Differential roles of *Yersinia* outer protein P-mediated inhibition of nuclear factor-kappa B in the induction of cell death in dendritic cells and macrophages

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Yersinia outer protein P (YopP) induces cell death in macrophages and dendritic cells (DC). In DC this YopP-dependent cell death coincides with the inhibition of nuclear factor-kappa B (NF- κ B) activation. However, as shown by measurement of propidium iodide uptake via disrupted cellular membranes, the preincubation of DC with several NF- κ B inhibitors prior to infection with *Yersinia* did not restore the death-inducing capacity of a YopP-deficient *Yersinia* mutant. These results suggest that in contrast to macrophages, in DC the YopP-dependent inhibition of NF- κ B activation is not causative for the induction of cell death. Instead, in DC, the inhibition of mitogen-activated protein kinases (MAPKs), in particular, p38 and c-Jun N-terminal kinase, prior to infection with a YopP-deficient *Yersinia* mutant substituted the death-inducing capacity of the *Yersinia* wild-type strain, indicating that the YopP-dependent inhibition of MAPKs mediates *Yersinia*-induced DC death. The differences between DC and macrophages in the mechanisms of cell death induction by YopP presented herein might be crucial for the function of these antigen-presenting cells.

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INTRODUCTION

Yersinia outer protein P (YopP) is injected by *Yersinia enterocolitica* into the cytosol of host cells by a type III secretion system, which causes cell death in macrophages and dendritic cells (DC) (Zhang *et al.*, 2005; Ruckdeschel *et al.*, 2001; Gröbner *et al.*, 2006, 2007a). In DC we have demonstrated that YopP-induced apoptosis is independent of the mitochondrial death pathway (Gröbner *et al.*, 2006) and that LPS can accelerate YopP-dependent DC death (Gröbner *et al.*, 2007a). In macrophages it was hypothesized that YopP (YopJ in *Yersinia pseudotuberculosis*) potentiates LPS-induced apoptosis by the inhibition of nuclear factor-kappa B (NF- κ B) activation (Ruckdeschel *et al.*, 1998, 2001; Haase *et al.*, 2005; Zhou *et al.*, 2005; Zhang & Bliska, 2003). Furthermore, it has been reported that in macrophages the inhibition of mitogen-activated protein kinases (MAPKs) together with NF- κ B is necessary for YopP-induced cell death (Zhang *et al.*, 2005). In the

present study we analysed the impact of YopP-dependent NF- κ B and MAPK inhibition on cell death induction in DC.

METHODS

Cultivation and infection of DC. Immature DC obtained from bone marrow of BALB/c mice (Harlan Winkelmann, Germany) (Gröbner *et al.*, 2006) or J774A.1 macrophages (ATCC TIB67) were infected with a *Y. enterocolitica* wild-type strain (pYV⁺) (Heesemann & Laufs, 1983) or a YopP-deficient mutant (*yopP*) (Ruckdeschel *et al.*, 2001). For this purpose overnight bacteria cultures grown at 27 °C were diluted 1:20 in fresh Luria–Bertani broth and grown for a further 1.5 h at 37 °C. The bacterial concentration was measured densitometrically at 600 nm. At 1 h post-infection, at a m.o.i. of 10:1, bacteria were killed by gentamicin (100 µg ml⁻¹) (Sigma). LPS was from *Salmonella typhimurium* (Sigma), the NF- κ B inhibitors MG-132, BAY-11-7082 and BAY-11-7085, and the MAPK inhibitors PD98059, SB203580 and SP600125 were all from Calbiochem.

Assessment of cell death by flow cytometry. Propidium iodide (PI) uptake (50 ng ml⁻¹) (Calbiochem) and the inner mitochondrial transmembrane potential $\Delta\Psi_m$ of DC stained by tetramethylrhodamine ethyl ester (40 nM; Molecular Probes) were determined by flow cytometry (FACSCalibur; BD Biosciences) and analysed by WinMDI

Abbreviations: CLSM, confocal laser scanning microscopy; DC, dendritic cells; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; NF- κ B, nuclear factor-kappa B; PI, propidium iodide.

version 2.8 software (J. Trotter, The Scripps Institute, La Jolla, CA, USA).

Confocal laser scanning microscopy (CLSM). DC or J774A.1 macrophages were seeded on coverslips and then incubated for 1 h with or without the NF- κ B inhibitors MG-132, BAY-11-7082 or BAY-11-7085. Subsequently, LPS was added for 1 h. After fixation, permeabilization and blocking with 100 % inactivated fetal calf serum (Sigma), samples were incubated with rabbit polyclonal p65 antibodies (sc-109; Santa Cruz) 1:50 in PBS for 60 min. After three washing steps with PBS, samples were incubated with secondary goat-anti-rabbit-Cy3 antibodies (Dianova) diluted 1:100 in PBS for 60 min. After three further washing steps with PBS, nuclei were stained with DAPI (Sigma; 1 μ g ml⁻¹) for 5 min and samples were analysed by CLSM using a Leica TCS SP2 ($\times 63$ objective). The images were subsequently processed in Photoshop 7.0.

NF- κ B ELISA. A total of 3×10^7 DC were lysed by MES buffer containing 0.2 mg digitonin ml⁻¹ as described previously (Gröbner *et al.*, 2006), and the pellets obtained after centrifugation, which contained nuclei and mitochondria, were used for NF- κ B ELISAs as recommended in the manufacturer's instructions (NF- κ B p65-kit and NF- κ B p50 kit; BD Clontech).

Immunoblot analysis. Extracts from 2×10^6 DC were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes (Schleicher & Schüll). Immunoblot analysis was performed with phospho-specific p38 MAPK antibodies (1:2500 dilution; BD) and (anti-mouse) peroxidase-conjugated secondary antibodies (1:1000 dilution; Dako) (Fig. 3a). As a loading control, membranes were stripped and reprobed with purified mouse anti-p38 α (1:1000 dilution; BD) and mouse peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by using enhanced chemoluminescence reagents (Amersham Biosciences).

Statistical analysis. The two-tailed Student's *t*-test was used to evaluate the difference in means between two groups.

RESULTS AND DISCUSSION

We analysed the differences in YopP-mediated cell death induction between DC and macrophages. LPS was reported to promote YopP-induced cell death in both these cell types. In DC, LPS contributes to YopP-induced cell death in the early phase of *Yersinia* infection by cleavage of caspase 8 (Gröbner *et al.*, 2007a). In J774A.1 macrophages, LPS potentiates apoptosis induced by the YopP-mediated inhibition of NF- κ B activation (Ruckdeschel *et al.*, 1998, 2001; Haase *et al.*, 2005; Zhou *et al.*, 2005; Zhang & Bliska, 2003).

Up to 60 min after infection with *Yersinia* wild-type, NF- κ B translocates to the nucleus of DC due to the LPS of *Yersinia*, whereas YopP does not inhibit NF- κ B activation until 90 min after infection (Erfurth *et al.*, 2004). However, it has been unclear, whether the inhibition of the NF- κ B signalling pathway is causative for YopP-induced DC death as has been described in macrophages. To analyse the role of YopP-dependent inhibition of NF- κ B activation in the induction of DC death, DC were infected with the *Yersinia* wild-type strain (pYV⁺) or the YopP-deficient mutant (*yopP*). As shown in Fig. 1(a), the mitochondrial transmembrane potential already was decreased 90 min after infection with YopP-secreting *Yersinia* (pYV⁺), but

not after infection with YopP-deficient *Yersinia* (*yopP*) confirming the findings that *Yersinia*-induced DC death is caused by YopP (Erfurth *et al.*, 2004; Gröbner *et al.*, 2006). Accordingly, the percentages of PI-positive DC, indicating the lack of cellular membrane integrity, increased as early as 90 min post-infection with pYV⁺ (Fig. 1b). In summary, these results suggest a simultaneous onset of YopP-dependent suppression of NF- κ B activation and induction of DC death.

To further assess whether the inhibition of NF- κ B induces death of DC in comparison to macrophages, DC or J774A.1 were preincubated with or without the proteasome inhibitor MG-132, which prevents degradation of the NF- κ B inhibitor I κ B α , or the NF- κ B inhibitors BAY-11-7082 or BAY-11-7085 (Nakanishi & Toi, 2005), and then treated with LPS for 1 h. As shown by Fig. 2(a), LPS caused the translocation of the NF- κ B p65 subunit from the cytosol into the nuclei, indicating the activation of NF- κ B in both DC and J774A.1. Preincubation of cells with the NF- κ B inhibitors distinctly inhibited the LPS-induced nuclear

Fig. 1. YopP of *Y. enterocolitica* rapidly induces death of DC. DC from BALB/c mice were infected with the *Yersinia* wild-type strain (pYV⁺) or the YopP-deficient mutant (*yopP*). (a) Flow cytometric analysis of the inner mitochondrial transmembrane potential ($\Delta\psi_m$) of DC stained with tetramethylrhodamine ethyl ester is shown. (b) Flow cytometric analysis of DC stained with PI is shown. Data are mean values $\pm$ SEM of three individual experiments. *, $P < 0.02$; **, $P < 0.002$; when compared to *yopP*-infected cells; medium, untreated cells. Staurosporine (stauro, 4 μ M) was used as a positive control.

Fig. 2. LPS-induced NF- κ B p65 activation is prevented by NF- κ B inhibitors. (a) DC from BALB/c mice or J774A.1 macrophages were seeded on coverslips, preincubated for 1 h with the NF- κ B inhibitors MG 132 (10 μ M), BAY-11-7082 (10 μ M) and BAY-11-7085 (10 μ M), and then treated with LPS (1 μ g ml⁻¹) for 1 h. After staining of cells with antibodies to NF- κ B p65 and Cy3-coupled secondary antibodies, and subsequent staining of nuclei with DAPI (1 μ g ml⁻¹), cells were analysed by CLSM. Data are representative of three independent experiments. Bar, 8 μ m. (b) After incubation of DC with or without the NF- κ B inhibitors, cells were incubated with LPS (1 μ g ml⁻¹) for 1 h, and nuclear extracts were analysed by ELISA for the NF- κ B p65 and the p50 subunits. Untreated cells (medium) were used as negative controls. Additionally, as an internal negative control, provided by the manufacturer of the analysis kit, nuclear extracts of LPS-treated DC were added to wells coated with a mutant DNA sequence indicating non-specific DNA binding (control). Data are means of two independent experiments performed in duplicate. (c, d) DC or J774A.1 macrophages were preincubated with NF- κ B inhibitors as described above and then infected with *Y. enterocolitica* (pYV⁺ or yopP). At 1 h post-infection NF- κ B p65 ELISA from nuclear extracts were performed as described above (c). Extracellular bacteria were killed by addition of gentamicin (100 μ g ml⁻¹) 1 h post-infection and, after staining with PI, cells were analysed by flow cytometry 4 h post-infection (d). Data in (c) and (d) are mean values $\pm$ SEM of four individual experiments. *, $P < 0.06$; **, $P < 0.006$; when compared to the correspondent DMSO control. Medium, untreated cells. White bars, DMSO; hatched bars, MG-132; grey bars, BAY-11-7082; black bars, BAY-11-7085.

translocation of NF- κ B p65, demonstrating the functionality of these inhibitors in DC and J774A.1. As DC spread out when cultivated on poly-L-lysine-coated coverslips, they appeared much larger than J774A.1 macrophages even though the same magnification was used. Additionally, NF- κ B activation in DC was quantified by NF- κ B ELISA. As shown in Fig. 2(b), the amount of NF- κ B p65 in nuclear extracts after stimulation of DC with LPS was about threefold higher compared to DC kept in medium, confirming the results shown in Fig. 2(a). The NF- κ B p50 subunit was unresponsive to LPS stimulation (Fig. 2b) confirming the results of a previous study performed in DC (Erfurth *et al.*, 2004). In nuclear extracts of DC infected for 1 h with pYV⁺ or *yopP* *Yersinia* the NF- κ B amount had doubled within the early phase (≤ 60 min) of infection suggesting that NF- κ B p65 nuclear translocation was caused by bacterial LPS (Fig. 2c), which is in accordance with our previous study (Erfurth *et al.*, 2004). However, when DC had been preincubated with several NF- κ B inhibitors 1 h prior to infection, the early *Yersinia* LPS-induced NF- κ B activation was reduced to levels similar to non-infected cells (Fig. 2c), thereby confirming the results shown in Fig. 2(a). Surprisingly, preincubation of DC for 1 h with the NF- κ B inhibitors even reduced the percentage of PI-positive cells after infection with *Yersinia* pYV⁺ for 4 h (Fig. 2d). Hence, it seems unlikely that YopP by inhibition of NF- κ B activation contributes to *Yersinia*-induced DC death. Instead, the reduction of YopP-mediated cell death by different NF- κ B inhibitors indicates that there was an inhibition of NF- κ B-dependent transactivation of proapoptotic genes, i.e. encoding death receptors, death receptor ligands or proapoptotic members of the Bcl2 family, or alternatively inhibition of the NF- κ B-dependent transcriptional repression of anti-apoptotic genes, i.e. encoding TRAF2 (Dutta *et al.*, 2006).

By contrast, cell death of J774A.1 macrophages was not reduced by pretreatment with NF- κ B inhibitors. In J774A.1 cells, treatment with MG-132 even increased the cell death rates after pYV⁺-infection. Furthermore, all inhibitors partly restored the cell death-inducing capacity of the *yopP* mutant in J774A.1 cells, indicating that the inhibition of NF- κ B activation is essential for the LPS-triggered induction of cell death in macrophages and confirming the results of former studies (Zhang *et al.*, 2005; Ruckdeschel *et al.*, 1998, 2001). When compared to macrophages only the treatment of DC with MG-132 prior to infection with *yopP* slightly increased cell death (Fig. 2d), whereas NF- κ B specific inhibitors did not. However, in DC infected with pYV⁺ the inhibitory effect of proteasome inhibition by MG-132 on YopP-induced cell death prevailed. Hence, the increase in cell death upon MG-132 treatment in *yopP*-infected DC might be triggered by reactive oxygen species, which recently have been reported to be overproduced in response to proteasome inhibition (Papa *et al.*, 2007). In summary, our data suggest, that in contrast to J774A.1 macrophages, the inhibition of NF- κ B activation is not essential for the induction of YopP-mediated DC death.

It has been reported that the YopP-mediated inhibition of the NF- κ B and the MAPK signalling pathways cooperate to promote cell death of macrophages (Zhang *et al.*, 2005). Therefore, we studied whether YopP-dependent DC death results from the inhibition of MAPK activity rather than the inhibition of NF- κ B activation. For this purpose, DC or J774A.1 were preincubated with several MAPK inhibitors and subsequently infected with *Y. enterocolitica*. As shown in Fig. 3(a), preincubation of DC with a p38 inhibitor (SB203580), a c-Jun N-terminal kinase (JNK) inhibitor (SP600125) or with a combination of these inhibitors, but not inhibition of the ERK signalling pathway (PD98059), partly, or even completely, restored the cell death-inducing capacity of the YopP-deficient mutant *yopP*. To confirm the inhibition of the p38-phosphorylation by YopP in pYV⁺-infected DC and by the p38 specific inhibitor SB203580 in *yopP*-infected cells, DC were treated with or without SB203580 for 30 min and subsequently infected with *Yersinia*. As shown by immunoblotting (Fig. 3b), phosphorylation of p38 was inhibited 15, 30 and 45 min post-infection with pYV⁺ (lanes 5, 9 and 13) when compared to DC infected with *yopP* (lanes 15, 19 and 23). By contrast, phosphorylation of p38 was detected in DC 30 and 45 min post-infection with the *yopP* mutant (lanes 19 and 23). Preincubation of cells with the p38 specific inhibitor SB203580 caused inhibition of p38 phosphorylation (lanes 20 and 24) in *yopP*-infected DC, whereas the JNK inhibitor SP600125 (lanes 21 and 25) or the NF- κ B inhibitor BAY-11-7082 (lanes 22 and 26), which were both used as controls, did not.

Our data suggest that in *Y. enterocolitica* YopP-dependent inhibition of MAPK kinases (MKKs) and bacterial LPS cooperate to induce cell death in DC. In particular, inhibitors for the MAPKs p38 or JNK, which are involved in the TNFR-signalling pathway, were sufficient to induce cell death in DC infected with the YopP-deficient strain *yopP*. Two recent studies describe the induction of apoptosis in cells treated with inhibitors of MAPK and tumour necrosis factor alpha (Furusu *et al.*, 2007; Ricote *et al.*, 2006).

By contrast, inhibition of p38, JNK and MEK1/2 MAPK in macrophages did not restore the cell death-inducing capacities of the *yopP* mutant. This is in accordance with a previous study performed in J774A.1 macrophages demonstrating that inhibition of MAPK contributes to *Yersinia*-induced cell death only in cells in which NF- κ B was inhibited (Zhang *et al.*, 2005).

Recently, it has been shown that YopJ of *Y. pseudotuberculosis*, which is homologous to YopP of *Y. enterocolitica*, catalyses serine/threonine acetylation, thereby mediating the inhibition of MKKs, i.e. MKK6, and the inhibition of NF- κ B signalling pathways (Mukherjee *et al.*, 2006; Mittal *et al.*, 2006; Bliska, 2006).

In the present study we provide evidence that YopP induces DC death by the inhibition of MAPKs, predominantly p38 and JNK. Moreover, we have recently demonstrated that YopP promotes the formation of the death inducing signalling complex (DISC), thereby causing the activation

Fig. 3. Inhibition of MAPKs restores the cell death-inducing capacity of a YopP-deficient *Yersinia* mutant in DC. DC from BALB/c mice or J774A.1 macrophages were preincubated for 30 min with the MAPK inhibitors PD98059 (20 μ M), SB203580 (10 μ M) and SP600125 (20 μ M), which inhibit MEK1/2, p38 and JNK, respectively, and then were infected with *Yersinia* (pYV⁺ or yopP). For the flow cytometric analysis extracellular bacteria were killed by the addition of gentamicin (100 μ g ml⁻¹) 1 h post-infection and DC (a) or J774A.1 (c) were stained with PI 4 h post-infection. Data in (a) and (c) are mean values $\pm$ SEM of three individual experiments. *, $P < 0.01$; when compared to the correspondent uninfected cells (DMSO); medium, uninfected cells. (b) Phospho-p38 immunoblot analysis from DC treated for 30 min with SB203580 (10 μ M), SP600125 (20 μ M) or BAY-11-7082 (10 μ M) prior to infection with *Yersinia* (pYV⁺, yopP). White bars, Medium; hatched bars, pYV⁺; black bars, yopP.

of caspase 8 (Gröbner *et al.*, 2007b). Hence, we speculate that YopP might promote the activation of caspase 8 at the level of DISC by acetylating MKK. This idea is supported by a study by Yeh *et al.* (1998) showing that MKK1 prevents apoptosis at this level.

DC are the most potent antigen-presenting cells. They have the unique ability to prime naive T cells and generate primary immune responses (Banchereau & Steinman, 1998). YopP of *Y. enterocolitica* induces cell death of mouse DC and thereby prevents DC from inducing T cell adaptive responses (Erfurth *et al.*, 2004). We observed that YopP involves caspase-dependent and, in contrast to macrophages, caspase-independent mechanisms of cell death induction in DC (Gröbner *et al.*, 2006; Ruckdeschel *et al.*, 2002). Moreover, we have shown in this study that in DC, in contrast to macrophages, YopP induces cell death by inhibiting MAPK activity.

This study supports the observation that there are differences between DC and macrophages in the mechanisms of cell death induction by YopP, which might be crucial for the function of these antigen-presenting cells. For example, in DC, which undergo terminal differentiation when infected with Gram-negative bacteria carrying LPS, the involvement of the inhibition of MAPK pathways by *Yersinia* YopP might allow the bacteria to limit the duration of DC activation and thereby subvert the immune response.

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