC was able not only to stimulate the secretion of cytokines but also to increase mRNA levels in a Ca^{2+} -dependent manner. The rise in intracellular Ca^{2+} , which occurred within a few seconds after VCC administration, showed a bi-modal behaviour: the first phase of VCC-induced Ca^{2+} increase was attributable to the ion release from ER (in a PLCγ- manner) and the second phase to the entry of extracellular Ca^{2+} . The inhibitory effect on cytokine synthesis and release following DIDS treatment can be interpreted by considering that SOCs, which carry Ca^{2+} into cells, conduct larger currents at negative membrane potentials (Hoth and Penner, 1992). Thus, the inhibition of the plasma membrane Cl^- channels would limit the massive entry of Ca^{2+} required for the activation of the mast cells. Obviously, we can not exclude that VCC might also contribute to the Cl^- entry by forming pores on the BMMC membranes and the addition of DIDS would inhibit this contribution. However, our finding that VCC can bind and activate BMMC through the engagement of a receptor strongly supports a pore-independent SOCs-mediated mechanism. Accordingly, a key finding of this study is that VCC is a TLR2 agonist

able to stimulate the phosphorylation and the subsequent degradation of I κ B in HEK TLR2-transfected cells. We hypothesize that VCC activates mast cells through the engagement of this PAMP receptor, which has been demonstrated to be involved in mast cell activation and cytokine production (Supajatura *et al.*, 2002). The link between TLRs and Src family kinase are also reasonably well established in various systems; for example it was recently demonstrated, in airway epithelial cells, that in response to bacterial ligands, c-Src initiates TLR2-associated signalling, followed by recruitment of phosphatidyl inositol 3 kinase (PI3K) and PLC γ to affect the release of Ca²⁺ from intracellular stores. The latter is important for the downstream activation of pro-inflammatory gene transcription (Chun and Prince, 2006). In mast cells, Fyn has been demonstrated to be important for *de novo* production of various cytokines (such as IL-4, IL-6 and TNF) after Fc ϵ RI activation; this Src kinase is also essential for I κ B- α phosphorylation and degradation, events required for NF- κ B nuclear translocation and gene transcription (Gomez *et al.*, 2005). On the basis of the collective findings we propose that both Fyn and Lyn are activated upon the engagement of the TLR2 by VCC, a model similar to that of antigen receptors, which merits further investigation. Moreover, while the link between Lyn and TLR2 has already been demonstrated (Kannan *et al.*, 2006), our finding provide the first evidence for the role of Fyn and its downstream signalling events in VCC-mediated TLR2 activation

Experimental procedures

Reagents

Vibrio cholerae cytolysin (VCC) was purified from culture supernatants of *V. cholerae* O1 El Tor 8731 (Hall and Drasar, 1990). The cleaved and active form of VCC was obtained by ethanol precipitation (final concentration, 40%), preparative isoelectric focusing in a sucrose density gradient, and hydroxyapatite chromatography (Zitser *et al.*, 1997; Zitser *et al.*, 1999). Cell culture media, FBS, and HBSS buffer were from Invitrogen/Gibco. FURA2-AM and pluronic acid were from Molecular Probes; Sulfinpyrazone, U73122, EGTA, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (disodium salt) (DIDS), were from Sigma. BAPTA-AM was from Calbiochem.

Generation of BMMC

Mice, *fyn*^{-/-} and *lyn*^{-/-}, were obtained as described (Odom *et al.*, 2004) and bone marrow was isolated from 5–6-wk-old wild-type and gene-disrupted mice as previously described (Saitoh *et al.*, 2000). BMNCs were grown in RPMI media supplemented with FBS, stem cell factor (SCF), and IL-3 as previously described (Saitoh *et al.*, 2000; Odom *et al.*, 2004). For BMMC cultures, Fc ϵ RI receptor expression was monitored weekly as previously described (Saitoh *et al.*, 2000) and cells were used for experiments when >95% of the population was Fc ϵ RI⁺. All the experiments were carried on in complete RPMI, as above, without SCF and IL-3.

Intracellular Ca²⁺ measurement

10⁶ BMMC were incubated with 5 μ M Fura-2 acetylmethyl ester (Fura2-AM), 250 μ M sulfinpyrazone and 32 μ M pluronic acid in HBSS for 40 min at room temperature before Ca²⁺ measurements. Cells, washed and suspended in 1.6 ml HBSS, were transferred to a cuvette. Measurements of the Fura-2 fluorescence were performed in a RF-5301PC spectrofluorophotometer (Shimadzu, Germany) equipped with a thermostat controlled cell holder and a stirring device. When indicated, immediately before addition of VCC (500 pM final concentration), 5 mM EGTA was added to chelate extracellular Ca²⁺. In the case of U73122 treatment, cells were pre-incubated for 10 min with the drug (30 μ M) at 37°C before starting fluorescence recording. In all experiments toxin was added directly to the cuvette. Cells were excited at λ 340 and 380 and emitted light was collected using a 520 nm band-pass filter.

Data are expressed as fluorimetric ratios considering that available calibration procedures of the cytosolic $[Ca^{2+}]$ provide very different values.

Detection of cytokines in culture supernatants

Supernatants were collected 4h after the incubation with the stimuli and the amount of IL-4, IL-5, IL-6 and TNF protein was quantified by Bioplex Cytokine Assays (Bio-Rad Laboratories), according to the manufacturer's protocol. For BAPTA-AM, U73122 and DIDS treatment, 10^6 cells/ml were pre-incubated at 37°C for 30 min, 10 min and 50 min respectively, before VCC intoxication. In the case of low Ca^{2+} condition, VCC intoxication was performed in RPMI plus 5 mM EGTA. Control cells were incubated with PBS (indicated as saline in table 1).

Real-time PCR analysis

Total RNA was isolated from 2×10^6 cells using SV Total RNA Isolation System (Promega Corporation) according to the manufacturer's instructions. RNA was reverse-transcribed and amplified with the following primers: 5'-GTCATCCTGCTCTTCTTCTCG-3' and 5'-TGTGGTGTTCTTCGTTGCTGTG-3' for IL-4; 5'-AAGAGAAGTGTGGCGAGGAG-3' and 5'-CAGTTTTGTGGGGTTTTTGC-3' for IL-5; 5'-TTCTGCAAGTGCATCATCGT-3' and 5'-CCGGAGAGGAGACTTCACAG-3' for IL-6; 5'-TACTGAACTTCGGGGTGATCGGTCC-3' and 5'-CAGCCTTGTCCTTGAAGAGAACC-3' or TNF; 5'-GATTACTGCTCTGGCTCCTA-3' and 5'-TCGTACTCCTGCTTGCTGAT-3' for β -actin. After amplification, data analysis was performed using the second derivative method algorithm. For each sample the amount of cytokine mRNA was expressed as n -fold of the normalized amount of mRNA from untreated cells (1 AU= mRNA cytokine concentration [fmol/ml]/mRNA β -actin [fmol/ml]).

MTS assay

MTS assay was done in 96-well plates using a CellTiter 96® kit (Promega). Briefly, 10^5 cells were plated in 96-well plates the day before experiment. At the end of treatment culture medium was removed and replaced by 100 μ l of complete medium w/o phenol red plus 20 μ l of MTS tetrazolium to each well. During an incubation period of 1.5 hours at 37°C, the MTS salt is metabolically reduced only by viable cells into an insoluble coloured formazan; the absorbance/optical density was read and recorded by a microplate reader at wavelength of 485 nm. Values are expressed as percent of viable cells compared to untreated cells, considered as 100%. This assay was performed in parallel with all the cell experiments to exclude any artefact resulting from differences in cell viability.

TLR screening

Human embryonic kidney (HEK) 293 cells constitutively expressing TLR2/CD14 (HEK293-TLR2/CD14), or TLR3 (HEK293-TLR3) or TLR4/CD14/MD2 (HEK293-TLR4/CD14/MD2) (Invivogen, San Diego, CA, USA) were grown in low-glucose Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Before the experiments, HEK293 cells were plated in 24-well tissue culture plates at a density of 5×10^5 cells/ml. Adherent cells were collected at various times after the addition of 25 μ g/ml Poly (I:C) (as positive control ligand of TLR3) or 100 ng/ml LPS (as positive control ligand of TLR4) or 1 μ M *Helicobacter pylori* HP-NAP (as positive control ligand of TLR2) (67) or 500 pM VCC. Monolayers were washed with ice-cold PBS. Cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 and 1 μ g/ml each of sodium orthovanadate, PMSF, leupeptin, pepstatin and aprotinin) by incubation on ice for 5 min. Lysates were cleared by centrifugation and proteins were separated in SDS-PAGE and transferred to nitrocellulose membranes; after being blocked with TBS-T (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.02%

Tween-20) supplemented with 3% milk, membranes were blotted with anti phospho-I κ B α antibody (Cell Signaling Technology, Danvers, MA). After stripping blots were re-probed with an anti- α actin antibody (Amersham Pharmacia Italia, Milan, Italy) used as a control for equal loading.

Statistical analyses

Data are means $\pm$ S.E.M. Student's *t*-test was used for statistical analysis of differences between experimental groups. In experiments on human colon each data set represents independent measurements on separate stripped mucosal preparation (one to four of these tissue preparations were obtained from the same colon). A p-value equal or below 0.05 was defined as a significant difference.

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Nonstandard abbreviations used

BMMC	bone marrow-derived mast cells
HEK	human embryonic kidney
HP-NAP	<i>Helicobacter pylori</i> neutrophil activating protein
IP3	inositol 1,4,5-triphosphate
PLCγ	phospholipase C γ
PTK	protein tyrosine kinases
SOCs	store-operated Ca ²⁺ channels
VacA	vacuolating cytotoxin A
VCC	<i>Vibrio cholerae</i> cytolysin

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Figure 1. Cytosolic Ca^{2+} increase induced by VCC

BMMCs were loaded with FURA2-AM, and the fluorescence ratio (340 nm/380 nm) was measured with a dual-wavelength modular fluorimeter as detailed in Materials and Methods. Arrow indicates the VCC addition (final concentration 500 pM). The experiment shown is representative of at least five individual experiments.

Figure 2. Pharmacological characterization of the Ca^{2+} response

BMMCs, loaded with FURA2-AM, were either incubated with 5mM EGTA just before starting the fluorescence recording (panel A), or pre-incubated for 10 min at 37°C with 30 μM U73122 (panel B). Arrows indicate VCC addition (final concentration 500 pM). Each panel shows one representative experiment from a minimum of three.

Figure 3. Role of Ca^{2+} on VCC-induced cytokine release

BMMC were incubated for 4 h with 500 pM VCC and cytokines secretion was measured in the culture supernatants with Bio-Plex™ cytokine assay. BAPTA-AM (10 μM) and U73122 (30 μM) were pre-administrated for 30 min and 10 min respectively, before adding the toxin. Data represent the average of three independent experiments with triplicate samples. Significance was determined by Student's t test (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$, versus VCC).

Figure 4. Effect of the chloride channel inhibitor on VCC-induced cytokine release

BMMCs were pre-incubated or not with 100 μ M DIDS for 50 min before being exposed to 500 pM VCC. After 4 h of incubation cytokines secretion was measured in the culture supernatants with Bio-PlexTM cytokine assay. Data represent the average of four independent experiments with triplicate samples. Significance was determined by Student's t test (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$, versus VCC).

Figure 5. Cytokine profile of BMMC from *Lyn*^{-/-} and *Fyn*^{-/-} mice after VCC treatment
 BMMC purified from wild type and *Lyn*- or *Fyn*-null mice, were incubated for 4 h with or without 500 pM VCC. Cytokines secretion was measured in the culture supernatants with Bio-PlexTM cytokine assay. The data shown is from five experiments conducted with different cell cultures derived from at least three different mice for each genotype. Significance of mutant versus wild-type BMBCs was determined by Student's *t* test (***, *p*<0.0005).

Figure 6. VCC-induced phosphorylation of IκBα in HEK 293 cells transfected with plasmid encoding distinct human TLRs

HEK 293 cells expressing the indicated TLR were incubated for different times with either VCC or with the specific agonists. After different times, cell lysates were subjected to SDS-PAGE immunoblotting with anti-phospho IκBα. Blot with α-actin antibody was used as a control for equal loading.

Table 1**Cytokines release by BMMC treated with VCC**

Treatment	IL-4 (pg/10⁶ cells) (mean ± SEM)	IL-5 (pg/10⁶ cells) (mean ± SEM)	IL-6 (pg/10⁶ cells) (mean ± SEM)	TNF (pg/10⁶ cells) (mean ± SEM)
Saline	0.141 ± 0.040	0.349 ± 0.134	3.559 ± 0.885	1.273 ± 0.551
VCC	46.466 ± 9.467***	1.101 ± 0.174**	1055.2 ± 188.9***	693 ± 85.586***

BMMC were incubated for 4 h with 500 pM VCC and cytokines secretion was measured in the culture supernatants with Bio-PlexTM cytokine assay. Data represent the average of three independent experiments with triplicate samples. Significance of VCC-treated versus control was determined by Student's t test (**, p<0.005; ***, p<0.0005).