Receptor-interacting protein 2 is a marker for resolution of peritoneal dialysis-associated peritonitis

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There are no predictive factors for peritoneal dialysis-associated peritonitis; however, its resolution correlates with a cell-mediated Th1 immune response. We tested the hypothesis that induction of receptor-interacting protein 2 (RIP2), an assumed kinase linked with Th1 responses, is a useful marker in this clinical setting. Basal RIP2 expression was measured in human immune cells and during dialysis-associated peritonitis. RIP2 increased with bacterial toxin cell activation and the temporal profile for this differed depending on immune cell involvement in the innate or adaptive phases of the response. Importantly, RIP2 expression increased in peritoneal immune cells during dialysis-associated peritonitis and this upregulation correlated with clinical outcome. An early induction in peritoneal CD14$^+$ cells correlated with rapid resolution, whereas minimal induction correlated with protracted infection and with catheter loss in 36% of patients. These latter patients had higher levels of MCP-1 consistent with a delayed transition from innate to adaptive immunity. Our study shows that upregulation of RIP2 is a useful marker to monitor dialysis-associated peritonitis and in predicting the clinical outcome of these infections.

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Resolution of an infection relies on the ability to trigger the appropriate pathogen-specific, adaptive immune response. Such a response can be broadly differentiated in two types based on the profile of cytokine production.[1] Cell-mediated or Th1 immune responses are characterized by predominant expression of interleukin-12 (IL-12) and interferon-γ (IFN-γ). In contrast, antibody-mediated or Th2 responses are characterized by predominant expression of IL-4. Many factors such as dose of antigen, route of entry, or primary antigen-presenting cell (APC) can determine the generation of either type of response.[2] It is becoming apparent that the effect of most of these factors is on cells involved in innate immunity which then influence the development of Th1 or Th2 responses, but the molecular events that link innate with adaptive immunity are largely uncharacterized.

In the case of peritoneal dialysis (PD)-associated peritonitis, clinical resolution is dependent on the development of a cell-mediated (Th1) immune response.[3–5] We currently do not have a robust clinical or molecular indicator for the imprinting of the response into a Th1 profile nor for the outcome of the infection.[6] Such an indicator would be highly beneficial in discriminating PD patients with peritonitis that can be treated ambulatory versus those requiring hospitalization early after diagnosis. To search for such an indicator, we investigated signaling molecules that have been associated with the differentiation of helper T cells into effector Th1 cells.

Receptor-interacting protein 2 (RIP2), also known as RICK or CARDIAK, is a member of the RIP family of proteins. This group of molecules includes at least four members (RIP1–RIP4), all of them sharing a high degree of sequence homology particularly at the N terminus, where they have a putative serine/threonine kinase domain.[7–9] In comparison with the other RIP family members, RIP2 stands out as the only member that has a caspase recruitment domain (CARD) in its C terminus. CARD-containing proteins generally interact with one another via CARD—CARD homotypic interactions, and their main function is in the activation of nuclear factor-κB (NF-κB) and/or caspases.[10–12] The extensive array of molecules that contain CARD domains and the implications of such molecules in many fundamental cell processes have focused significant attention on RIP2.

The function of RIP2 is unknown. Although it was initially identified as a molecule involved in cell death through the tumor necrosis factor (TNF) receptor family,[7] overexpression studies linked RIP2 with cell proliferation and differentiation through the NF-κB and the JNK signaling pathways.[8,9,13] This idea was strengthened by subsequent studies using *Rip2*$^{-/-}$ mice[14,15] that exhibit impaired NF-κB-dependent macrophage responses, marked decrease in thymocyte and CD4^{+} T-cell proliferation upon stimulation, and diminished IFN-γ production by natural killer cells and Th1 cells. Such a phenotype implicated RIP2 in the activation of cells that participate in the innate and adaptive phases of immunity, and in the generation of cell-mediated, Th1-driven immune responses.

On the basis of these data, we examined RIP2 expression during PD-associated peritonitis. Here, we report that RIP2 is expressed at low levels in all peripheral blood immune cells under resting conditions, but its expression is significantly increased upon activation with bacterial toxins acting on toll-like receptor 4 (TLR4) or TLR2, both *in vitro* and *in vivo* during the course of PD-associated peritonitis. The increase in RIP2 expression occurs in neutrophil polymorphonuclear (PMN) cells, monocytes, and T lymphocytes in a sequential fashion during the transition of innate to adaptive immunity. More importantly, the level of RIP2 expression in peritoneal immune cells in the initial stages of PD-associated peritonitis correlates with clinical outcome. Specifically, early upregulation of RIP2 in peritoneal CD14^{+} cells correlates with rapid resolution of infection, whereas minimal or no upregulation of RIP2 expression correlates with protracted peritonitis, characterized by significantly higher monocyte chemoattract protein 1 (MCP-1) levels and resulted in catheter loss in 36% of the patients.

RESULTS

Activation-induced upregulation of RIP2 expression

To study the regulation of RIP2 expression, we first assessed the effect of activation on RIP2 mRNA and protein expression in human peripheral blood mononuclear cells (PBMC). Remarkably for a signaling molecule, we found that, in the absence of stimulation, RIP2 expression in PBMC was very low but increased significantly upon mitogenic stimulation. Such an increase was observed at the mRNA level (on average 6-fold over baseline) as detected by Northern blot analysis (Figure 1a) and by real-time polymerase chain reaction (PCR; Figure 1b), and at the protein level (Figure 1c). The presence of two bands representing transcripts of approximately 2.5 and 1.9 kb in the Northern blot is consistent with previous data suggesting differential splicing for RIP2 RNA.[7–9] Interestingly, stimulation with either phorbol-12-myristate-13-acetate or ionomycin alone induced RIP2 protein expression, and the effect was slightly increased when added together. The change in RIP2 expression was specific and not the result of an overall increase in gene transcription as it was not observed for RIP3, another RIP family member (Figure 1d).[16]

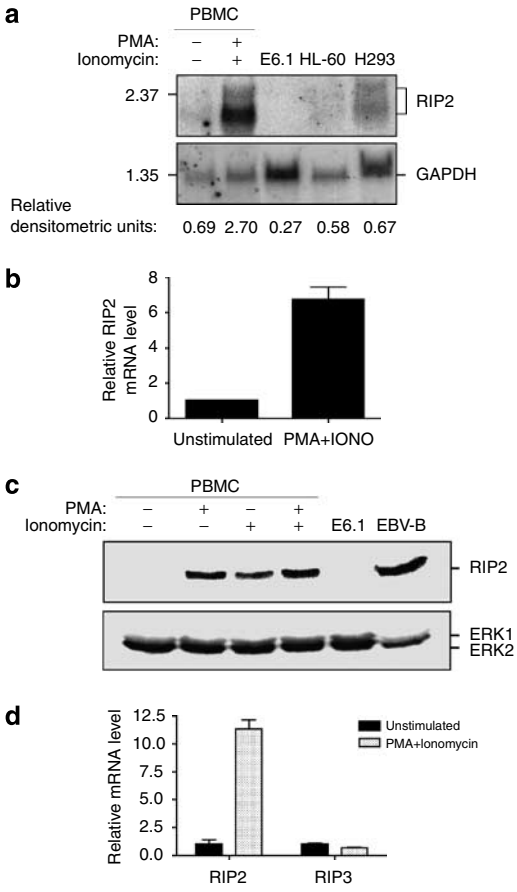

Figure 1 | Activation-induced upregulation of RIP2 mRNA and protein in PBMC. (**a–d**) PBMCs were left unstimulated or stimulated *ex vivo* with phorbol-12-myristate-13-acetate (100 ng/ml) and ionomycin (2 μg/ml) for 18 h. RIP2 expression was determined at the RNA level by (**a**) Northern Blot analysis and (**b**) real-time PCR, and at the protein level by (**c**) Western blot analysis. RNA and protein lysates from E6.1 cells served as a negative control, whereas HL-60, H293, and EBV-B served as positive controls. Band intensities in (**a**) were quantified and expressed as relative densitometric units. Results in (**b**) are graphed as the mean ± s.d. of RIP2 mRNA expression levels, as determined by real-time PCR, for three independent experiments. (**d**) Comparison of RIP2 and RIP3 mRNA expression (mean ± s.d.) as determined by real-time PCR. Results are representative of at least two independent experiments.

Time-dependent upregulation of RIP2 expression in blood cell subsets upon activation

To dissect the origin of RIP2 upregulation upon PBMC activation, we sorted the various immune cell subsets from peripheral blood and stimulated them with cell-specific stimuli. Upregulation of RIP2 mRNA expression was observed in peripheral blood monocytes in response to lipopolysaccharide (LPS), concomitantly to a TNF-α response (Figure 2a). In these cells, RIP2 upregulation occurred much faster than in PMN or T cells, reaching maximum levels after 2 h of stimulation. In PMN, LPS induced a moderate increase (four- to five-fold over baseline) in RIP2 expression, reaching maximum expression at 8 h (Figure 2b). In contrast, the upregulation of RIP2 in

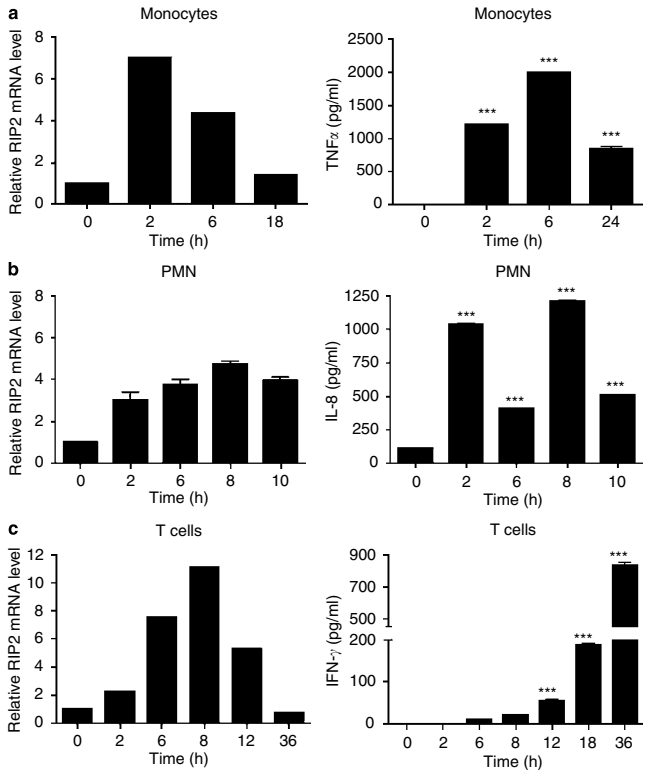

Figure 2 | Activation-induced upregulation of RIP2 mRNA expression is cell-specific and time-dependent. Total RNA was collected from (**a**) purified blood monocytes stimulated with LPS (2 μg/ml); (**b**) purified PMN cells stimulated with LPS (2 μg/ml); and (**c**) peripheral blood T cells stimulated with plate-bound anti-CD3 (5 μg/ml) + IL-2 (20 U/ml) for the various time intervals and RIP2 expression was analyzed by real-time PCR. Levels of (**a**) TNF-α, (**b**) IL-8, and (**c**) IFN-γ were measured by enzyme-linked immunosorbent assay and served as positive controls for cell activation. Results shown are plotted as mean $\pm$ s.d. and are representative of at least three independent experiments. ***$P \leqslant 0.001$.

T lymphocytes in response to plate-bound anti-CD3 antibodies plus IL-2 followed a different time course with a steady increase to peak levels (11-fold over baseline) occurring at 8 h (Figure 2c). Activation of PMN and of T cells was confirmed by detection of IL-8 and IFN-γ, respectively. The different temporal profiles were not due to differences in the magnitude of response to each stimulus as maximal response was achieved for each cell type.

Upregulation of RIP2 expression can be induced through TLRs

Next, we examined whether microbial toxins, a more physiologically relevant stimulation, could induce such a response (Figure 3). We observed that the Gram-negative-associated toxin LPS, and the Gram-positive-associated toxins peptidoglycan (PGN) and lipoteichoic acid (LTA) induced significant upregulation of RIP2 (8- to 25-fold over baseline levels) (Figure 3a). Because these pathogen-derived molecules utilize TLRs to activate immune cells (TLR4 for LPS and TLR2 for PGN and LTA),[17] we next tested the dependence of RIP2 expression on TLR signaling with the use of TLR blocking antibodies. As expected, pretreatment of 1,25-dihydroxyvitamin D_3-differentiated U937 cells with either anti-TLR4 or anti-TLR2 blocking antibodies completely abrogated the upregulation of RIP2 mRNA in response to *Escherichia coli* LPS and *Staphylococcus aureus* PGN, respectively (Figure 3b and c). Our data imply that the increase in RIP2 expression in response to bacterial challenge is the result of signaling from TLRs.

Upregulation of RIP2 expression during a clinical infection

Once demonstrated that RIP2 expression was upregulated upon immune cell activation by microbial toxins, and given the temporal profile of RIP2 upregulation in different immune cell subsets, we proceeded to test if upregulation of RIP2 expression was occurring in the course of PD-associated peritonitis, a clinical immune response with a well-defined innate immune component that evolves into a Th1 immune response.[3–5]

As previously reported, during the first 2 days of PD-associated peritonitis, we observed a very significant increase in the number of PMN cells in the peritoneal cavity.[18] After this time, the number of PMN cells decreased, whereas the percentage of $CD14^+$ macrophages/dendritic cells in the effluents increased. On the basis of these cellular dynamics, we analyzed the expression of RIP2 mRNA by real-time PCR in PMN and $CD14^+$ cells (not enough T cells were consistently obtained for RNA isolation in these clinical samples). We found that, during PD-associated peritonitis, RIP2 mRNA levels in PMN were increased over baseline on the first 2 days of infection decreasing afterwards in most cases (Figure 4a). RIP2 mRNA levels were also increased in $CD14^+$ cells, with 80% of patients reaching their maximum RIP2 mRNA expression level within the first 48 h of infection (Figure 4b). The upregulation of RIP2 in the course of PD-associated peritonitis was corroborated at the protein level, with an increase in RIP2 protein expression in peritoneal mononuclear cells (mainly $CD14^+$ cells and T lymphocytes) from days 1 to 3 of infection (Figure 4c), a profile that covers the innate response and its transition to a predominant adaptive response.

Upregulation of RIP2 expression during PD-associated peritonitis correlates with clinical outcome

Next, we compared the level of RIP2 upregulation with the clinical outcome of PD-associated peritonitis. We observed that patients who failed to upregulate RIP2 in peritoneal $CD14^+$ cells during the early stages of infection ($<$2-fold above basal level) had protracted peritonitis as defined by the need for hospitalization, catheter removal (four patients, 36%; *E. coli, Citrobacter fruendii/Candida tropicalis, S. aureus, Pseudomonas aeruginosa*), and death (one patient; *S. aureus* infection) (Figure 5a). In direct contrast, all patients who upregulated RIP2 ($>$2-fold above basal levels) within the first 48 h of infection had conventional peritonitis (as defined by the lack of hospital admission) with rapid resolution. The

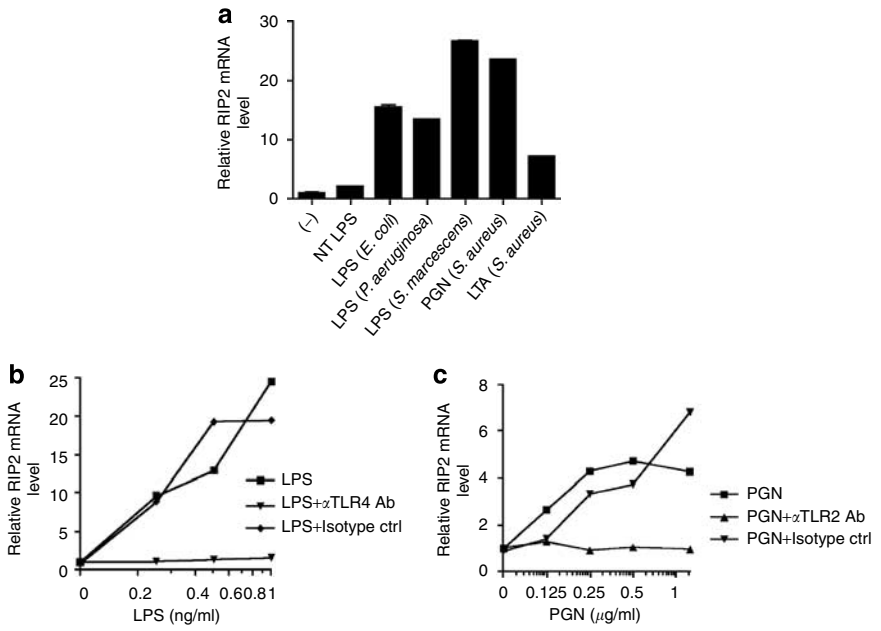

Figure 3 | Upregulation of RIP2 in response to bacterial toxins is pattern recognition receptor-dependent. (**a**) 1,25-Dihydroxyvitamin D_3-differentiated U937 cells were stimulated with 2 μg/ml of nontoxic (NT) LPS, *E. coli* LPS, *P. aeruginosa* LPS, *S. marcescens* LPS, 10 μg/ml *S. aureus* PGN, and *S. aureus* LTA for 2 h and RIP2 mRNA expression measured by real-time PCR. (**b**) 1,25-Dihydroxyvitamin D_3-differentiated U937 cells were stimulated with titrating amounts of *E. coli* LPS in the presence/absence of anti-TLR4 antibody (10 μg/ml) or (**c**) titrating amounts of *S. aureus* PGN in the presence/absence of anti-TLR2 antibodies (10 μg/ml). Total RNA was isolated and RIP2 expression was analyzed by real-time PCR. Results are plotted as mean $\pm$ s.d. and are representative of three independent experiments.

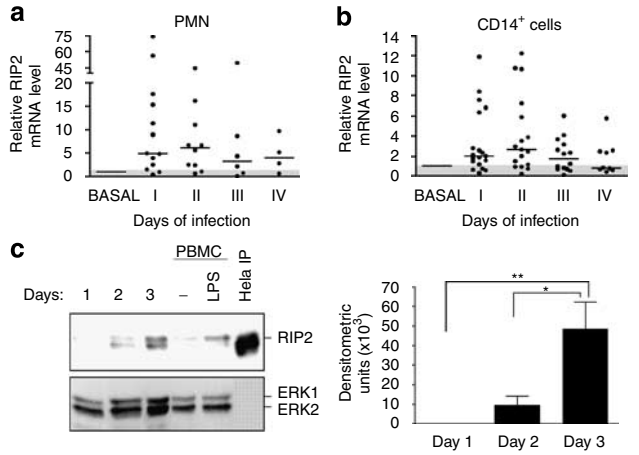

Figure 4 | Upregulation of RIP2 in peritoneal immune cells during PD-associated peritonitis. Long dwelling PD effluents from patients with peritonitis were collected each day after diagnosis. Total RNA was collected from (**a**) purified PMN ($n = 14$) and (**b**) $CD14^+$ peritoneal cells ($n = 20$) every day during the course of PD-associated peritonitis and analyzed for the expression of RIP2 mRNA by real-time PCR. Results are plotted as the relative RIP2 mRNA level for each patient during the first 4 days of infection. (**c**) Whole-cell lysates were prepared from peritoneal mononuclear cells (i.e., depleted of PMN) during the first 3 days of PD-associated peritonitis and immunoblotted for RIP2. Immunoblotting for total ERK1/2 expression served as loading controls. Right panel: band density was quantified from the immunoblots of six separate infections and are plotted as mean $\pm$ s.d. *$P \leqslant 0.05$, **$P \leqslant 0.01$.

levels of RIP2 RNA (median $\pm$ s.d.) by real-time PCR in $CD14^+$ cells of peritoneal effluents were also significantly higher in cases of conventional peritonitis than in protracted ones ($P = 0.04$; Mann–Whitney *U*-test). This is in direct contrast with the clinical values obtained on day 1 for the total cell count (Figure 5b) and the percentage of neutrophils (Figure 5c), which do not show any significant differences between conventional and protracted peritonitis patients at the same time point. We also failed to see a significant difference in the levels of the proinflammatory cytokine IL-6 in the effluent at time of diagnosis (Figure 5d). It is important to note that differences in RIP2 levels seen on the first day of infection are not due to differences in antibiotic treatment as the day 1 effluent was drained before the administration of antibiotics, nor is it due to increased numbers of $CD14^+$ cells in the effluents, as the conventional peritonitis group had fewer $CD14^+$ cells than the protracted peritonitis group (data not shown, $P = 0.036$). In addition, no significant differences between groups were observed in terms of medications, time on dialysis, age, previous episodes of peritonitis, or causal pathogen ($P = 0.1492$; Table 1).

To address formally the suitability of RIP2 mRNA levels in the prognosis of peritonitis outcome, we plotted the receiver-operator characteristic curve for the RIP2 mRNA values, the total cell count, and the dialysate IL-6 levels. With this test, we found that only the receiver-operator characteristic curve of the RIP2 mRNA levels gave an area under the curve that was significantly different from the line of identity ($P = 0.039$; Figure 6). The same test for total cell count or IL-6 show lower areas under the curve that were not statistically significant and that fell on the line of identity. On the basis of these data, we concluded that RIP2 expression is a good indicator of clinical outcome for PD-associated peritonitis.

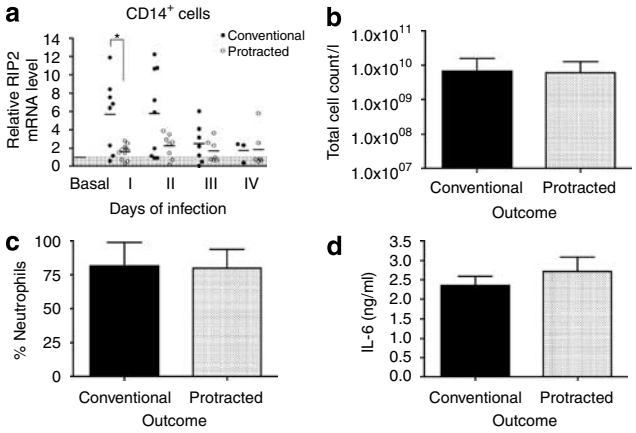

Figure 5 | Early upregulation of RIP2 mRNA in peritoneal CD14$^+$ cells correlates with outcome of PD-associated peritonitis. (**a**) RIP2 mRNA levels of patients with episodes of conventional peritonitis ($n=9$; ●) versus patients with protracted peritonitis ($n=11$; ○) during the first 4 days of clinical peritonitis. The day 1 clinical laboratory values for the (**b**) total cell counts per litre of effluent, the (**c**) percentage of neutrophils, and (**d**) the day 1 dialysate IL-6 levels, plotted as mean ± s.d., for patients with conventional peritonitis versus patients with protracted peritonitis. *$P<0.05$.

Because RIP2 expression has been linked to the ability to mount a Th1 response, we assessed the levels of IL-12, a Th1-inducing cytokine, in conventional vs protracted peritonitis. For this analysis, we excluded those cases of fungal peritonitis given their unique pathogenesis and clinical presentation. We found a trend toward higher levels of IL-12p40 in the peritoneal effluents from conventional PD-associated peritonitis when compared with the levels in peritoneal effluents of patients with protracted PD-associated peritonitis ($P=0.059$) (Figure 7). Conversely, the levels of IL-8 showed a trend toward higher levels in the protracted peritonitis group ($P=0.067$). More importantly, levels of MCP-1 were significantly higher in the protracted group on day 2 ($P=0.036$) consistent with the idea of a delayed clearance of activated innate immune cells (Figure 7 and data not shown). This finding is in agreement with the claim that RIP2 expression in PD-associated peritonitis is needed for the development of a Th1-type immune response, which has been associated with the clearance of inflammation and a favorable outcome for PD-associated peritonitis.[5,19] Although we cannot make direct conclusions between RIP2 and IL-12p40 levels due to the initial design of the study, the fact that both correlate with the clinical outcome implicates RIP2 as being necessary for the development of a protective immune response. Future studies are needed to determine if there exists a direct correlation in IL-12 production and RIP2 levels in PD patients during episodes of peritonitis.

Expression of IL-12p35 in response to LPS and IFN-γ in blood monocytes is RIP2-dependent

To document further the link between RIP2 upregulation in the APC and the imprinting of an immune response into the Th1 profile,[20] we directly tested whether the ability of an APC to upregulate RIP2 expression determines its capacity to produce IL-12p35.[21] To do this, we used RNA interference for RIP2. As expected, LPS and IFN-γ stimulation of peripheral blood monocytes upregulated RIP2 and IL-12p35 expression (Figure 8). More importantly, RIP2 silencing with siRNA (as shown in the bottom graph of Figure 8) significantly inhibited the upregulation of IL-12p35 expression in response to stimulation with LPS and IFN-γ (Figure 8, top graph). Of interest, down-modulation of RIP2 with siRNA correlated with a slight increase of background IL-12p40 in

Table 1 | Demographics of the patients with PD-associated peritonitis included in this study

Parameter	Conventional peritonitis (n=21)	Protracted peritonitis (n=14)
Age (years)	57.14 ± 15.48 (20–78)	57 ± 18.5 (29–92)
Sex		
Male/Female	15/6	11/3
Time on PD (months)	0.75–76	0.25–81
Primary kidney disease		
Glomerulonephritis	2	4
Diabetic nephropathy	10	4
Hypertension	3	1
Other	6	5
Residual renal function (K_r)	0.33 ± 0.6	0.12 ± 0.23
nPNA (g/kg/day)	0.81 ± 0.22	0.85 ± 0.25
Serum albumin (g/l)	31.58 ± 4.4	32.85 ± 3.29
Icodextran use (number of patients)	5	5
Infectious agent		
Gram-positive	**13**	**5**
Staphylococcus aureus	3	2[a]
Staphylococcus epidermidis	4[b]	
Staphylococcus warneri	1	
Enterococcus faecalis	4	
α-haemolytic Streptococcus		2[a]
Corynebacterium diphtheriae	1	1
Gram-negative	**6**	**8**
Acinetobacter baumannii	2[b]	1
Escherichia coli	1	1
Serratia marcescens		2
Citrobacter freundii	1	1[c]
Pseudomonas aeruginosa	1	3
Pantoea agglomerans	1	
Culture negative	**3**	**2**
Fungal		1[c]
Hospitalization		13
Death		1[d]
Catheter removal		6[e]

nPNA, normalized protein equivalent of nitrogen appearance; PD, peritoneal dialysis; RIP2, receptor-interacting protein 2.
[a]One *α-haemolytic Streptococcus* infection was concomitant with *S. aureus* infection.
[b]One *S. epidermidis* infection was concomitant with *Acinetobacter baumannii* infection.
[c]*Candida tropicalis* infection was concomitant with *C. freundii* infection.
[d]Patient died approximately 48 h after initial onset of peritonitis symptoms (*S. aureus* infection). Patient was not hospitalized and did not have catheter removed.
[e]Infections resulting in catheter removal included one *E. coli*, one *C. fruendii* concomitant with *C. tropicalis*, one *S. aureus*, and three *P. aeruginosa* infections (four were studied for RIP2 expression – one *E. coli*, one *C. fruendii/C. tropicalis*, one *S. aureus*, and one *P. aeruginosa*).

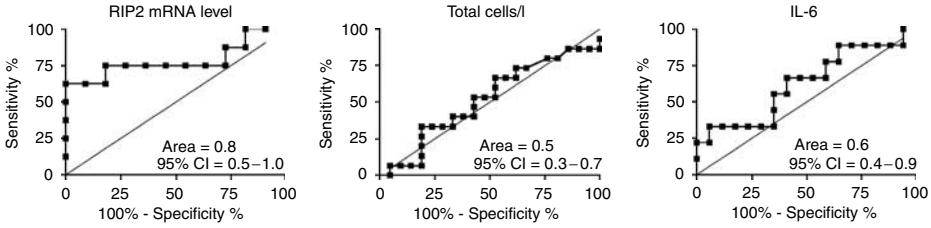

Figure 6 | RIP2 mRNA levels at time of diagnosis are a good predictor of peritonitis outcome. Receiver-operator characteristic curves for day 1 RIP2 mRNA levels ($P = 0.039$), total cell count/l of effluent ($P = 0.847$), and IL-6 levels in dialysate ($P = 0.293$) are shown. The area under the curve and the 95% confidence interval are indicated in each graph.

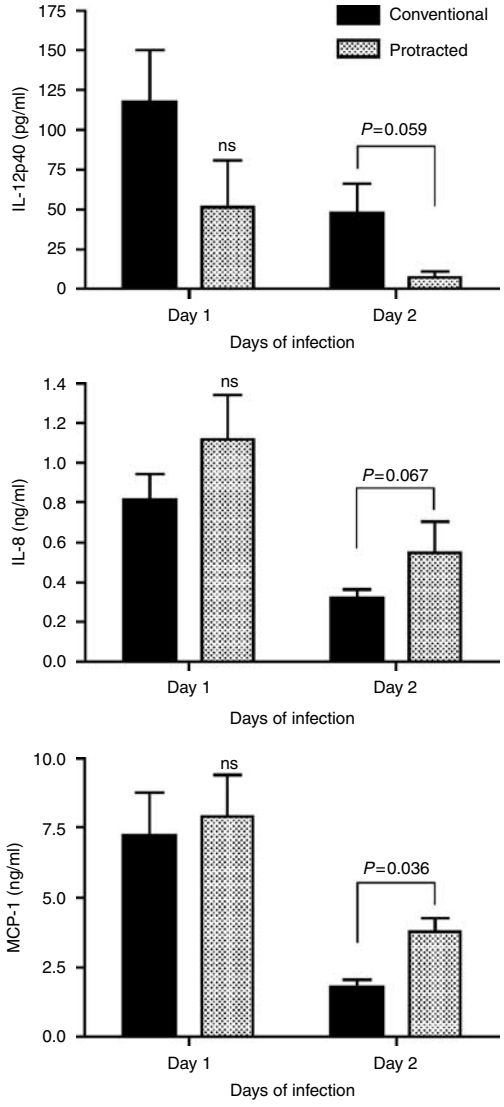

Figure 7 | Levels of IL-12p40, IL-8, and MCP-1 in the effluents of PD patients with peritonitis. Levels of IL-12p40, IL-8, and MCP-1 in the effluents of patients with episodes of conventional peritonitis ($n = 17$; black bars) and patients with protracted peritonitis ($n = 6$; shaded bars) as measured by enzyme-linked immunosorbent assay. Results are plotted as mean ± s.e.m. NS, not significant.

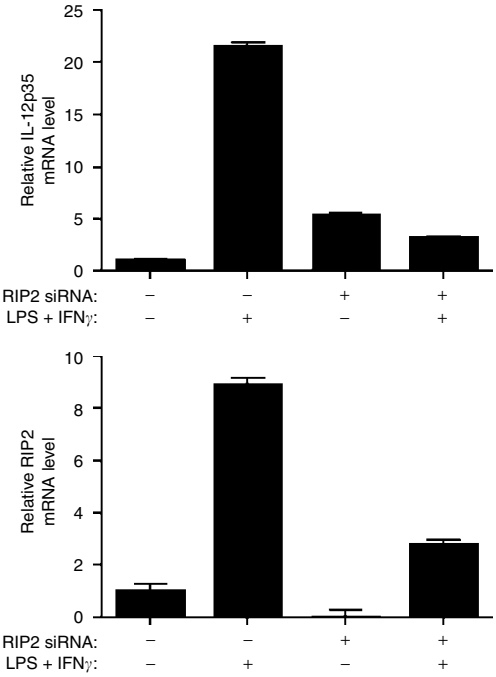

Figure 8 | Upregulation of IL-12p35 mRNA in blood monocytes is RIP2-dependent. Purified peripheral blood monocytes were transfected with or without RIP2 siRNA and left for 18 h at 37° before stimulation with 1 μg/ml LPS and 25 ng/ml IFN-γ for 6 h. Total RNA was collected and analyzed for the expression of IL-12p35 by real-time PCR. The bottom graph documents the downregulation of RIP2 by RNA interference using real-time PCR. Results are plotted as mean ± s.d. and are representative of two independent experiments.

the absence of stimulation, suggesting an involvement of RIP2 in a regulatory feedback of the signaling pathway leading to production of this cytokine. Further studies on this observation are currently underway. Together, our findings show that APCs that cannot upregulate RIP2 expression fail to produce the Th1-inducing cytokine IL-12 upon stimulation and thus will not generate a protective Th1 immune response, providing a mechanistic explanation for the clinical data on PD-associated peritonitis.

DISCUSSION

The results of this study show that RIP2 expression in human immune cells increases in response to activation through different receptors following a time-dependent, cell-type-specific profile. Specifically, monocytes reached peak RIP2 mRNA levels earlier than PMN and T cells in line with the notion that resident tissue macrophages initiate the response

to infection.[22] We also show that activation-induced upregulation of RIP2 is required for IL-12 production by APCs. More importantly, we demonstrate that RIP2 expression is upregulated during the course of PD-associated peritonitis, a clinical infection stimulating a protective Th1 immune response, and that this upregulation correlates with the clinical outcome of such an infection. Thus, RIP2 upregulation may be a useful monitoring tool and prognostic factor of PD-associated peritonitis.

The precise pathway involved in RIP2 upregulation is currently unknown. Our results imply that such pathway(s) emanate from different receptors, and that the type of receptor engaged dictates the timing and peak levels of RIP2 expression. One candidate pathway is the NF-κB cascade, shared by many receptors including T-cell receptor (TCR) and pattern recognition receptors (PRRs) such as TLR2, TLR4, and NOD2.[23–27] The results presented here support a role for TLR2 and TLR4, and for TCR in inducing RIP2 expression. A molecular mapping of the pathways upregulating RIP2 is currently undergoing, because it may have biological implications on the immunomodulation of inflammatory/adaptive responses.

What is the function of RIP2 in cell signaling? RIP2 has been linked to activation in response to stimulation from different receptors and pathways, but its precise role remains unclear. RIP2 seems critical for NF-κB activation in response to LPS-induced TLR4 signaling,[14,15,28] muramyl dipeptide (MDP)-induced NOD2 signaling,[14,26,29] TCR signaling,[14,15,30] and to cytokine (e.g., TNF-α and IL-1) receptor signaling.[31] Such a role seems to operate on the inhibitor of NF-κB kinase (IKK) complex, involving IRAK1 and TRAF6 for TLR-4 signaling, Bcl10 for TCR signaling, and TRAF2 and TRIP6 for cytokine signaling. Given the arguments presented above and our finding that TNF-α can also induce RIP2 expression, it is plausible to conclude that RIP2 regulates its own expression through its effects on NF-κB activation. It has also been claimed that RIP2 has mitogen-activated protein kinase kinase activity.[32] However, the putative serine/threonine kinase activity of RIP2 is not required for all these effects because macrophages expressing a kinase-dead RIP2 still respond to LPS-induced, TLR4 signaling.[28] This suggests that RIP2 may also act as an adaptor and/or have an activity other than kinase activity. In line with this assumption, it has been reported that RIP2 may facilitate a cross-talk between activators and regulators of the NF-κB pathway as reported for CARD6 or TAK1 on NOD protein signaling.[31,33–35] Also, Abbott *et al.*[29] have reported that RIP2 is required for NOD2-dependent ubiquitinylation of NF-κB essential modulator (NEMO) and NF-κB activation.

Although there is much evidence to support a role of RIP2 in the induction of proinflammatory genes,[11,14,15,28,36] little evidence exists delineating the role of RIP2 in regulating antigen presentation and T-cell polarization. Here, we report for the first time a direct role for RIP2 in the induction of IL-12p35 expression by monocytes. This finding indicates that the induction of Th1 immune responses is not only regulated at the levels of the T cell as reported previously,[14,15] but is also regulated by RIP2 at the level of APC. In addition, our findings also implicate RIP2-dependent NF-κB activation in the regulation of a number of genes whose expression is also dependent on the activation of interferon regulatory factors, such as IFN-β and IL-12p35,[37,38] further emphasizing the need for RIP2 expression during infection to obtain optimal immune responses.

The study of the *in vivo* regulation of human RIP2 expression has identified this molecule as a potential clinically relevant tool. Our data show that, during episodes of PD-associated peritonitis, patients who have high levels of RIP2 expression in peritoneal $CD14^+$ cells within the first 48 h of the onset of clinical symptoms have a good prognosis (correlating with the clinical diagnosis of conventional PD-associated peritonitis), and clear the infection within 4 days of initial onset without complications. In sharp contrast, 11 out of the 13 patients who failed to upregulate RIP2 expression early on in the disease required hospitalization and had a protracted evolution with one death and 36% requiring catheter removal. This dichotomy is independent of significant differences in total cells between the two groups or the causal pathogen, although there were a significantly greater number of $CD14^+$ cells in the effluents of patients with protracted peritonitis on day 1 and a significantly higher level of MCP-1 in the effluents of patients with protracted peritonitis on day 2. The ability for PD patients to mount Th1-mediated adaptive immune responses correlates with peritonitis outcome and in some cases has been associated with a lower risk of peritonitis,[5,39] likely because Th1 responses enhance the bactericidal function of macrophages, the antigen-presenting capacity of mesothelial cells, the clearance of neutrophils, and the development of memory T cells.[40–43] As such, our findings provide a molecular explanation for such clinical observation, that is, that the generation of an effective Th1 response is contingent upon RIP2 upregulation and IL-12 production, a conclusion consistent with the claim that *Rip2*$^{-/-}$ mice have defective Th1 immune responses[14,15] and *in vitro* data that RIP2 enhances the anti-cytomegalovirus cell response.[38]

What determines the ability to upregulate RIP2 in PD patients is unknown. The existence of allelic variations in the regulation of RIP2, which translate in differential ability to mount a Th1 response, is a possible explanation. There is evidence for NOD2 polymorphisms that determine the susceptibility or resistance to Crohn's disease, likely through differential regulation of the NF-κB pathway.[44] Given the regulatory role that NOD2 plays on some Th1 responses[45] and its capacity to interact with RIP2,[26] it is plausible to propose that the inability of some patients to mount Th1 responses to PD-associated peritonitis is a reflection of polymorphisms in the NOD2/RIP2/NF-κB axis.

Finally, our findings suggest that modulation of RIP2 expression may have therapeutic value to enhance beneficial Th1 responses or downregulate those that are harmful, as suggested by the reported therapeutic effect of RIP2 inhibition in a mouse model of Crohn's disease.[46] In the specific case of

PD-associated peritonitis, Th1 responses are protective while the lack of a Th1 response correlates with prolonged inflammation that is detrimental to the patient as it enhances peritoneal membrane fibrosis and loss of ultrafiltration capacity.[47]

MATERIALS AND METHODS

Reagents

Phorbol-12-Myristate-13-Acetate, ionomycin, LPS (*E. coli* 0111:B4; *Serratia marcescens*, *P. aeruginosa*), PGN (*S. aureus*), and LTA (*S. aureus*) were obtained from Sigma-Aldrich (Oakville, Canada). Non-toxic LPS from *Rhodobacter capsulatus* was provided by Dr MA Valvano (University of Western Ontario, London, ON, Canada). Recombinant human IL-2 was obtained from Roche Diagnostics (Laval, Canada). Cell lineage was determined with directly labelled monoclonal antibodies against CD3 (UCHT1) and CD19 (HIB19) (eBioscience, San Diego, CA), CD14 (M5E2) and CD16 (3G8) (BD Biosciences, Mississauga, Canada). For blocking experiments, 1,25-dihydroxyvitamin D_3-differentiated U937 cells were pretreated with anti-TLR4 (HTA125) and anti-TLR2 (TL2.1) (eBioscience) for 30 min before stimulation.

Cells

Human cell lines U937, HL-60, Jurkat E6.1, HEK293; and the EBV-B cell-transformed cell line (GM4672) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 containing 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 10 mM HEPES, and 10% fetal bovine serum. U937 cell differentiation was induced with 0.1 μM of 1,25-dihydroxyvitamin D_3 for 3 days.[48]

Human PBMC were isolated using Ficoll-Paque Plus (Amersham Biosciences, Baie D'urfe, Canada). PMN cells were isolated by dextran sedimentation and hypotonic lysis of red blood cells. Further purification was done by magnetic cell sorting (Miltenyi Biotec, Auburn, CA, USA), and evaluated by flow cytometry. Purity ranged from 94–99%.

PD-associated peritonitis

Patients were recruited from the London Health Sciences Centre Peritoneal Dialysis Unit. Two-liter, long dwell (between 6 and 12 h for day 1; and between 6 and 8 h for days 2 to 4) PD effluents were collected from 35 patients on automated or cycler PD using either Baxter Dianeal or Icodextran solutions, over the course of 30 months. Diagnosis of PD-associated peritonitis was based on the presence of abdominal pain, and a cloudy PD effluent with a leukocyte count above 100×10^6/l of effluent. During episodes of PD-associated peritonitis, patients had their long duration dwell effluents (6–12 h for day 1–8 h for days 2–4) collected every 24 h for 4 days, whereby the first drainage bag after the onset of symptoms and before the initiation of antibiotic treatment was classified as day 1. Patient demographics are representative of current PD program trends and are outlined in Table 1. No significant differences in the distribution of Gram-positive and -negative organisms in conventional versus protracted peritonitis were observed. Patient participation in the study was voluntary and research protocols were approved by the Office of Research Ethics at the University of Western Ontario. Peritonitis outcome was defined based on the need for hospitalization; patients not requiring hospitalization were considered to have conventional peritonitis, whereas patients requiring hospitalization within the first 5 days of infection onset were considered to have protracted peritonitis. Cells were isolated from PD effluents by centrifugation at 1600 r.p.m. for 15 min at 4°C.[49]

Quantitative real-time PCR

Expression of gene transcripts was quantified on the Mx4000 multiplex quantitative PCR (Stratagene, La Jolla, CA, USA) using Brilliant SYBR Green QPCR Core Reagent kit. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Mississauga, Canada) or Trizol reagent (Invitrogen Life Technologies, Burlington, Canada). Complementary DNA was synthesized using the Omniscript reverse transcriptase kit (Qiagen) and oligo-dT 12–18 primer (Invitrogen). Primers used were RIP2 F1, 5′-CCATTGAGATTTCG CATCCT-3′ and RIP2 R1, 5′-ATGCGCCACTTTGATAAACC-3′ defining a product of 161 bp; GAPDH F1, 5′-TGCACCACC AACTGCTTAGC-3′ and GAPDH R1, 5′-GGCATGGACTGTGGTC ATGAG-3′ (100 bp); RIP3 F1, 5′-GGCCCCAGAACTGTTTGTTA-3′ and RIP3 R1, 5′-CGGTTGGCAACTCAACTTCT-3′ (115 bp); and IL-12p35 F1, 5′-GTTCCCATGCCTTCACCACT-3′ and IL-12p35 R1, 5′-TGTCTGGCCTTCTGGAGC-3′ (69 bp). Each PCR product was sequenced to confirm specificity. The relative expression levels, based on the threshold values (C_t) of the RIP2, RIP3, or IL-12p35 gene normalized to the glyceraldehyde 3-phosphate dehydrogenase gene, were quantified using the delta C_t method. The amplification efficiency was calculated for each real-time PCR reaction and data from experiments for which the efficiency of reaction was between 80 and 110% were used.

Northern blotting

Total RNA (25–30 μg) from resting or activated PBMC was used for Northern blot analysis[50] using a human RIP2 cDNA probe corresponding to nucleotides 561–1259. Membranes were hybridized with a β-actin probe (ATCC) as a control for RNA levels.

Western blotting

Whole-cell lysates from unstimulated and stimulated PBMC and peritoneal mononuclear cells were prepared using standard lysis buffer. Detection of RIP2 was performed with a rabbit polyclonal antibody against RIP2 (Cayman, Cedarlane, Hornby, Canada; eBioscience) and signals acquired and quantified using the Fluorchem 8000 unit (Alpha Innotech, Fisher Scientific, Ottawa, Canada).

Cytokine production

Levels of TNF-α, IL-8, IFN-γ, IL-12p40, and MCP-1 (BD Biosciences, Mississauga, Canada) were determined by enzyme-linked immunosorbent assay. Each supernatant was assayed in triplicate.

RNA interference

Transfection of blood monocytes was carried out by the Amaxa Nucleofection System (ESBE Scientific, Markham, Canada). Validated duplexes of 21-oligonucleotide siRNA targeting RIP2 mRNA were obtained from Ambion (Austin, TX) and used at a final concentration of 100 nM.

Statistics

Unpaired Student's *t*-test with Welch's correction, Mann–Whitney *U*-test, one-way ANOVA with Tukey post-test, Fisher's exact test, and receiver-operator characteristic curves were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A difference between groups was considered significant when $P < 0.05$.

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