Prokineticin 1 induces CCL4, CXCL1 and CXCL8 in human monocytes but not in macrophages and dendritic cells

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ABSTRACT. Prokineticin 1 and 2 (PROK1 and PROK2) are two small proteins largely expressed in inflammatory tissues and involved in monocyte activation and differentiation. The focus of this study was to evaluate whether PROK1 was able to induce chemokine secretion in human monocytes, in monocyte-derived macrophages and in monocyte-derived dendritic cells, an aspect not addressed thus far. Here, we show for the first time, using flow cytometry, that PROK receptors 1 and 2 are present on the surface of human monocytes. Subsequently, monocytes were selected to investigate the chemokine response after stimulation by PROK1. Our results show that only three chemokines (CCL4, CXCL1 and CXCL8) were significantly induced at both the transcript and protein level, and that PROK1 induces most potently CXCL8, in a dose-dependent manner. From a mechanistic point of view, by blocking independently Gαi protein or intracellular calcium, monocytes lose the ability to secrete CXCL8 in response to PROK1. Finally, we observed that CCL4, CXCL1 and CXCL8 secretion, following PROK1 induction, is only observed in monocytes and not in monocyte-derived macrophages and dendritic cells. Our results demonstrate that, *in vitro*, the differentiation status of monocytes influences chemokine production after stimulation by PROK1, and that this chemokine production is geared toward a pro-inflammatory response. This could represent a novel amplification loop of leukocyte recruitment, extravasation and tissue invasion.

Keywords: prokineticin, CXCL8, chemokine, monocytes

Endocrine gland-VEGF (EG-VEGF) and Bv8, also referred as prokineticin 1 (PROK1) and prokineticin 2 (PROK2) respectively, are two, small, secreted peptides of 8kDa that belong to a larger class of peptides called the AVIT protein family [1]. PROK1 and PROK2 have been reported to be involved in a variety of biological mechanisms such as endothelial cell proliferation [2], neuron survival [3], gastrointestinal motility [4], circadian rhythm [5], pain sensation [6, 7], hematopoiesis [8] and macrophage differentiation [9]. Interestingly, both prokineticins bind non-selectively and with similar affinity to their two, known, G protein-coupled receptors, prokineticin receptor 1 and 2 (PROKR1 and PROKR2). Binding of prokineticin ligand to its receptor induced mobilization of calcium, activation of mitogen-activated protein kinase (MAPK) signaling pathways, phosphoinositol turn-over, and Akt kinase activation [3, 10-13].

Prokineticins are expressed in inflamed tissues and are involved in many aspects of the immune response. It seems that their cellular source within the immune system is specific to cells from the monocyte-granulocyte lineage [8, 9, 14, 15]. This was observed in a screening of a panel of leukocytes where innate immune cells such as monocytes and neutrophils expressed high transcript levels of both PROK2 and PROKR2, suggesting autocrine and paracrine regulation [8]. Furthermore, infiltrating neutrophils in tonsillitis and appendicitis samples expressed high levels of PROK2 [8], macrophages of bovine corpus luteum regression and follicular atresia were positive for both PROK1 and PROKR1 protein [14]. We recently showed that in human liver PROK2 and its receptors were expressed specifically by Kuppfer cells, the liver's resident macrophages [16].

Based on the literature, it seems that the prokineticin activities within immunity are highly conserved across evolution, and are specific to the innate immune and hematopoietic systems [17]. In invertebrates, a prokineticin-like cytokine (astakine), induced a strong hematopoietic response both *in vitro* and *in vivo* in freshwater crayfish [18], and in vertebrates, mice systemically exposed to either PROK1 or PROK2 showed an increase in the number of circulating neutrophils and monocytes [8]. Furthermore, *in vitro* assays showed that PROK2 induced chemoattraction and migration of human monocytes [8], and PROK1 promoted human monocyte differentiation into macrophage-like cells [9]. Interestingly, no

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report has shown a direct production of cytokines in response to prokineticins in monocyte/macrophage cells. However, when macrophages are primed by PROK1, LPS is able to induce production of pro-inflammatory cytokines geared towards a TH1 response, such as tumor necrosis factor α (TNF-α), interleukin 12 (IL-12), and by suppressing interleukin 10 (IL-10) [9]. Furthermore, in murine macrophages, PROK2 stimulates lipopolysaccharide-induced production of the pro-inflammatory cytokines, interleukin-1 (IL-1) and IL-12, and reduced the production of IL-10 [19].

Thus, taken altogether, these data support the idea that PROK2 and PROK1 are involved in leukocyte production, monocyte recruitment towards normal or inflammatory tissue, monocyte differentiation, and release of pro-inflammatory cytokines. Here we investigate for the first time, whether prokineticins are able to induce the production of chemokines in cells from the monocyte/macrophage lineage, and if there is a differential response according to the differentiation state of the monocyte. The trafficking of leukocytes in development, homeostasis and inflammation is considered to be dependent upon the chemokine protein family. The chemokine protein superfamily, which includes more than 40 members, is organized into four different sub-families on the basis of the relative position of the cysteine residues in the mature protein (CC, CXC, CXXXC and XC). They exert their biological function through interaction with 7-transmembrane G-protein-coupled receptors that are differentially expressed on sub-groups of leukocytes [20]. In this study, we identified the chemokines responsive to PROK1 by measuring all presently known chemokines (n = 44) using real time PCR. Subsequently, for selected chemokines, protein production was validated by ELISA. Finally, intracellular pathways involved in CXCL8 protein production by PROK1 were studied using inhibitors of Gαi, and calcium signaling.

METHODS AND MATERIALS

Reagents

Endotoxin-free recombinant human endocrine gland-vascular endothelial growth factor/prokineticin 1 (PROK1) produced in the mouse myeloma cell line NS0, was obtained from R&D systems (Minneapolis, MN, USA). Polyclonal rabbit antibodies against PROKR1 and PROKR2 were generously provided by Dr. Feige, INSERM U878, Grenoble [21]. Granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) were obtained from Cellgenix (Cellgenix, Antioch, IL, USA). Lipopolysaccharide (LPS) from *Salmonella typhimurium,* intracellular calcium chelator 1, 2-bis (2-aminophenoxy) ethane-*N*, *N*, *N*', *N*'-tetraacetic acid (BAPTA), and Pertussis toxin (PTX, a Gαi inhibitor) were all obtained from Sigma (Sigma Aldrich, Saint-Quentin Fallavier, France).

Cell isolation and culture

Leukocytes were harvested as a secondary product from informed donors undergoing platelet apheresis on a COBE Spectra Leucoreduction System LRS (Gambro BCT). At the end of the collection, the LRS chamber was disconnected from the system and mononuclear cells were isolated by density gradient centrifugation. Monocytes were then purified by elutriation in PBS (Lonza, France), supplemented with 0.8% human serum albumin (Vialebex, France), using a Beckman J6-MC centrifuge in a 40 mL elutriation chamber, at a constant speed of 2 500 rpm at 4°C. The initial buffer flow was set to 40 mL/min, and mononuclear cells were loaded into the elutriation chamber. The flow of elutriation medium was then increased step by step and cells were washed out of the chamber depending on cell size and cell density (65, 70, 77, 85, 90 and 95 mL/min – each step for 4 min). To collect the monocyte-enriched fraction from the elutriation chamber ("rotor off fraction"), the centrifuge was stopped and the cells were pushed out from the chamber. Monocyte purity after elutriation was monitored by flow cytometry using CD14 monoclonal antibody (BD biosciences, Franklin Lakes, NJ, USA), and cells were, on average, 95% CD14 positive. Monocytes were cultured the same day they underwent platelet apheresis at 3 x 10^6 cells/mL, at a density of 300 000 cells/cm^2 in RPMI 1640 GlutaMAX™-I (Gibco, Paisley, Scotland), supplemented with 10% complement-inactivated fetal calf serum (FCS) and streptomycin antibiotic. Monocyte-derived macrophages were differentiated for six days by adding GM-CSF at 400 UI/mL. Monocyte-derived dendritic cells were produced by incubating monocytes with 200 UI/mL IL-4 and 1 000 UI/mL GM-CSF for six days. Cells were incubated at 37°C and 5% CO_2.

Flow cytometry

Prokineticin receptor 1 and 2 expression on the surface of freshly isolated monocytes, macrophages and dendritic cells was evaluated by flow cytometry using rabbit polyclonal antibodies against PROKR1 and PROKR2, and rabbit pre-immune sera were used as a negative control. Cell labeling for flow cytometry was performed in 2% PBS/BSA at 4°C using 1 million cells. Cells were incubated for 45 min at 4°C with primary antibodies at an IgG concentration of 100 ng/mL for anti-PROKR1 and its corresponding pre-immune serum, and at 200 ng/mL for PROKR2 and its appropriate control serum. Following washing, cells were incubated with phycoerythrin-conjugated goat anti rabbit IgG (5 µg/mL, Jackson Immunotech, Suffolk, England) for 45 min at 4°C in 2% PBS/BSA. After a final washing step, cells were analyzed by flow cytometry using FACScalibur (Becton Dickinson, Grenoble, France). Alternatively, the blocking Fc receptor step was achieved by a pre-incubation with human IgG at 50 µg/mL.

Quantification of chemokine gene expression by real-time quantitative PCR

After 8 h of cell stimulation of monocytes by PROK1 (1 µg/mL or 125 nM) and/or LPS (1 000 ng/mL), total RNA was extracted using the SV Total RNA isolation Kit® (Promega, Charbonnières-les-Bains, France), and 1µg of total extracted RNA was subjected to a reverse

transcription reaction using a high capacity cDNA archive Kit® (Applied Biosystem, Foster City, CA, USA). Primers having the same melting temperature (Tm) were designed on two different exons using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer38www.cgi).
PCR amplification reactions were carried out in duplicate with iQ-SYBR Green Supermix (Bio-Rad) in a 15 µL reaction volume containing 200 nM primer and 5 ng cDNA, and using a Chromo4™ System (Bio-Rad). Thermal cycling was initiated with 3 min incubation at 95°C followed by 40 cycles of 95°C for 10 s, 55 °C for 15 s and 72°C for 15 s. The ΔCt method was retained for quantification and GAPDH, HPRT1 and HSPCB housekeeping genes used for multiple normalization as described previously [22].

Chemokine protein measurement by ELISA

After 24 h stimulation of monocytes, macrophages and dendritic cells with variable concentrations, of PROK1 (0.5 µg/mL and 1 µg/mL) and/or LPS (0.05, 0.5, 5, 50 and 1 000 ng/mL) depending on the experiment, supernatants were collected. Treatment of monocytes with selective inhibitors (PTX, or BAPTA) required a 2hr pre-incubation period to allow target saturation. Following a washing step, fresh medium with inhibitors was added to the cells in the presence or absence of PROK1 (1 µg/mL or 125 nM). After a 24 h incubation, the supernatant was collected free of cells for measurement of secreted chemokines. Chemokine concentrations were measured using a sandwich ELISA for human CCL3/MIP-1α, CCL4/MIP-1β, CCL18/PARC, CCL20/MIP-3α, CXCL8/IL-8 (R&D systems) and for human CXCL1/GROα (PeproTech, Rocky Hill, NJ, USA).

STATISTICAL ANALYSES

Significance between groups was calculated using a non-parametric test for matched samples, Friedman's ANOVA, to exclude that difference between groups was due to random sampling, and subsequently exact group to group difference was calculated with Wilcoxon's non-parametric test for paired samples. A level of $p < 0.5$ was considered significant. Calculations were made with the commercially available software Statistica (StatSoft, Maisons-Alfort, France).

RESULTS

PROK receptor expression on surface of human monocytes, macrophages and dendritic cells

Before studying the chemokine secretion in response to PROK1, we evaluated, using flow cytometry, the presence of PROKR1 and PROKR2 receptors on the surface of human monocytes, *in vitro*-differentiated macrophages and dendritic cells. Monocytes, obtained from human donors undergoing platelet apheresis, were highly purified by elutriation, reaching 95% purity as assessed by CD14 labeling. Macrophages and dendritic cells were differentiated from such monocytes by growing them for six days in the presence of GM-CSF and respectively in the presence or absence of IL-4. Using rabbit polyclonal anti-PROKR1 and anti-PROKR2, our results revealed that PROKR1 and PROKR2 are present on the surface of fresh human monocytes, since means for peak fluorescence of monocytes were 1.90 ± 0.20 ($n = 4$) and 2.85 ± 0.95 ($n = 4$) higher with anti-PROKR1 and anti-PROKR2 respectively, than the IgG control *(figure 1)*. When monocytes were differentiated into macrophages and dendritic cells, no difference between peak fluorescence means was observed (data not shown). Fc receptor interaction with anti-PROKR has been ruled out by appropriated pre-incubation step (data not shown). Thus, monocytes were selected as the model to study the chemokine response after PROK1 induction.

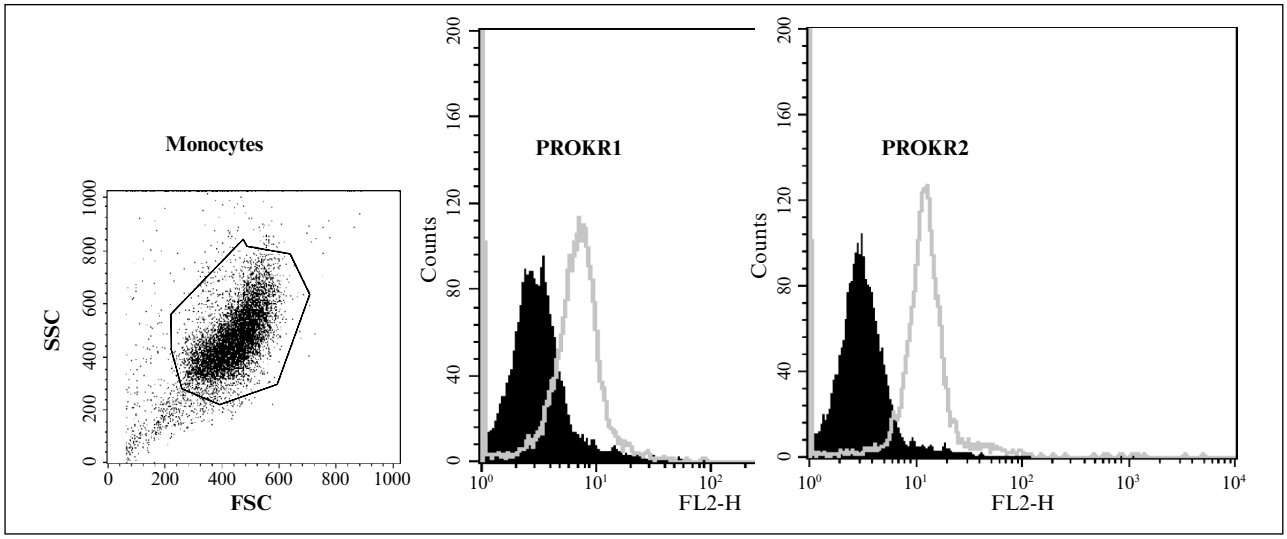

Figure 1
Flow cytometry analysis of PROKR1 and PROKR2 expression in human monocytes. Scatter plot of monocyte population. Histogram representing monocyte-gated population labeled with anti-PROKR1 (no fill) or pre-immune serum (black fill), or labeled with anti-PROKR2 (no fill) or pre-immune serum (black fill).

Global chemokine expression profile induced by PROK1 in human monocytes

Based on the facts that PROK1 and PROK2 activate, with a similar range of affinity, both receptors [11, 13] and that only human recombinant PROK1 from a non-bacterial expression system was commercially available when this study was begun, we used PROK1 as an activator of prokineticin receptors. The chemokine profile obtained in human monocytes after PROK1 induction was analyzed using real time PCR. Primers were designed for amplification of all known human chemokines (n = 44). *Figure 2A* shows chemokine induction levels by PROK1 (1 μg/mL) after 8 h in purified monocytes from three healthy donors. By way of comparison, monocytes were also stimulated with LPS (1 μg/mL), a pro-inflammatory activation signal for mononuclear phagocytes, and by combining LPS (1 μg/mL) with PROK1 (1 μg/mL) for 8 h, respectively shown in *figure 2B and C*. Although donor to donor variation was observed, monocyte exposure to PROK1 resulted in the regulation of a restricted subset of chemokines. Only chemokines with induction levels greater than four in at least two of the three donors were considered significant, hence out of the 44 measured, only 20 chemokines were induced at significant levels, (CCL1-3, CCL3L1, CCL4, CCL4L1, CCL7-8, CCL15, CCL18-20, CCL23, CXCL1-3, CXCL5-6, CXCL8-9, and CXCL12, CXCL14), 13 were not significantly induced by a PROK1 stimulus (CCL5, CCL22, CCL24, CCL26, MEC, CXCL4, CXCL7, CXCL10-11, CXCL13, CXCL16, and XCL1-2) and nine were undetectable (CCL11, CCL13, CCL16, CCL17, CCL21, CCL25, CCL27, PPBPL1, and CX3CL1). No chemokines were observed to be significantly down-regulated by PROK1. Concerning induction by LPS alone, 27 chemokines (CCL1-3, CCL3L1, CCL4, CCL4L1, CCL5, CCL7-8, CCL15, CCL18-20, CCL23, CXCL1-3, and CXCL5-14) were induced, eight chemo-

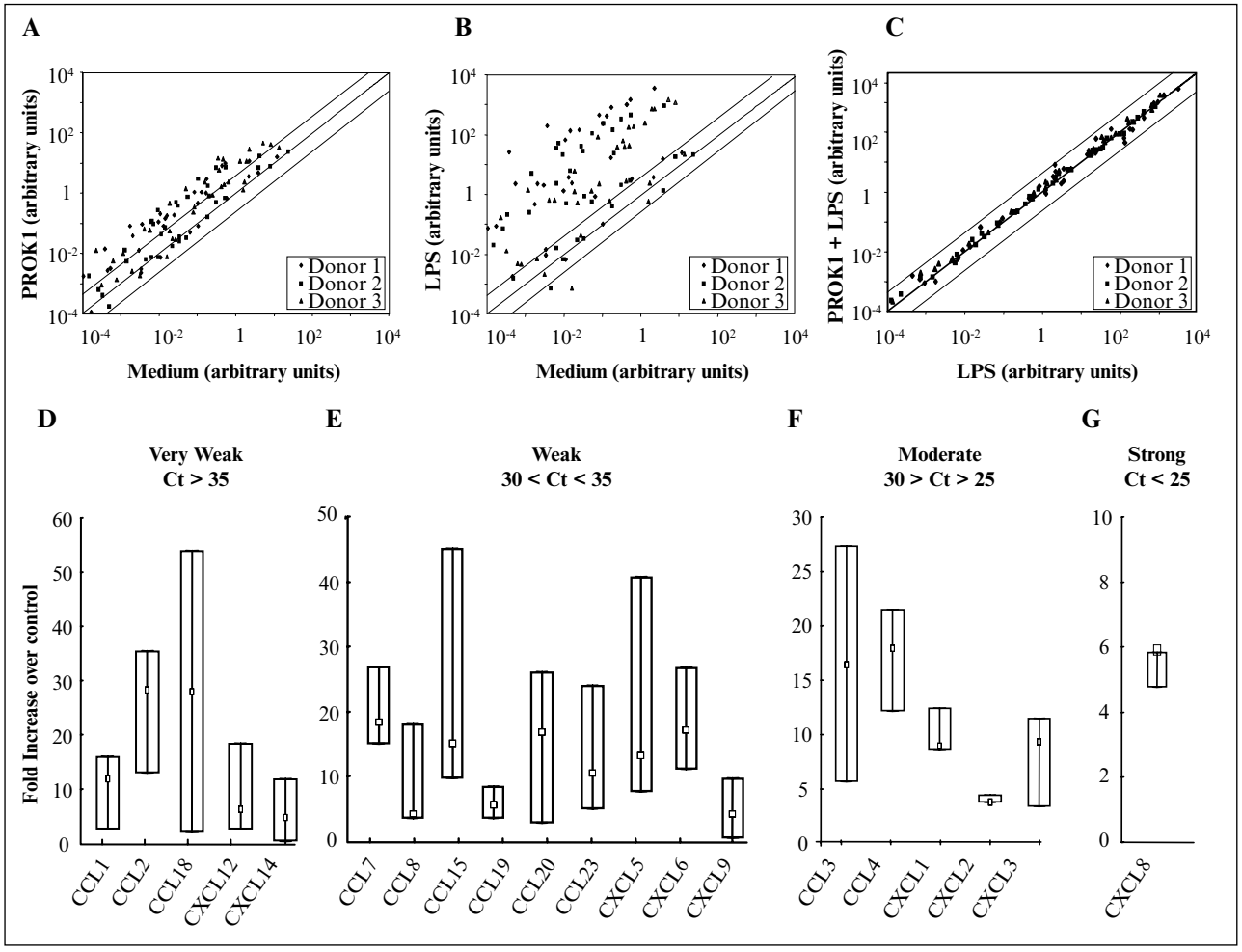

Figure 2

Expression profile of chemokines induced by PROK1 alone, LPS alone and LPS plus PROK1. Logarithmic plot representing chemokine mRNA induction by monocytes from three different donors stimulated for 8 h with (**A**) PROK1 (1 μg/mL) *versus* medium, (**B**) LPS (1 μg/mL) *versus* medium, and (**C**) PROK1+LPS (1 μg/mL each) *versus* LPS (1 μg/mL). Induction of mRNA of all 44 chemokines was measured by real time PCR. Each point was performed in duplicate. Chemokine induction levels were calculated using the ΔCt method for quantification and GAPDH, HPRT1 and HSPCB housekeeping genes were used for multiple normalization. Full lines represent the 4 and 0.25 boundary values of activated/control ratio. The bottom panel shows the box plot representing mRNA induction measured by real time PCR of chemokines with an induction factor greater than 4 in at least two out of the three donors after PROK1 stimulation; these chemokines were further selected through a classification based on their basal cycle threshold (Ct) expression level; (**D**) very weak Ct > 35, (**E**) weak Ct [30-35], (**F**) moderate Ct [30-25], and (**G**) strong Ct < 25. The *y*-axis values represent the induction of each gene relative to control after multiple normalization using GAPDH, HPRT1 and HSPCB housekeeping genes.

kines did not respond significantly to LPS (CCL22, CCL24, CCL26, MEC, XCL1-2, CXCL4, CXCL16), and nine were undetectable (CCL11, CCL13, CCL16, CCL17, CCL21, CCL25, CCL27, PPBPL1, CX3CL1). The LPS-induced chemokine expression profile obtained was in agreement with previous, similar studies [23, 24]. No chemokines were observed to be significantly down-regulated by LPS. When comparing the expression profile induced by LPS alone, the combination of PROK1 with LPS did not stimulate differentially any chemokines *(figure 2C)*.

Chemokine transcript induction and protein secretion in PROK1-induced human monocytes

In order to select among the 20 chemokines significantly induced by PROK1 and susceptible to being detected at the protein level, we classified chemokines into four groups according to their basal level, very weak $Ct > 35$, weak Ct [30-35], moderate Ct [30-25], and strong $Ct < 25$ *(figure 2D-G)*. Thus, chemokines which displayed moderate (CCL3, CCL4 and CXCL1), or high (CXCL8) basal expression and that were most strongly induced, were extensively studied *(figure 2F, G)*. Moreover, to support and validate these chemokine expression profiles, we enrolled more donors ($n \geq 6$), and analyzed both transcript and protein expression. For validation of our classification system, we also selected chemokines that exhibited the highest induction level among the groups that showed very weak (CCL18) or weak (CCL20) basal expression *(figure 2D, E)*.

As illustrated in *figure 3*, induction of both mRNA and protein were compared in monocytes incubated with PROK1 *versus* medium, and with LPS + PROK1 *versus* LPS alone. All treatments were relative to medium alone; however the graphics were separated into PROK1 *versus* medium and PROK1+LPS *versus* LPS because of the overwhelming induction-fold of chemokines by LPS, which would have overshadowed induction by PROK1 alone. Our results show that transcript induction by PROK1 was significant for CCL20, CCL3, CCL4, CXCL1 and CXCL8 *(figure 3A)*, while protein secretion in supernatant was significant for only CCL4, CXCL1 and CXCL8 *(figure 3C)*. These results confirm our classification system where only chemokines with moderate to high basal expression were detectable by ELISA. It is worth noting that because CXCL1, CXCL2 and CXCL3 share such high sequence homology, the ELISA kit does not distinguish between the three proteins. Since our PCR data show that CXCL1 is most strongly induced by PROK1, we speculate that CXCL1 is the most abundant immunoreactive chemokine in the supernatant. Nevertheless, we cannot exclude the possible participation of CXCL2 and CXCL3.

Monocytes stimulated by LPS alone or LPS + PROK1 showed no significant difference in chemokine mRNA induction, however, the overall induction-fold was far superior to PROK1 alone *(figure 3B)*. Incubation of monocytes with LPS resulted in a very strong induction of protein secretion for CCL3, CCL4, CXCL1 and CXCL8 *(figure 3D)*. In contrast, although CCL18 and CCL20 protein was undetectable in both the supernatant of monocytes treated with medium and PROK1, addition of PROK1 to LPS consistently resulted in synergistic production of CCL18 and CCL20 compared to those induced by LPS alone *(figure 3D)*.

Dose-dependent analysis of the synergistic effect of PROK1 with LPS on monocyte stimulation

LPS is such a potent inducer of chemokine secretion that it might have overshadowed the input of PROK1 during co-stimulation between LPS and PROK1, on secretion of CCL3, CCL4, CXCL1 and CXCL8. Therefore, protein secretion was measured after gradually decreasing the concentration of LPS from 1 000 ng/mL to 0.05 ng/mL, while keeping the concentration of PROK1 constant at 1 µg/mL. Our results showed that all chemokines measured (CCL18, CCL20, CCL3, CCL4, CXCL1 and CXCL8) are LPS-dose dependent *(figure 4A-F)*. However, addition of PROK1 with LPS, significantly increased CCL18 and CCL20 secretion *(figure 4A, B)*, but did not affect production of CCL3, CCL4, CXCL1 and CXCL8 *(figure 4C-F)*. This synergistic effect of PROK1 on CCL18 and CCL20 production is observed for every concentration of LPS.

Pathways involved in CXCL8 production by PROK1

Out of all the chemokines measured in monocyte supernatant, CXCL8 was the chemokine most strongly induced by PROK1, and in a clear dose-dependent manner *(figure 5A)*. We further investigated the functionality of the receptor, by blocking independently different pathways involved in prokineticin receptor signaling events. Firstly, PTX specifically blocks G protein Gαi downstream signaling of G protein couple receptors, which include PROKR1 and PROKR2 [8, 11]. Here, monocytes pretreated with PTX, lose completely, in a dose-dependent manner, the ability to induce CXCL8 protein secretion when stimulated by PROK1 *(figure 5B1*, *B2*). Secondly, intra-cellular calcium, which has also been shown to play an important role in prokineticin signaling [11-13, 25], can be buffered using a calcium chelator, BAPTA. *Figure 5C1* and *C2* show that PROK1-mediated CXCL8 protein production is blocked in a dose-dependent manner by BAPTA.

Chemokines CXCL1 and CXCL8 are specifically produced in monocytes after PROK1 induction, but not in macrophages or dendritic cells

Finally, based on our flow cytometry results which showed a decrease of receptor expression on the surface of macrophages and dendritic cells, we hypothesized that this decrease might have an impact on the ability of PROK1 to induce chemokine production in these cells. Monocytes, as well as macrophages and dendritic cells differentiated from the same monocytes, were stimulated by PROK1 for 24 hours. CXCL1 and CXCL8, the chemokines most strongly induced by PROK1, were measured by ELISA in the conditioned media collected. Our results show that induction of CXCL1 and CXCL8 by PROK1 is significant only in human monocytes, and no differential induction is observed in macrophages or dendritic cells *(figure 6A, B)*.

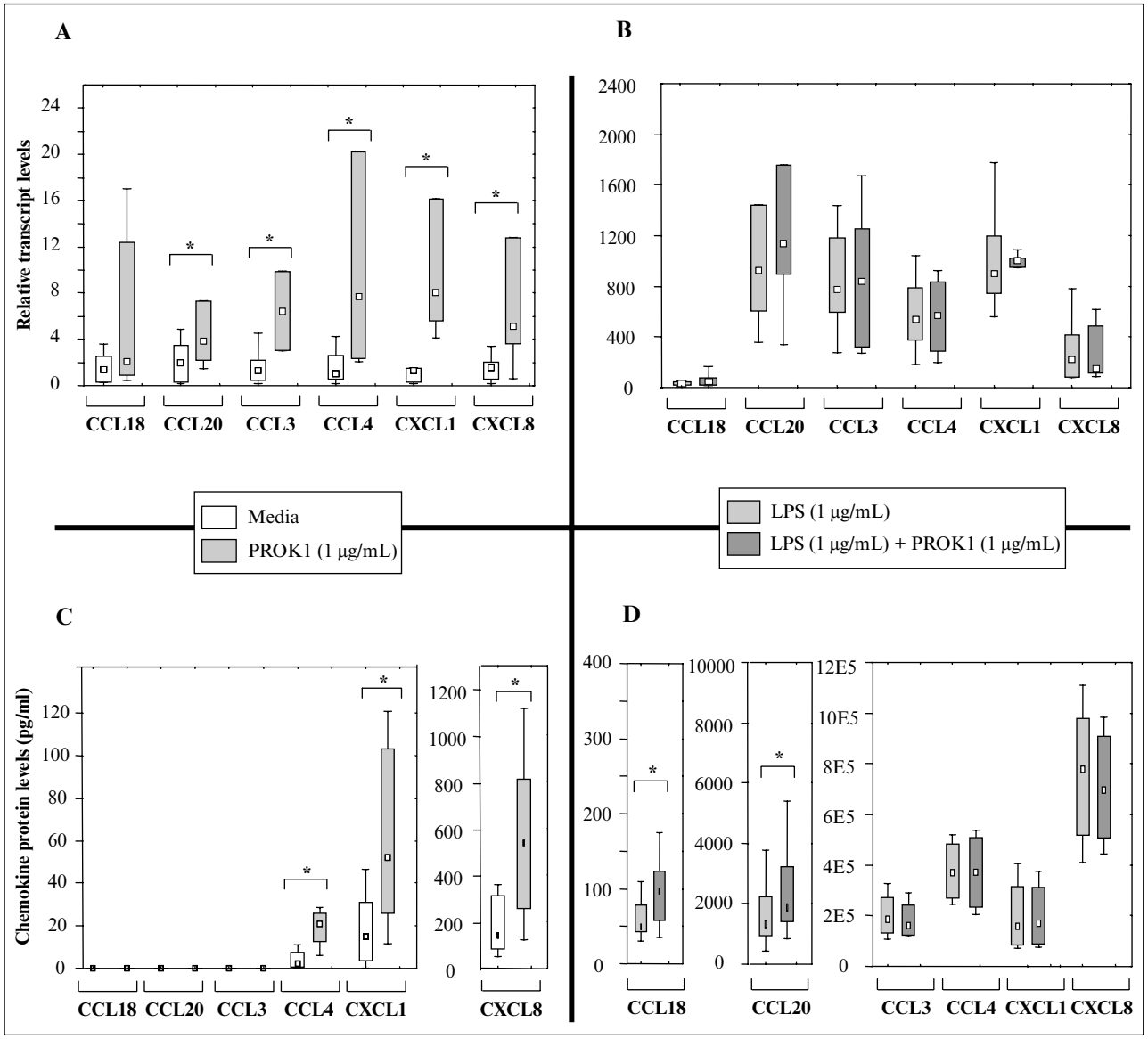

Figure 3

Transcript *versus* protein induction of chemokines. Box plot representing mRNA induction measured by real time PCR of chemokines; CCL18, CCL20, CCL3, CCL4, CXCL1, CXCL8, after 8h of stimulation with (A) medium alone *versus* PROK1 (1 µg/mL),and (B) LPS (1 µg/mL) *versus* LPS plus PROK1 (1 µg/mL each). The *y*-axis values represent the induction of each gene relative to control after multiple normalization using GAPDH, HPRT1 and HSPCB housekeeping genes. Experiments were performed using monocytes from six different donors and each point was performed in duplicate. Box plot represents protein secretion measured by ELISA of chemokines; CCL18, CCL20, CCL3, CCL4, CXCL1, CXCL8 after 24 h stimulation with (C) medium alone *versus* PROK1 (1 µg/mL), (D) LPS (1 µg/mL) *versus* LPS plus PROK1 (1 µg/mL each). The *y*-axis values represent the concentration of each protein expressed in pg/ml. Experiments was performed using monocytes from seven different donors and each point was performed in duplicate. The bars of the box plot correspond to the atypical variations, the boxes to the values between 25% and 75%, and the small square to the median. Significant difference of expression was noted: $p < 0.05$ = *.

DISCUSSION

Before studying chemokine induction by PROK1 in cells from the monocyte/macrophage lineage, we ensured that prokineticin receptors were present on the surface of human monocytes, monocyte-derived macrophages and monocyte-derived dendritic cells; even though transcript expression had already been reported in all these cells [8, 19]. Using flow cytometry analysis, we confirmed for the first time the presence of both receptors on the surface of monocytes, however, we observed a strong decrease in receptor expression in macrophages and dendritic cells, suggesting monocytes were the cells most inducible by the PROK1 ligand. The loss of PROKR1 and PROKR2 receptors on the surface of macrophages and dendritic cells may be caused by either the cytokines added to the monocytes (GM-CSF or IL-4) or to the differentiation state of the cells.

Subsequently, we evaluated the chemokine response in monocytes after exposure to PROK1 for 8h, by measuring all 44, known chemokines using real time PCR, and then validating protein induction using ELISA. Our data show that induction at both the mRNA and protein level is only observed for chemokines, CCL4, CXCL1 and CXCL8, and in addition, CXCL8 is the chemokine most potently induced responding to PROK1 dose-

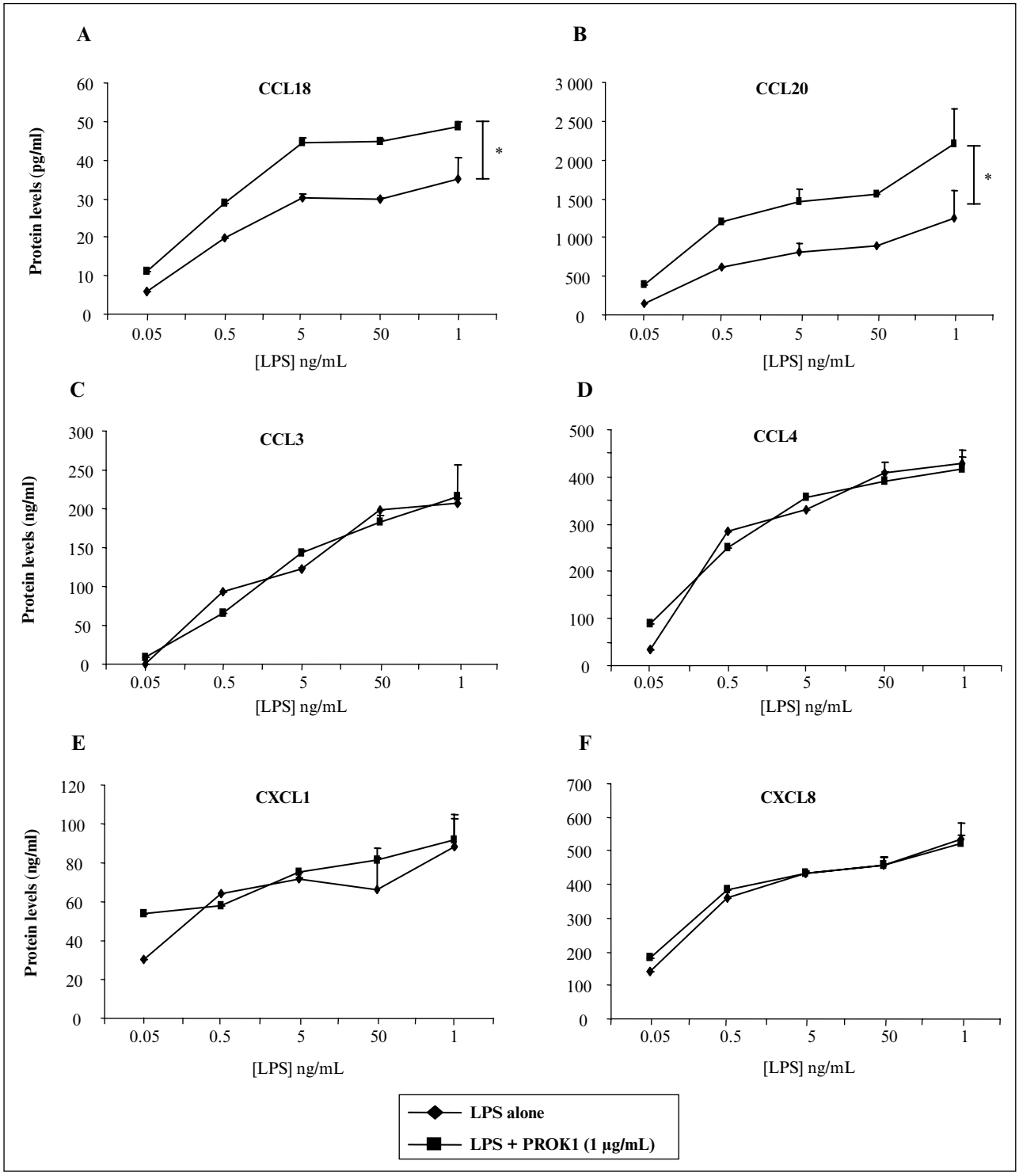

Figure 4

Synergistic effect of PROK1 on LPS dose-dependent induction of chemokine in monocytes. CCL18 (**A**), CCL20 (**B**), CCL3 (**C**), CCL4 (**D**), CXCL1 (**E**) and CXCL8 (**F**) protein concentrations were measured by ELISA in culture supernatant of monocytes stimulated for 24 h with decreasing concentrations of LPS; 1 000 ng/mL, 50 ng/mL, 5 ng/mL, 0.5 ng/mL, 0.05 ng/mL, in the presence (squares) or absence (diamonds) of PROK1 (1 µg/mL). The *y*-axis values represent the concentration of each protein in the supernatant, expressed in pg/ml for CCL18 and CCL20 and in ng/mL for CCL3, CCL4, CXCL1 and CXCL8. Results shown are representative of three independent experiments and each point was performed in duplicate. Significant difference of expression was noted: $p < 0.05 = *$.

dependently. The fact that we observed, for the first time, a direct expression of cytokines in response to PROK1 alone, it allowed us to investigate the pathways involved in PROK1 receptor activation. This was studied by using selective inhibitors for different intracellular signals involved in prokineticin receptor activation. Receptors for prokineticins are G-coupled 7-domain transmembrane receptors and according to the literature, PROK receptors couple to Gi, Gq and even Go, thus activating distinct pathways [26]. Our results support the fact that Gi and Gq pathways are implicated in PROK1 signalling, since PTX, and BAPTA (a calcium chelator), which inhibit

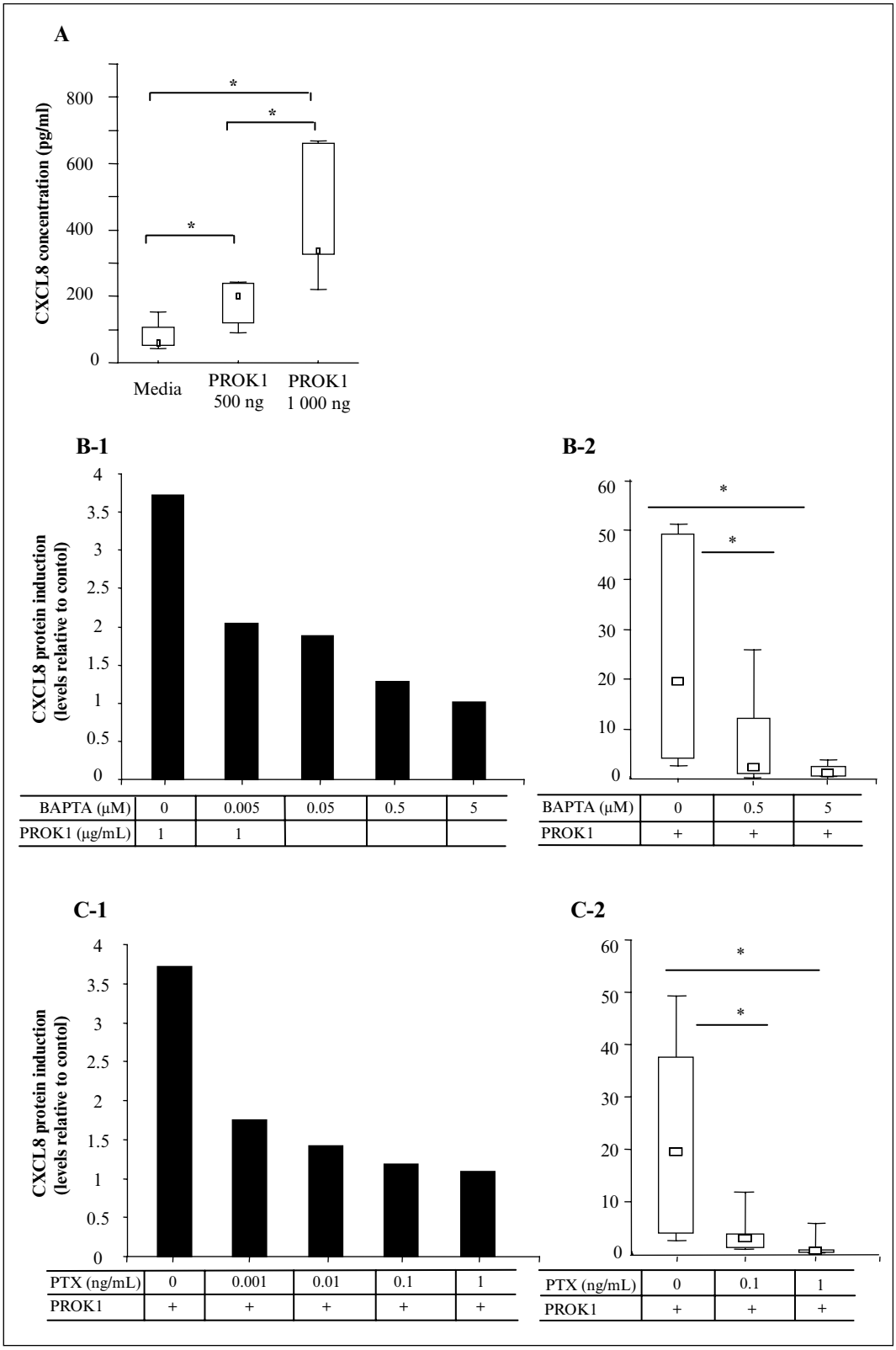

Figure 5

PROK1 dose-dependent induction of CXCL8 in human monocytes and its inhibition by BAPTA or PTX. (**A**) Box plot represents CXCL8 protein concentration (pg/mL) measured by ELISA in monocyte culture supernatants after 24 h stimulation with medium alone, or with 0.5 μg/mL PROK1 and 1 μg/mL PROK1. Experiments were performed using monocytes from five different donors, and each point was performed in duplicate. The bars of the box plot correspond to the atypical variations, the boxes to the values between 25% and 75%, and the small square to the median. Significant difference of expression was noted: $p < 0.05$ = *. CXCL8 protein production was measured by ELISA in supernatants of monocytes pre-incubated for two hours with either (**B**) intra-cellular calcium chelator (BAPTA) or (**C**) Gαi protein inhibitor, PTX, followed by stimulation for 24h with PROK1 (1 μg/mL) in the presence of each inhibitor. The *y*-axis values represent the induction of CXCL8 protein over the basal control level. (**B1**) and (**C1**) are typical dose response experiments. (**B2**) and (**C2**) present mean results for two doses of inhibitors from six independent experiments, each point was performed in duplicate. Significant difference of expression was noted: $p < 0.05$ = *.

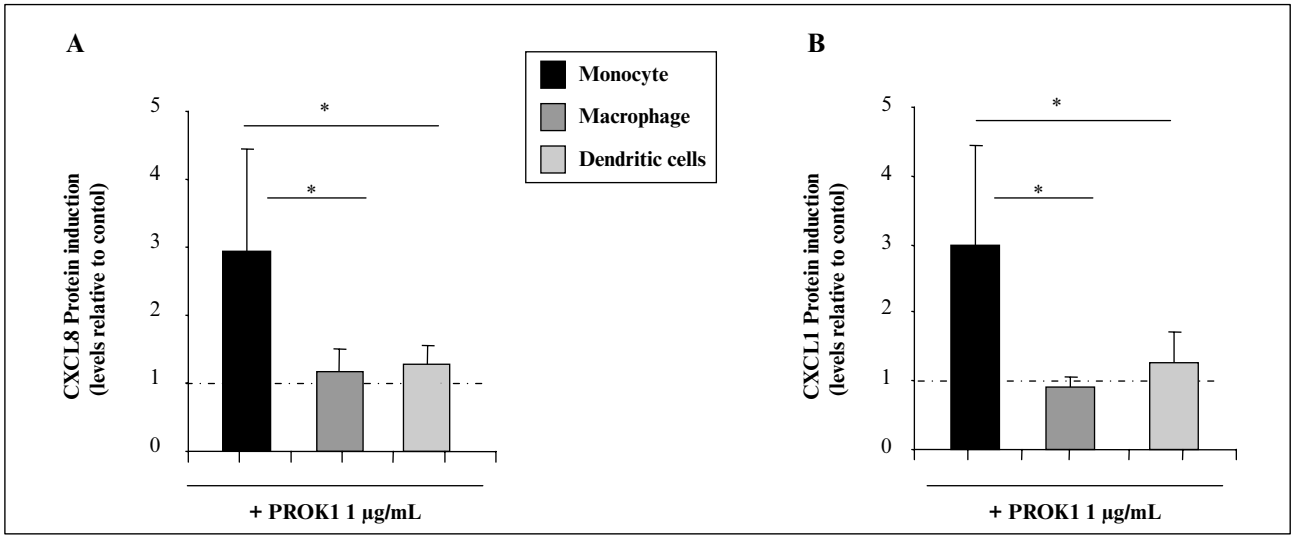

Figure 6

Comparison of CXCL1 and CXCL8 induction by PROK1 in human monocytes, macrophages and dendritic cells. CXCL1 and CXCL8 protein production was measured by ELISA in culture supernatants of monocytes, macrophages or dendritic cells after 24 h stimulation with PROK1 (1 µg/mL). Results are presented as induction of CXCL8 (**A**) or CXCL1 (**B**) over the basal control level. Mean values are from five independent experiments, each point was performed in duplicate. Significant difference of expression was noted: $p < 0.05 = *$.

respectively Gi and Gq pathways, abolish CXCL8 production by PROK1 in a dose-dependent manner.

Several studies evaluating the role of prokineticins in the immune system and in inflammatory processes, have demonstrated that prokineticins function as pro-inflammatory mediators [8, 9, 19]. Our results are consistent with these previous reports since we have demonstrated PROK1 alone can induce secretion of the powerful pro-inflammatory chemokines CCL4, CXCL1 and CXCL8. Indeed, CCL4, is primarily chemotactic for monocytes and T cells [27-30], whereas CXCL8 and CXCL1 are primarily chemotactic for neutrophils [27-29]. In the context of an inflammatory environment, we show that combination of PROK1 with LPS consistently resulted in a synergy only for CCL18 and CCL20 protein secretion, even at LPS concentrations as low as 50 pg/mL. Both CCL18 and CCL20 are characterized by their ability to attract immature dendritic cells and naïve lymphocytes [31, 32], suggesting that PROK1 might participate further in activation and recruitment of leukocytes involved in specific immunity.

Furthermore, other studies evaluating the cytokines induced by prokineticins in the monocyte/macrophage lineage, mainly used macrophages as their cellular model and cytokine induction was never observed by PROK1 alone but in combination with a cofactor (LPS or IFNγ) [9, 19]. Here, we studied the direct response of monocytes, monocyte-derived macrophages and monocyte-derived dendritic cells to PROK1, by measuring chemokine secretion in conditioned media. Our results show that induction of pro-inflammatory chemokines CXCL1 and CXCL8 by PROK1 is only detected in monocytes, most likely due to the down-regulation of surface receptors during the differentiation process. Thus, our data in combination with other reports, suggest that the differentiation status of monocytes leads to a differential response to PROK1.

Recently it was shown that in myeloid-cell-dependent tumors, CD11b+GR1+ cells (consisting primarily of neutrophils and cells from the macrophage lineage) had an important role in tumor angiogenesis which was mediated in part by secretion of PROK2 in the tumor stroma [15]. However, the molecular mechanisms underlying the angiogenic activity of PROK2 is not entirely clear. When considering that CXCL1 and CXCL8 are also highly potent angiogenic factors by signalling through their common receptor CXCR2 on endothelial cells [33], we speculate that PROK2 might influence angiogenesis by inducing CXCL1 and CXCL8 in mononuclear phagocytes expressing PROK receptors.

In conclusion, this study demonstrates that, *in vitro*, PROK1 alone preferentially activates monocytes compared to macrophages and dendritic cells, and could participate in an amplification loop of leukocyte recruitment, extravasation and tissue invasion by inducing in monocytes a restricted set of pro-inflammatory chemokines.

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REFERENCES

1. Kaser A, Winklmayr M, Lepperdinger G, Kreil G. The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. *EMBO Rep* 2003; 4: 469.
2. LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, *et al.* Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* 2001; 412: 877.
3. Melchiorri D, Bruno V, Besong G, Ngomba RT, Cuomo L, De Blasi A, *et al.* The mammalian homologue of the novel peptide Bv8 is expressed in the central nervous system and supports neu-

ronal survival by activating the MAP kinase/PI-3-kinase pathways. *Eur J Neurosci* 2001; 13: 1694.

4. Negri L, Lattanzi R, Giannini E, De Felice M, Colucci A, Melchiorri P. Bv8, the amphibian homologue of the mammalian prokineticins, modulates ingestive behaviour in rats. *Br J Pharmacol* 2004; 142: 181.
5. Cheng MY, Bullock CM, Li C, Lee AG, Bermak JC, Belluzzi J, *et al.* Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* 2002; 417: 405.
6. Mollay C, Wechselberger C, Mignogna G, Negri L, Melchiorri P, Barra D, *et al.* Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. *Eur J Pharmacol* 1999; 374: 189.
7. Negri L, Lattanzi R, Giannini E, Colucci M, Margheriti F, Melchiorri P, *et al.* Impaired nociception and inflammatory pain sensation in mice lacking the prokineticin receptor PKR1: focus on interaction between PKR1 and the capsaicin receptor TRPV1 in pain behavior. *J Neurosci* 2006; 26: 6716.
8. LeCouter J, Zlot C, Tejada M, Peale F, Ferrara N. Bv8 and endocrine gland-derived vascular endothelial growth factor stimulate hematopoiesis and hematopoietic cell mobilization. *Proc Natl Acad Sci USA* 2004; 101: 16813.
9. Dorsch M, Qiu Y, Soler D, Frank N, Duong T, Goodearl A, *et al.* PK1/EG-VEGF induces monocyte differentiation and activation. *J Leukoc Biol* 2005; 78: 426.
10. Lin R, LeCouter J, Kowalski J, Ferrara N. Characterization of endocrine gland-derived vascular endothelial growth factor signaling in adrenal cortex capillary endothelial cells. *J Biol Chem* 2002; 277: 8724.
11. Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY. Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *J Biol Chem* 2002; 277: 19276.
12. Soga T, Matsumoto S, Oda T, Saito T, Hiyama H, Takasaki J, *et al.* Molecular cloning and characterization of prokineticin receptors. *Biochim Biophys Acta* 2002; 1579: 173.
13. Masuda Y, Takatsu Y, Terao Y, Kumano S, Ishibashi Y, Suenaga M, *et al.* Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochem Biophys Res Commun* 2002; 293: 396.
14. Kisliouk T, Friedman A, Klipper E, Zhou QY, Schams D, Alfaidy N, *et al.* Expression pattern of prokineticin 1 and its receptors in bovine ovaries during the estrous cycle: involvement in corpus luteum regression and follicular atresia. *Biol Reprod* 2007; 76: 749.
15. Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, *et al.* Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 2007; 450: 825.
16. Monnier J, Piquet-Pellorce C, Feige JJ, Musso O, Clement B, Turlin B, *et al.* Prokineticin 2/Bv8 is expressed in Kupffer cells in liver and is down regulated in human hepatocellular carcinoma. *World J Gastroenterol* 2008; 14: 1182.
17. Monnier J, Samson M. Cytokine properties of prokineticins. *Febs J* 2008; 275: 4014.
18. Soderhall I, Kim YA, Jiravanichpaisal P, Lee SY, Soderhall K. An ancient role for a prokineticin domain in invertebrate hematopoiesis. *J Immunol* 2005; 174: 6153.
19. Martucci C, Franchi S, Giannini E, Tian H, Melchiorri P, Negri L, *et al.* Bv8, the amphibian homologue of the mammalian prokineticins, induces a proinflammatory phenotype of mouse macrophages. *Br J Pharmacol* 2006; 147: 225.
20. Luster AD. Chemokines-chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; 338: 436.
21. Hoffmann P, Feige JJ, Alfaidy N. Placental expression of EG-VEGF and its receptors PKR1 (Prokineticin Receptor-1) and PKR2 throughout mouse gestation. *Placenta* 2007; 28: 1049.
22. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3 (RESEARCH0034).
23. Locati M, Deuschle U, Massardi ML, Martinez FO, Sironi M, Sozzani S, *et al.* Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. *J Immunol* 2002; 168: 3557.
24. Suzuki T, Hashimoto S, Toyoda N, Nagai S, Yamazaki N, Dong HY, *et al.* Comprehensive gene expression profile of LPS-stimulated human monocytes by SAGE. *Blood* 2000; 96: 2584.
25. Chen J, Kuei C, Sutton S, Wilson S, Yu J, Kamme F, *et al.* Identification and pharmacological characterization of prokineticin 2 beta as a selective ligand for prokineticin receptor 1. *Mol Pharmacol* 2005; 67: 2070.
26. Zhou QY. The prokineticins: a novel pair of regulatory peptides. *Mol Interv* 2006; 6: 330.
27. Proost P, Wuyts A, Van Damme J. Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1. *J Leukoc Biol* 1996; 59: 67.
28. Standiford TJ, Kunkel SL, Greenberger MJ, Laichalk LL, Strieter RM. Expression and regulation of chemokines in bacterial pneumonia. *J Leukoc Biol* 1996; 59: 24.
29. Kopydlowski KM, Salkowski CA, Cody MJ, van Rooijen N, Major J, Hamilton TA, *et al.* Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J Immunol* 1999; 163: 1537.
30. Robertson MJ. Role of chemokines in the biology of natural killer cells. *J Leukoc Biol* 2002; 71: 173.
31. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* 2003; 14: 409.
32. Schutyser E, Richmond A, Van Damme J. Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J Leukoc Biol* 2005; 78: 14.
33. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. *Eur J Cancer* 2006; 42: 768.