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Bv8, the amphibian homologue of the mammalian prokineticins, induces a proinflammatory phenotype of mouse macrophages

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1 The small protein Bv8, isolated from the amphibian skin, belongs to a novel family of secreted proteins linked to several biological effects. We describe the expression of Bv8/prokineticins and their receptors in mouse macrophages, and characterize their proinflammatory activities.

2 The rodent analogue of Bv8, prokineticin-2, is expressed by macrophages, as well as its G-protein-coupled receptor prokineticin receptor (PKR-1 and PKR-2). PKR-1 is expressed more abundantly.

3 Bv8 induces potent chemotaxis of macrophages at concentrations as low as 10^{-12} M.

4 It stimulates lipopolysaccharide-induced production of the proinflammatory cytokines IL-1 and IL-12, reducing that of the anti-inflammatory cytokine IL-10. The effects are observed starting at the very low concentration of 10^{-11} M.

5 Effects on chemotaxis and cytokine are not pertussis-toxin sensitive, but are completely prevented by addition of the phospholipase inhibitor U73122, suggesting a G_q protein is involved in the Bv8-induced effects.

6 Studies in PKR-1 knockout mice indicate that all the activities exerted by Bv8 on macrophages are mediated by the PKR-1 receptor.

7 In conclusion, Bv8 appears to be able to induce the macrophage to migrate and to acquire a proinflammatory phenotype.
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Abbreviations: EG-VEGF, endocrine gland-derived vascular endothelial growth factor; fMLP, formyl-met-leu-phe; IL, interleukin; LPS, lipopolysaccharide; MIT1, mamba intestinal toxin; PK, prokineticin; PKR, prokineticin receptor; PTX, pertussis toxin; TNF, tumor necrosis factor; U73122, (1-[6-[[(17b)-3-methoxyestra-1-3-5(10)-trien-17-yl]amino]hexyl])-1H pyrrole-2.5-dione

Introduction

The small 77-amino-acid (aa) protein Bv8, isolated from the amphibian skin (Mollay *et al*., 1999) belongs to a novel family of secreted proteins whose homologues have been found in snakes (VPRA, MIT1) (Joubert & Strydom, 1980; Schweitz *et al*., 1999), rodents (mouse Bv8 or prokineticin (PK) 1 and 2) (Melchiorri *et al*., 2001) and humans (PK 1 or EG-VEGF and PK 2) (Masuda *et al*., 2002). This peptide family has structural shared motives, such as the 20-aa terminal sequence and a pattern of cysteine sequence that folds the molecules into a globular form.

The mRNA of murine Bv8-like protein, PK 1 and 2 (PK-1 and PK-2), has been detected in the brain, spinal cord, dorsal root ganglia, gastrointestinal tract, endocrine glands, spleen and circulating leukocytes of mice, rats and humans (Li *et al*., 2001; Melchiorri *et al*., 2001; Masuda *et al*., 2002; Le Couter *et al*., 2004). Two receptors for this family of secretory proteins have been identified in humans, rats and mice (Lin *et al*., 2002; Masuda *et al*., 2002). These receptors, PKR-1 and PKR-2, belong to the G-protein-coupled receptor and share approximately 85% aa identity. They are distributed in the brain and peripheral organs (Melchiorri *et al*., 2001; Negri *et al*., 2002), including the spleen and leukocytes (Le Couter *et al*., 2004).

The list of biological activities associated with Bv8/PK peptides is rapidly growing. They seem to influence complex behaviors such as feeding and drinking (Negri *et al*., 2004), and circadian rhythms (Cheng *et al*., 2002), and are involved in neuronal survival (Melchiorri *et al*., 2001), angiogenesis (Le Couter *et al*., 2001; Le Couter & Ferrara, 2003), and the reproductive cycle (Wechselberger *et al*., 1999). Bv8 and EG-VEGF are also related to regulation of hematopoiesis and hematopoietic cell mobilization (Le Couter *et al*., 2004). Moreover, an hyperalgesic activity of Bv8 has been clearly demonstrated. When injected intravenously or subcutaneously in rats, Bv8 induces intense systemic nociceptive sensitization to mechanical and thermal stimuli applied to the tail and paws (Mollay *et al*., 1999; Negri *et al*., 2002).

The cytokines interleukin (IL-)1β and tumor necrosis factor alpha (TNF-α) are closely involved in peripheral nerve and neural hyperexcitability, leading to the development of persistent hyperalgesia (Watkins & Maier, 1999; 2002). However, anti-inflammatory cytokines have been reported to limit the hyperalgesic responses induced by inflammatory stimuli and by IL-1β and TNF (Poole *et al*., 1995).

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Considering that lymphoid organs, circulating leukocytes and hematopoietic cells all express high levels of Bv8-like protein (Le Couter *et al*., 2004), we assessed whether Bv8 could influence the physiology of the macrophage, the main cell involved in inflammatory responses. Macrophages play a central role in both innate and adaptive immunity (Schiffmann, 1982; Beutler, 2004). They are fundamental to the innate immune response and their ability to be chemotactically attracted to the site of initial microbial invasion or to an inflammatory focus is crucial for full activation of the immune/inflammatory response that follows (Beutler, 2004). They are the main producers of the proinflammatory cytokines IL-1β and TNF and the major anti-inflammatory cytokine IL-10 (Moore *et al*., 1993; Beutler, 2004; Hoebe *et al*., 2004). Macrophages also synthesize and release IL-12, the critical factor driving the development of T helper (Th)-1 cells (Moore *et al*., 1993; Trincheri, 1995; Mosman & Sad, 1996; Hoebe *et al*., 2004), linked to cellular immune responses and tissue injury.

Therefore, considering the importance of macrophages in all aspects of the inflammatory response, we evaluated the effect of Bv8 *in vitro* on several macrophage functions.

First of all we investigated whether murine peritoneal macrophages expressed PK receptors and PK 1/2. We then analyzed how Bv8 affected macrophage migration and the production of the pro-inflammatory cytokines IL-1β, TNF and IL-12 and the anti-inflammatory cytokine IL-10. In order to identify the type of receptor involved, we designed some experiments with macrophages from PKR-1 knockout (KO) mice, and tried to characterize the type of G-protein signaling in the effects observed.

Methods

Animals

Balb/C male mice, 18–20 g body weight (Charles River, Calco, Italy), were used. They were kept on a 12-h light–dark cycle with water and food *ad libitum* and were housed six to a cage. When indicated, PKR-1 KO mice and wild-type (WT) controls were used.

PKR-1-deficient mice were generated by Lexicon Genetics Incorporated (The Woodlands, TX, U.S.A.). A targeting vector was constructed in which exon 1 of the PKR-1 gene was replaced with a neomycin resistance gene derived from LacZ/Neo vector. Lex-1 embryonic stem (ES) cells were electroporated with the targeting vector before selection of the cells expressing the targeted allele for the generation of chimeric mice. PKR-1 deficient mice were generated by breeding chimeric mice with C57BL/6 mice.

Progeny were genotyped with PCR, which permitted the amplification of the WT PKR-1 gene (5′-GGTGACTATGA CATGCCCCTGG-3′, 5′-CTCTCGGAAAGGGAGAGGCA AGG-3′) and the neomycin-resistant gene cassette, which was inserted to disrupt the PKR-1 coding region (5′-CAGCGC ATCGCCTTCTATC-3′, 5′-CTCTCGGAAAGGGAGAGG CAAGG-3′). Genomic DNA was isolated from tail samples by proteinase K (Sigma, St Louis, MO, U.S.A.) digestion and ethanol precipitation, and 200 ng DNA was amplified (HotStarTaq DNA Polymerase, Qiagen, Milan, Italy) with the following cycle parameters: 95°C 3 min (one cycle); 95°C 1 min, 55°C 1 min, 72°C 1 min (30 cycles); 72°C 10 min (one cycle). Amplified products were resolved on 2% agarose gel. WT littermates were used as controls.

All the animal procedures were approved by the Institutional Review Board of the Department of Pharmacology of the University of Milan.

Drugs

Bv8 was extracted from the skin secretion of electrically-stimulated *Bombina variegata* and purified to 98% (HPLC) as previously described (Mollay *et al*., 1999). Formyl-met-leu-phe (fMLP), lipopolysaccharide (LPS), the G_i inhibitor pertussis toxin (PTX), U73122, inhibitor of all phospholipase C isoforms and its inactive analogue U73343 were purchased from Sigma. All substances were diluted to the concentrations required in endotoxin-free sterile medium.

Harvesting elicited peritoneal macrophages

Mice were inoculated intraperitoneally with 2 ml of 3% Brewer's thioglycollate medium (Difco, Detroit, MI, U.S.A.) for macrophage elicitation. Peritoneal exudate cells (PEC) were harvested in cold RPMI-1640 medium (Sigma) plus 10% fetal calf serum (FCS) (Gibco BRL, Life Technology, Italy) 3 days after elicitation (Limiroli *et al*., 2002, Major *et al*., 2002). PEC from 4–8 mice were pooled. Cell viability was checked by the Trypan blue exclusion test.

Harvesting resident peritoneal macrophages

Peritoneal cells from naive mice were harvested in cold RPMI-1640 plus 10% FCS (Limiroli *et al*., 2002). Cells from 20–40 mice were pooled in order to obtain enough cells for the experiments.

Gene expression analysis of PKR-1, PKR-2, PK-1 and PK-2 by RT–PCR

PEC were resuspended in RPMI plus 10% FCS at 4×10^6 ml^{-1} and 1 ml aliquots were dispensed into wells of a 24-well culture plate. Macrophages were isolated and purified by adherence to culture plates. After 2 h, nonadherent cells were removed with the medium and adherent cells were washed twice with warm RPMI plus 10% FCS (Limiroli *et al*., 2002). Adherent macrophages were used for RNA extraction.

Extracted RNA was purified using RNeasy columns (Qiagen, Milan, Italy) and checked for accidental degradation on agarose gel. Two micrograms of purified RNA were used for cDNA synthesis with reverse transcriptase (Promega, Milan, Italy) and the reaction product was diluted to 100 μl with deionized water; 2.5 μl of cDNA solution was used for PCR amplification (iCycler, Bio-Rad, Milan, Italy).

Beta-actin (housekeeping gene) gene expression was used as internal control. Specific sense and antisense primers were synthesized (Biogen, Rome, Italy), to PCR-amplify the mouse PKR-1, PKR-2, PK-1 and PK-2 cDNA, according to the following sequences:

PKR-1: 5′-ATTCTCGGACTTTCTTTGC-3′
5′-GCGGCTGTTTGACACTTC-3′ (379 bp);
PKR-2: 5′-GGCCATCGCTATTGACAGAT-3′
5′-GCGGTGAGGTAGTGCTTCTC-3′ (501 bp);

PK-1: 5′-AAGATCCCCTTCTTGAGGAAAC-3′
5′-CCAGAAAGGTTGCTTTAGGAAG-3′ (420 bp);
PK-2, 5′-AGCTGCCACCCCCTGACTCG-3′
5′-TTCCGGGCCAAGCAAATAAACC-3′(125 bp);

PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and the fluorescent bands were revealed with the Versa Doc 3000 imaging system (Bio-Rad, Milan, Italy), excised from the gel, eluted (CFXPCR Kit, Amersham Pharmacia, Italy) and sequenced (Primm s.r.l., Italy) to confirm the expected product.

Chemotaxis

Elicited or resident peritoneal macrophages were collected as described above and pooled. Cells and chemoattractant substances were suspended in RPMI-1640 + BSA 1%.

Chemotaxis was measured using a Boyden modified 48-well microchemotaxis chamber, with the upper and the lower compartments separated by a polycarbonate filter (Biomap, Agrate Brianza, Italy), with a pore diameter of 5 μm. Cells (2×10^6 cells ml^{-1}, 10^5 macrophages/well) were placed in the upper chamber, and samples of either medium (in order to assess spontaneous mobility), of the chemoattractant fMLP at the fixed concentration of 10^{-8} M, or Bv8 at concentrations from 10^{-8} to 10^{-14} M, were added to the lower chamber. The chambers were incubated for 100 min at 37°C in an atmosphere of 5% CO_2, then the migrated cells adhering to the distal part of the filters were fixed and stained. These cells were quantitated by microscopically counting random fields by a scorer blind to the experimental conditions (Sacerdote *et al*., 2005).

To distinguish better between true chemotaxis, cell migration following a chemical gradient and chemokinesis, random activation of movement due to the presence of a chemical, a 'checkerboard analysis' was performed, in which different concentrations of Bv8 were placed in the upper and lower compartments of the Boyden chamber.

In the experiments aimed at characterizing the type of G-protein involved, macrophages were preincubated at 37°C for 1 h with 1 μg ml^{-1} PTX, 10 μM of U73122 or the inactive molecule U73343, then tested for chemotaxis in the Boyden chamber with either Bv8 (10^{-11} M) or fMLP (10^{-8} M). As controls, the same macrophages were incubated for 1 h only with medium. To examine the chemotaxis of resident macrophages, Bv8 was used at the optimal concentration of 10^{-11} M.

In another series of experiments elicited peritoneal macrophages were isolated, as above, from PKR-1 KO mice and their WT controls. Cells were tested in the Boyden chamber for their ability to migrate in the presence of either Bv8 (10^{-11} M) or fMLP (10^{-8} M).

Results are reported either as chemotactic index, that is, cells that migrated in the presence of the chemoattractant/cell that migrated with medium only, or as the number of macrophages migrated/microscopic field.

All experiments were repeated 2–4 times.

Cytokine production

Macrophages were collected as above and pooled. Cells were resuspended in RPMI plus 10% FCS at either 1×10^6 cells ml^{-1} (elicited cells) or 2×10^6 ml^{-1} (resident macrophages), and 1-ml portions were dispensed into a 24-well culture plate. Macrophages were isolated and purified by adherence to culture plates. After 2 h, nonadherent cells were removed with the medium and adherent cells were washed twice with warm RPMI plus 10% FCS (Limiroli *et al*., 2002; Major *et al*., 2002; Cailhier *et al*., 2005).

Differential staining with Diff-Quick (Dade, Biomap, Milan, Italy) was used to establish the percentage of macrophages in the adherent cells and to rule out the presence of different cell types. The proportion of macrophages in the adherent cells was always $\geqslant 98\%$.

Elicited macrophages were primed with 1 μg ml^{-1} of LPS (Sigma) for TNF, IL-1 and IL-10 production or with 1 μg ml^{-1} LPS and 50 U ml^{-1} interferon (IFN)-γ (Pharmingen, San Diego, CA, U.S.A.) for IL-12 stimulation. The stimuli were added to the macrophage cultures in a final volume of 1 ml/well of RPMI plus 10% FCS, 1% glutamine (Sigma), 2% penicillin/streptomycin solution (Sigma), 0.1% 2-mercaptoethanol (Sigma) (complete medium).

Nonelicited macrophages were stimulated with 1 μg ml^{-1} of LPS for TNF, IL-1β and IL-10 production or with 10 μg ml^{-1} of LPS and 100 U ml^{-1} of IFN-γ for IL-12 (Limiroli *et al*., 2002). Bv8 was added to macrophage cultures at the same time as LPS at concentrations ranging from 10^{-7} to 10^{-13} M.

The plates were incubated at 37°C and 5% CO_2, and supernatants were collected after 24 h culture and stored frozen at −80°C for cytokine analysis (Limiroli *et al*., 2002). In the experiments with the inhibitors, macrophages were separated by adherence as described, and preincubated for 1 h in the presence of PTX (1 μg ml^{-1}), U73122 or U73343 (10 μM). At the end of the preincubation the stimuli for the production of cytokines (1 μg ml^{-1} LPS for TNF, IL-1β and IL-10 production, and 1 μg ml^{-1} LPS plus 50 U ml^{-1} IFN-γ for IL-12 stimulation) were added together with Bv8 at the concentration of 10^{-9} M. Supernatants for cytokine evaluation were collected 24 h later (Sacerdote *et al*., 2000).

When macrophages from PKR-1 KO and WT mice were used, the cells were isolated as described and Bv8 was added at the concentration of 10^{-9} M.

Cytokine ELISA

IL-12 p70 protein levels were determined by an enzyme-linked immunosorbent assay (ELISA) protocol standardized by Pharmingen (San Diego, CA, U.S.A.). The anti-IL-12 capture monoclonal antibody (mAb) (4 μg ml^{-1}) was absorbed on a polystyrene 96-well plate and the IL-12 in the sample was bound to the antibody-coated wells. The biotinylated anti-IL-12 detecting mAb (0.25 μg ml^{-1}) was added to bind the IL-12 captured by the first antibody. After washing, avidin–peroxidase (Sigma) was added to the wells to detect the biotinylated detecting antibody and finally 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) substrate was added. A colored product formed in proportion to the amount of IL-12 in the sample and was measured at optical density 405 nm. The amount of cytokine in each supernatant was extrapolated from the standard curve. The standards were recombinant cytokine curves generated in doubling dilutions from 30 to 4000 pg ml^{-1}.

IL-10 production was measured with the same ELISA protocol except for the use of anti-IL-10 capture mAb at 2 μg ml^{-1}, biotinylated anti-IL-10 detecting mAb at 0.5 μg ml^{-1}

and a standard curve from 15 to 2000 pg ml^{-1} (all mAbs and recombinant cytokines were from Pharmingen).

TNF-α concentrations in culture media were measured with an OptEIA set for mouse TNF, with standard curves ranging from 15 to 1000 pg ml^{-1} (BD Biosciences, Milan, Italy). Streptavidin–peroxidase and tetramethylbenzidine were used for color development. The color reaction was stopped with 2 N H_2SO_4 and read at an optical density of 450 nm.

For IL-1β measurements, a CytoSet Elisa kit for mouse IL-1β was used (Biosource, Prodotti Gianni, Milan, Italy). The concentrations of the capture and of the secondary biotinylated antibodies were 1.25 and 0.125 μg ml^{-1}, respectively. Standard curves generated from recombinant protein ranged from 15 to 1000 pg ml^{-1}. Color was developed as for TNF.

Statistical analysis

Results are expressed as the mean $\pm$ s.d. Data were analyzed by one-way analysis of variance followed by Bonferroni's *t*-test for multiple comparisons. Significance was accepted at $P<0.05$.

Results

Expression of PK receptors and of PKs in murine macrophages

Both PKR isoforms were expressed in thioglycollate-elicited mouse macrophages, but PKR-1 was more abundant than PKR-2 (Figure 1). Moreover, these cells only expressed PK-2. PK-2 mRNA was always present as a tissue-specific double-splice variant: the short form encodes for a protein similar to amphibian Bv8 but the long form encodes for an additional 21 aa domain, inserted in the center of the polypeptide chain, already described only in mouse and human testis (Wechselberger *et al*., 1999).

Chemotaxis in Balb/c mice

To test whether Bv8 induced macrophage migration, the protein was added in the lower chemotaxis chamber in a wide range of concentrations and elicited macrophages were seeded in the upper chamber. Bv8 was a potent and efficient stimulator of macrophage chemotaxis (Figure 2), with efficacy comparable to that of the chemotactic peptide fMLP, reaching a maximum chemotaxis index of 3 at the concentration of 10^{-12} M. Its potency in inducing chemotaxis is clear from the significant effect already present at the very low concentration of 10^{-14} M. The bell-shaped response curve is typical of most chemotactic substances, and is probably due to rupture of the gradient, that happens with the highest concentrations.

To better distinguish between true chemotaxis, cell migration following a chemical gradient and chemokinesis, random activation of movement due to the presence of a chemical, a checkerboard analysis was performed, placing different concentrations of Bv8 in both the upper and lower chambers. Table 1 shows cell migration primarily in response to a positive chemical gradient, indicating the true chemotactic activity of the protein.

To identify the type of G-protein coupled to the chemotactic effect of Bv8, we used the inhibitor of the G_i protein PTX , the phospholipase inhibitor U73122 and its inactive control reagent U73343. Elicited macrophages were preincubated for 1 h together with 1 μg ml^{-1} PTX, 10 μM U73122 or 10 μM U73343, and checked for their ability to migrate in the presence of an optimal active concentration (10^{-11} M) of Bv8 or fMLP (10^{-8} M).

As reported in the upper panel of Figure 3, PTX pretreatment did not affect the migration induced by Bv8

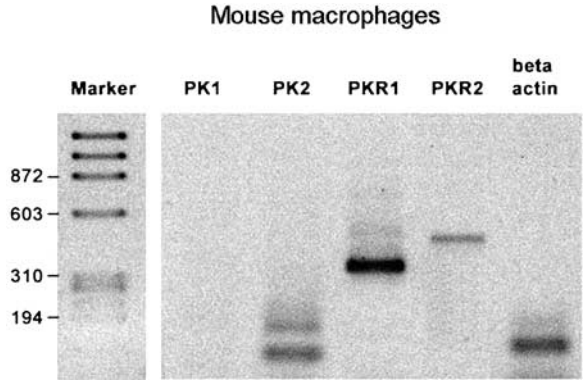

Figure 1 RT–PCR amplification of mRNAs for prokineticin receptors (PKR) PKR-1 and PKR-2 and of prokineticin-1 (PK-1) and -2 (PK-2) in elicited purified peritoneal macrophages. The left lane indicates the molecular marker.

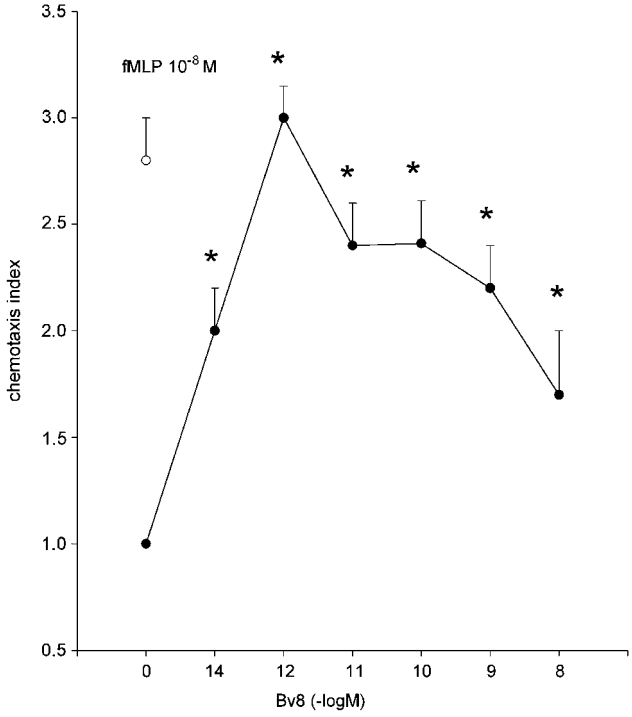

Figure 2 Macrophage chemotactic activity of Bv8, added in the lower Boyden chamber at the concentrations indicated. Thioglycollate-elicited macrophages were added in the upper chamber. The chemotactic activity of the standard chemoattractant peptide fMLP at the concentration of 10^{-8} M is also indicated. Results are reported as chemotactic index, that is, the number of cells migrated with Bv8/number of cells migrated in the presence of medium only. Means $\pm$ s.d. of four experiments. *$P<0.05$ vs background migration (chemotactic index = 1).

but, as expected, the chemotactic activity of fMLP was completely abolished in PTX-treated macrophages. In contrast (lower panel of Figure 3), after pretreatment with the phospholipase C inhibitor U73122, fMLP and Bv8-stimulated chemotaxis was significantly reduced, while U73343, its structurally close analogue that lacks the phospholipase-C inhibiting activity, had no such effect.

In order to rule out a toxic effect of the inhibitors on macrophages, at the end of pretreatment, before incubating the cells in the Boyden chamber, we checked the viability by the Trypan blue exclusion test. The percentage of viable cells was not affected by the inhibitors (data not shown).

Although thioglycollate-elicited macrophages are commonly utilized for macrophage studies (Limiroli *et al*., 2002; Major *et al*., 2002; Cook *et al*., 2003; Sacerdote *et al*., 2005), the possibility exists that thioglycollate-mediated stimulation might interfere with the results, affecting the stage of macrophage activation. To rule this out, we examined whether Bv8 also stimulated chemotaxis of resident, not elicited macrophages. As reported in Table 2, Bv8 (10^{-11} M) induced significant migration of resident macrophages. This effect was not blocked by PTX pretreatment, while the phospholipase C inhibitor U73122, but not U73343, prevented it.

Chemotaxis in PKR-1 KO and WT control mice

Mice knocked-out for PKR-1 have recently been generated. As this receptor is the most widely expressed on macrophages, we tested whether macrophages from these animals were still able to respond chemotactically to Bv8. Because the previous chemotaxis experiments were performed in Balb/C mice, and the PKR-1 KO animals had been generated on a C5/7BL6 background, we used WT littermates as controls. In these experiments, we used only thioglycollate-elicited macrophages. The results are described in Figures 4. Macrophages from WT animals showed chemotactic activity towards both fMLP and Bv8 similar to that with cells from the BalbC/J mice (see Figures 1 and 2). Macrophages from the KO animals also gave a significant chemotactic response when stimulated with

Table 1 Checkerboard analysis of Bv8-induced chemotaxis

Lower chamber	*Upper chamber*		
Bv8 (−log M)	*0*	*11*	*10*
0	28.5±3.2	22.5±3.4	26.3±3.9
11	90.0±13.0*	26.0±4.6	30.0±1.5
10	88.0±6.0*	30.0±2.3	28.1±3.6

Chemotactic responsiveness (migration towards a chemical gradient) of thioglycollate-elicited macrophages to Bv8, placed in both the upper and lower compartments of a Boyden chamber, as indicated. Results are cells/fields±s.d. from triplicate determinations.
*$P<0.01$ vs medium only (without Bv8 in either chamber).

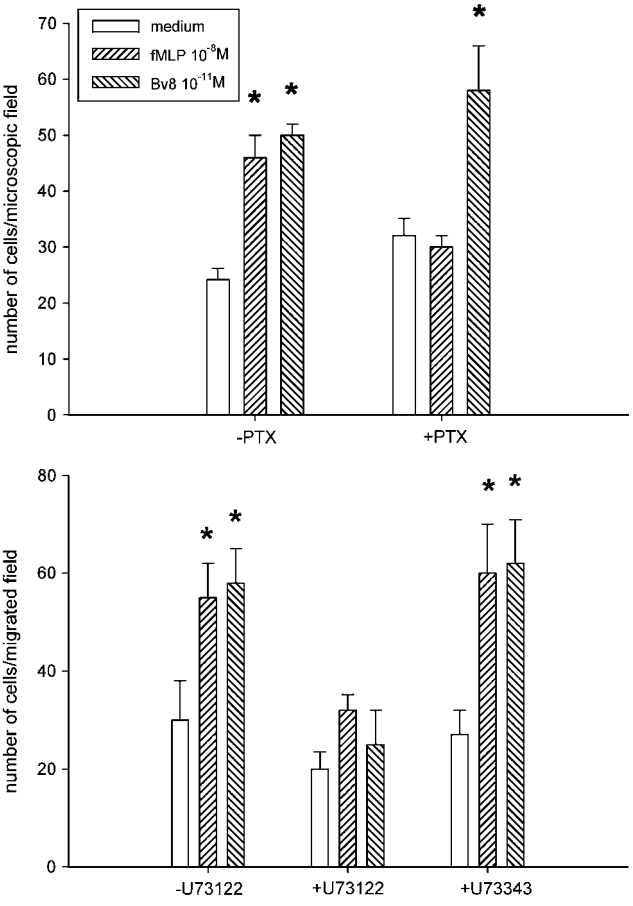

Figure 3 Effect of pretreatment with pertussis toxin, PTX, (upper panel) or the phospholipase inhibitors U73122 and U73343 (lower panel) on Bv8 and fMLP-induced chemotaxis. Elicited macrophages were preincubated with 1 μg ml^{-1} PTX, 10 μM U73122 or U73343 or medium only for 1 h. The viability of macrophages was checked and they were added in the chemotaxis chamber in the presence of medium only or with optimal concentrations of fMLP (10^{-8} M) or Bv8 (10^{-11} M). The chambers were then incubated for 100 min as indicated in Methods. The preincubation with PTX and U73122 prevented fMLP-chemotaxis, while only U73122 blocked Bv8's effect. Results are the number of cells migrated/microscopic field. *$P<0.01$ vs medium, that is, spontaneous migration.

Table 2 Resident peritoneal macrophage chemotaxis induced by fMLP and Bv8

Chemotactic stimuli	*Pretreatment (1 h)*			
	Medium	*+PTX*	*+U73122*	*+U73343*
Medium	15.2±8.1	17.0±9.5	12.2±8.1	16.4±6.3
fMLP 10^{-8} M	39.3±6.3*	21.1±6.7	15.0±4.2	44.0±8.0*
Bv8 10^{-11} M	43.1±6.2*	45.0±5.8*	16.1±9.4	41.2±9.6*

Effect of pretreatment with PTX, the phospholipase inhibitor U73122 and its inactive control U73343 on Bv8 and fMLP-induced chemotaxis of resident macrophages. Cells were preincubated with 1 μg ml^{-1} PTX, 10 μM U73122, 10 μM U73343 or medium only for 1 h before testing in the chemotaxis chamber with medium only, fMLP or Bv8. Results are cells/field±s.d. from triplicate determinations.
*$P<0.01$ vs medium/medium (spontaneous migration).

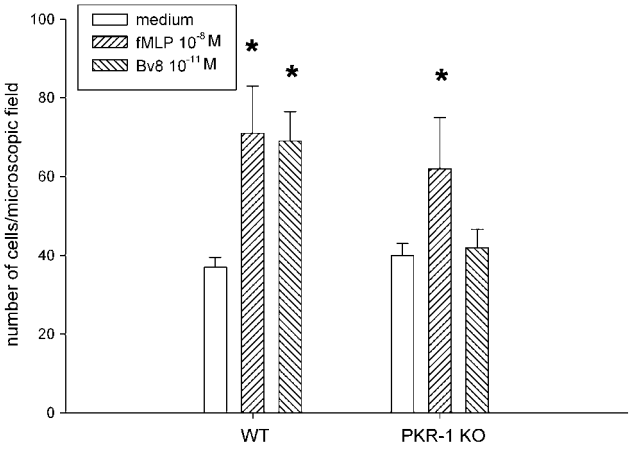

Figure 4 Migration of macrophages from wild-type (WT) or PKR-1 knockout (KO) mice. Thioglycollate-elicited macrophages were incubated in the Boyden chamber with medium only, fMLP (10^{-8} M) or Bv8 (10^{-11} M). Results are the number of cells migrated/microscopic field. Macrophages from WT mice were chemotactically attracted by both fMLP and Bv8 (*$P<0.01$ vs number of cells migrated in the present of medium only) while the macrophages from PKR-1 KO mice responded chemotactically to fMLP but not to BV8 (*$P<0.01$ vs number of cells migrated in the presence of medium only). Means ± s.d. of two experiments.

fMLP. In contrast, Bv8 completely lost its ability to induce chemotaxis with the macrophages from the PKR-1 KO animals.

Effect of Bv8 on cytokines in Balb/C mice

Bv8 was added *in vitro* for 24 h to thioglycollate-elicited macrophage cultures with or without LPS for the stimulation of IL-1β, TNF and IL-10 production, and LPS + IFN-γ for IL-12. As reported in panel (a) of Figure 5, Bv8 never affected spontaneous production of IL-1β. However, the protein significantly boosted the production of this cytokine in LPS-stimulated macrophages. The increase was evident at 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M, but lower and higher concentrations were not effective. Panel (b) of the Figure 5 shows the levels of TNF. When cells were cultured without LPS, this cytokine was not detectable. LPS induced TNF production but Bv8 never had any such effect at the concentrations tested. In contrast, as shown in the panel (c) of Figure 5, Bv8 added *in vitro* to macrophages significantly reduced LPS-induced production of the antiinflammatory cytokine IL-10. This effect was significant at the concentrations of 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M, while lower and higher concentrations had no noticeable activity.

Unstimulated cells, with or without Bv8, did not produce measurable amounts of IL-12. LPS + IFN-γ stimulated significant production of IL-12 and the addition of Bv8, at the concentrations of 10^{-11} and 10^{-9} M, significantly enhanced this output (Figure 5, panel d).

In order to rule out that thioglycollate elicitation could interfere with the results, we evaluated the ability of Bv8 to alter stimulated cytokine production also by resident, not elicited macrophages. Cells from 20/40 mice were pooled for each experiment.

As shown in Table 3, the levels of IL-1β, TNF and IL-10 produced by resident macrophages after *in vitro* stimulation with LPS (1 μg ml^{-1}) were comparable in thioglycollate-elicited and resident macrophages, while the LPS + IFN-γ-stimulated production of IL-12 was low in resident macrophages. However, BV8 at the concentration of 10^{-11} and 10^{-9} M affected the cytokine levels similarly to thioglycollate-elicited macrophages: IL-1β and IL-12 production was increased, IL-10 production decreased and TNF was not affected.

Considering that Bv8 has similar effects on elicited and resident macrophages, subsequent experiments were performed only with elicited macrophages, as this enabled us to use far fewer animals.

PTX and the phospholipase inhibitor U73122 were used to investigate the type of G-protein involved in Bv8-induced cytokine modulation. Purified thioglicollate-elicited macrophages were preincubated for 1 h in the presence of 1 μg ml^{-1} PTX or 10 μM U73122 and U73343 before the addition of LPS and 10^{-9} M Bv8. As reported in Figure 6, PTX did not affect the cytokines' responses to Bv8. With PTX treatment Bv8 significantly increased the concentrations of IL-1β (upper panel) and IL-12 (lower panel), while IL-10 (middle panel) remained low. The figure also shows that pretreatment with U73122, but not with its inactive analogue U73343, completely prevented Bv8's effects on the three cytokines.

Effect of Bv8 on cytokines in PKR-1 KO and WT control mice

Figure 7 shows the effect of Bv8 on cytokine production in PKR-1 KO mice and their WT controls. In these experiments, we used only thioglycollate-elicited macrophages. Like in Balb/C mice, Bv8 (10^{-9} M) increased IL-1β and IL-12 but lowered IL-10 concentrations in WT controls. These effects were completely abolished in the PKR-1 KO mice, demonstrating its dependency on this receptor.

Discussion

The macrophage is a fundamental effector cell of innate immune responses, but it also constitutes the interface between innate and adaptive immunity thanks to its ability to produce important regulatory cytokines such as IL-12 and IL-10 (Beutler, 2004; Hoebe *et al*., 2004). The present study found that Bv8 strongly influences all aspects of macrophage physiology, driving a proinflammatory macrophage phenotype.

The recruitment of macrophages and monocytes to sites of inflammation, injury and infection is a crucial step in inflammation and antimicrobial immune responses. The ability to migrate towards chemoattractants is a first, important event in macrophage physiology (Schiffmann, 1982; Beutler, 2004; Mantovani *et al*., 2004). Bv8 promotes substantial macrophage migration at very low concentrations indeed – as low as 10^{-12} M – with an effect similar to that of the classical chemoattractant fMLP.

High levels of Bv8 expression have been detected in infiltrating neutrophils at sites of inflammation (Le Couter *et al*., 2004). Neutrophils are the first cells to be recruited at sites of inflammation and injury, followed by macrophages. It is therefore possible that Bv8's potent chemotactic activity plays an important role in directing macrophage migration. In the same study, Le Couter *et al*. (2004) also showed that Bv8

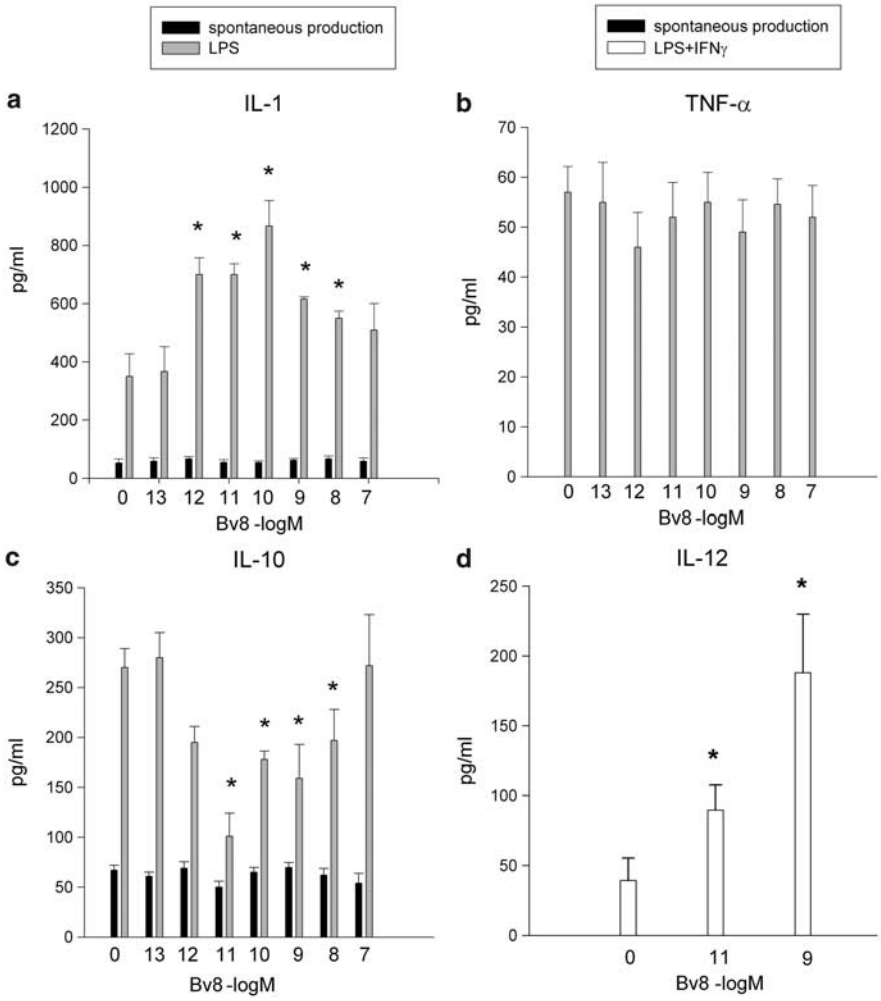

Figure 5 Effect of the addition of Bv8 on IL-1β (a), TNF-α (b), IL-10 (c) and IL-12 (d) production by macrophages. Thioglycollate-elicited macrophages (1×10^6 ml^{-1}), purified by adherence, were used. Bv8 was added alone (effect on spontaneous production) or together with stimuli to induce cytokine production. IL-1β, TNF-α and IL-10 were stimulated with 1 μg ml^{-1} of LPS. The production of IL-12 was stimulated with 1 μg ml^{-1} of LPS + 50 U ml^{-1} INF-γ. After 24 h incubation, culture media were collected and cytokines measured by specific ELISA. TNF and IL-12 levels were undetectable in unstimulated cells. Means $\pm$ s.d. of four experiments. *$P<0.01$ vs 0 Bv8 (i.e. macrophage cultures without Bv8 addition).

Table 3 Effect of Bv8 on stimulated cytokine production by resident peritoneal macrophages

	IL-1 (pg ml^{-1})	*TNF* (pg ml^{-1})	*IL-10* (pg ml^{-1})	*IL-12* (pg ml^{-1})
Stimuli alone	376 $\pm$ 58	65.7 $\pm$ 9.5	552.6 $\pm$ 80	32 $\pm$ 2
Bv8 10^{-11} M	564 $\pm$ 34*	72.9 $\pm$ 6.7	234.7 $\pm$ 42*	65 $\pm$ 11*
Bv8 10^{-9} M	669 $\pm$ 74*	71.3 $\pm$ 5.8	324.4 $\pm$ 25*	83 $\pm$ 15*

Resident peritoneal macrophages were purified by adherence. The production of IL-1, TNF and IL-10 was stimulated with 1 μg ml^{-1} LPS, and IL-12 production was stimulated with 10 μg ml^{-1} of LPS and 100 U ml^{-1} IFN-γ . Bv8 was added together with the stimuli. After 24-h incubation, culture media were collected and cytokines measured by specific ELISA.
Mean $\pm$ s.d. from triplicate determinations.
*$P<0.01$ vs stimuli alone.

can induce migration of human monocytes, indicating that the chemotactic properties are common to myeloid cells of different species.

However, besides attracting macrophages, Bv8 further stimulates them. The addition of the protein boosts the production of the proinflammatory cytokines IL-1β and IL-12 induced by LPS. Like chemotaxis, cytokine production is increased by Bv8 at low doses.

The observation that TNF production was not affected by Bv8 is unexpected, but it is known that in the proinflammatory cytokine cascade TNF is frequently the first to be synthesized and secreted, followed by IL-1β (Cavaillon *et al*., 2003; Cunha *et al*., 2005). It is therefore possible that Bv8 acts on specific mechanisms for IL-1β, downstream of TNF regulation, and not on common pathways (Beutler, 2004). Mirroring the Bv8-induced increase of IL-1β and IL-12, there was also a

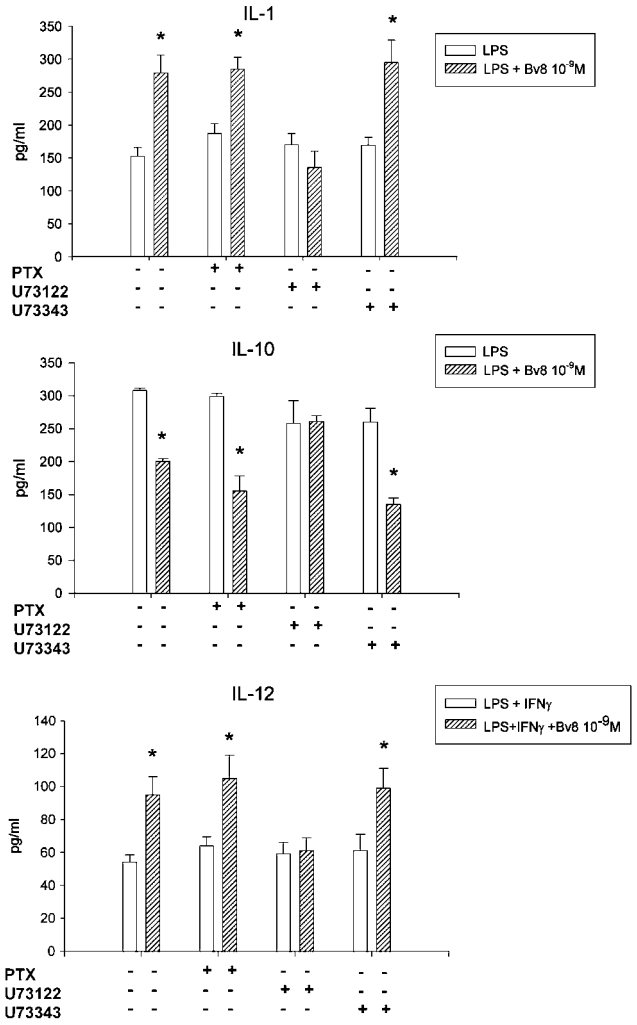

Figure 6 Effect of pretreatment with PTX, the phospholipase inhibitor U73122 and its inactive control U73343 on Bv8's effects on IL-1β (upper panel), IL-10 (middle panel) and IL-12 (lower panel) production by macrophages. Thioglycollate-elicited macrophages ($1 \times 10^6\,\mathrm{ml}^{-1}$) were purified by adherence. Cells were preincubated with $1\,\mu\mathrm{g\,ml}^{-1}$ PTX, 10 μM U73122 or U73343 or medium only for 1 h. At the end of the preincubation, IL-1β, TNF-α and IL-10 were stimulated with $1\,\mu\mathrm{g\,ml}^{-1}$ of LPS. The production of IL-12 was stimulated with $1\,\mu\mathrm{g\,ml}^{-1}$ of LPS + $50\,\mathrm{U\,ml}^{-1}$ INF-γ. Bv8 at the concentration of 10^{-9} M was added together with the stimuli. After 24 h incubation, culture media were collected and cytokines measured by specific ELISA. *$P<0.01$ vs blank columns, representing LPS alone (IL-1β, and IL-10) or LPS + $50\,\mathrm{U\,ml}^{-1}$ INF-γ (IL-12).

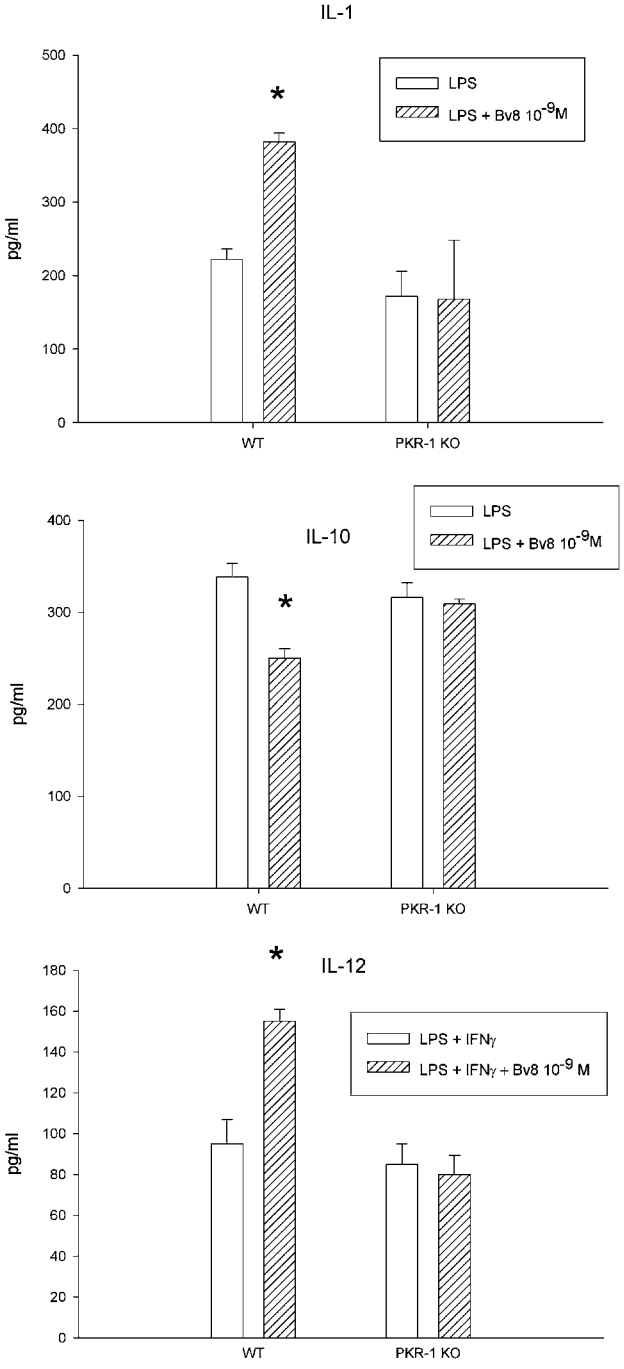

Figure 7 Effect of Bv8 (10^{-9} M) on IL-1β (upper panel), IL-10 (middle panel) and IL-12 (lower panel) production by macrophages from wild type and PKR-1 KO mice. Thioglycollate-elicited macrophages ($1 \times 10^6\,\mathrm{ml}^{-1}$) were purified by adherence and were stimulated with $1\,\mu\mathrm{g\,ml}^{-1}$ of LPS for IL-1β and IL-10, and $1\,\mu\mathrm{g\,ml}^{-1}$ of LPS + $50\,\mathrm{U\,ml}^{-1}$ INF-γ for IL-12. After 24 h incubation, culture media were collected and cytokines measured by specific ELISA. Bv8 influenced stimulated cytokine production by macrophages from WT animals but not from PKR-1 KO mice. Means $\pm$ s.d. of two experiments. *$P<0.01$ vs blank columns, representing LPS alone (IL-1β, and IL-10) or LPS + $50\,\mathrm{U\,ml}^{-1}$ INF-γ (IL-12).

significant decrease in the anti-inflammatory cytokine IL-10. Bv8 therefore strongly skews the pro inflammatory macrophage profile by raising IL-1β and lowering IL-10.

IL-12, produced by antigen-presenting cells, links the innate and cell-mediated components of the immune response by inducing CD4+ T-cell differentiation to Th1-like cells, while IL-10 underlies the development of a Th2 profile (Mosman & Sad, 1996). Hypothetically, therefore, Bv8's effects on the macrophage might have an indirect impact on the Th1/Th2 balance, influencing the development of immune responses. This interesting aspect is being evaluated (Sacerdote *et al*., in preparation).

Bv8 and its mammalian analogues are ligands for two G-protein-coupled receptors, PKR-1 and PKR-2, which show distinct expression patterns in different organs. Although the PCR analysis did showed that macrophages express both

PKR-1 and PKR-2 transcript, PKR-1 is expressed more abundantly. This observation is in agreement with other reports that PKR-2 is the form expressed mostly in the central nervous system (Melchiorri *et al*., 2001; Negri *et al*., 2002), while PKR-1 is largely present on immune tissue (Melchiorri *et al*., 2001; Le Couter *et al*., 2004).

In this study, we used, for the first time, recently generated PKR-1-deficient mice, clearly demonstrating that all the activities exerted by Bv8 on macrophages are completely mediated by the PKR-1 receptor. Bv8-induced chemotaxis and cytokine modulation were in fact completely abolished in these PKR-1 KO animals. We also characterized the early signal mechanisms activated immediately after Bv8 binds to its receptor. Pretreatment with PTX toxin did not affect either Bv8-induced chemotaxis or cytokine modulation. These results seem to rule out the coupling of macrophage PKR-1 receptors to a $G_{\alpha i/0}$ protein. The complete blockade of Bv8 activity on macrophage with the phospholipase inhibitor U73122 suggests there is a G_q-coupled PKR-1 acting through the IP-3/phospholipase-C signaling pathways. The phospholipase-C isoenzymes hydrolyze PI 4,5-bisphosphate (PIP2) to produce DAG and inositol 1,4,5 triphosphate (IP3), a calcium mobilizer, second messenger. Mammalian phospholipase-C comprises four subtypes, β, δ, γ and ε. Classically, phospholipase C-β is considered to be regulated by G protein-coupled receptors through a $G\alpha_q$ protein (Rhee, 2001).

It also appears that different events in macrophage activation, such as chemotaxis and cytokine production, are governed by the same intracellular signaling, quite likely activated by Bv8 binding to PKR-1. The involvement of a G protein α subunit of the q type has been reported for other Bv8 effects too, such as the increase of $[Ca^{2+}]$ concentrations and the p42/p44 MAPK phosphorylation in CHO cells expressing PKRs and in the dorsal root ganglia cells, whereas PTX did not affect these phenomena (Negri *et al*., 2002). In contrast to our results, Le Couter *et al*. (2004) found that PTX blocked Bv8-induced migration of human monocytes. However, some explanations can be offered. First of all, the type of receptor involved in Bv8-induced human monocyte migration was not determined, and PKR-1 or PKR-2 could have different coupling systems. In addition, although monocytes and macrophages derive from a common myeloid lineage, tissue macrophages have different characteristics from monocytes.

It is also increasingly emerging that different macrophage subtypes might have different 'inflammatory' functions (Cook *et al*., 2003). In our study, however, we found similar effects on both chemotaxis and cytokine production when Bv8 was administered to thioglycollate-elicited inflammatory macrophages, or to resident peritoneal macrophages from naive animals, the sentinel cells that respond to injury and orchestrate the innate immune responses (Cailhier *et al*., 2005). Bv8's ability to induce an inflammatory macrophage phenotype seems therefore general in macrophages at different stages and states of maturation or activation. In a previous study, we found a similar response as regards the effect on cytokines, using either resident or elicited macrophages (Limiroli *et al*., 2002).

Besides PK-receptors, macrophages also express high levels of PK-2 mRNA, which was always present as a tissue-specific double-splice variant: the short form encodes for a protein similar to amphibian Bv8 while the long form encodes for an additional 21 aa domain, inserted in the center of the polypeptide chain, already described only in mouse and human testis (Wechselberger *et al*., 1999). The insert was sequenced and was rich in basic residues, arginine and lysine, containing several potential cleavage sites for prohormone convertases. Nothing is known about the function of the longer PK-2 variant. Bullock *et al*. (2004) produced a recombinant protein of this splice variant that is about 200 times less potent than PK-2, in an aequorin-based assay for $[Ca^{2+}]$ mobilization in PKR-transfected CHO cells.

We have found a similar PK-2 long variant in rat macrophages and granulocytes, in human circulating granulocytes and in the promyelocytic cell line HL-60 (Negri *et al*., in preparation). Interestingly, coexpression of the ligand and receptors of this family of proteins has been observed in most immune cell types (Le Couter *et al*., 2004).

Recently, evidence has emerged that cytokines link the immune and the nervous systems and may be involved in the generation of pain and hyperalgesia. IL-1β has been associated with hyperalgesic states, acting directly on sensory neurons to increase their susceptibility to noxious stimuli (Safie Garabedian *et al*., 1995; Sommer & Kress, 2004). In contrast, IL-10 lowers nociceptive thresholds in several pain models (Poole *et al*., 1995). In view of Bv8's potent hyperalgesic activity (Negri *et al*., 2002), it is conceivable that this might be related to its effects on cytokines.

In conclusion, both resident and inflammatory macrophages seem to be targets for the Bv8/PK protein family. As these cells have functional PKR-1 receptors and produce Bv8, this molecule might possibly have some paracrine/autocrine role in regulating macrophage function.

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