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# A novel inflammatory pathway involved in leukocyte recruitment: role for the kinin B1 receptor and the chemokine CXCL5

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## Abstract

The kinin B1 receptor is an inducible receptor not normally expressed but induced by inflammatory stimuli and plays a major role in neutrophil recruitment, particularly in response to the cytokine interleukin-1β (IL-1β). However, the exact mechanism involved in this response is unclear. The aim of this study was to dissect the molecular mechanism involved, in particular to determine whether specific ELR-CXCL chemokines (specific neutrophil chemoattractants) played a role. Using intravital microscopy, we demonstrated that IL-1 β induced leukocyte rolling, adherence and emigration in mesenteric venules of wild type (WT) mice, associated with an increase of B1 receptor mRNA expression, was substantially attenuated (>80%) in B1 receptor knockout mice (B1KO). This effect in B1KO mice was correlated with a selective down regulation of IL-1β-induced CXCL5 mRNA and protein expression compared to WT mice. Furthermore a selective neutralizing CXCL5 antibody caused profound suppression of leukocyte emigration in IL-1β treated WT mice. Finally, treatment of human endothelial cells with IL-1β enhanced mRNA expression of B1 receptor and the human CXCL5 homologues (hCXCL5 and hCXCL6). This response was suppressed by ~50% when cells were pretreated with the B1 receptor antagonist des-Arg$^{9}$[Leu]$^{8}$BK whilst treatment with des-Arg$^{9}$-BK, the B1 receptor agonist, caused a concentration-dependent increase in hCXCL5 and hCXCL6 mRNA expression. This study unveils a pro-inflammatory pathway centred on kinin B1 receptor activation of CXCL5 leading to leukocyte trafficking, and highlights the B1 receptor as a potential target in the therapeutics of inflammatory disease.

### Keywords

Neutrophils; Chemokines; Inflammation; Endothelials cells; cell trafficking

## INTRODUCTION

Leukocyte recruitment at sites of tissue injury is an important facet of an inflammatory response (1). Polymorphonuclear neutrophils (PMNs) are the first cells recruited to the inflammatory site and their uncontrolled accumulation is thought to contribute to organ

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dysfunction (2). One of the integral cytokines involved in cell recruitment is interleukin-1β (IL-1β) (3). IL-1β brings about its effects by activating a number of pro-inflammatory pathways. In particular this cytokine induces the expression of several acute response proteins, one of which is the kinin B1 receptor. Our previous work demonstrates that this receptor plays an important role in mediating IL-1β-induced leukocyte recruitment (4), (5).

The kinins are commonly recognised as a family of inflammatory peptides (6-8); the effects of which are mediated by the endogenous agonists bradykinin (BK) and one of the main metabolites of BK, des-Arg$^9$BK (DABK). The biological effects of the kinins are brought about by their interaction with specific G protein-coupled receptors. At present, there are two clearly defined and cloned kinin receptors: B1 and B2. The B2 receptor, which is activated by BK, is constitutively expressed, suffers rapid desensitisation following activation, and mediates many of the acute actions of the kinins including edema, increased blood flow and pain (6-8). In contrast the B1 receptor, activated by DABK, is normally absent but is induced under inflammatory conditions, often hand-in-hand with an enhancement of the circulating levels of the endogenous B1 agonist, DABK, and does not undergo desensitisation upon activation (6-8). This receptor is induced during inflammation by certain immunostimulants; the optimal inducer being IL-1β (5, 7-9). In addition to a number of the inflammatory functions exhibited by B2 receptor activation (6-8), B1 receptor activation also stimulates leukocyte recruitment by promoting interaction between leukocytes and the endothelium, resulting in increased rolling, adhesion and migration of PMNs (4, 5). Significantly antagonism of B1 receptors, *in vivo*, attenuates IL-1β-induced leukocyte accumulation (5), and inflammatory responses dependent upon leukocyte recruitment are attenuated in kinin B1 receptor knockout (B1KO) mice (10) however, the exact downstream mechanisms involved in this response have yet to be determined.

Chemokines are proinflammatory cytokines that stimulate leukocyte chemoattraction and are produced in response to infectious and other inflammatory stimuli by a number of different cell types, including endothelial cells (11). That endothelial cells produce chemokines is of particular significance since within the vasculature the endothelial cell is the site for leukocyte recruitment, and expression of chemokines on the endothelial cell surface plays a pivotal role in leukocyte migration by facilitating the direct interaction of endothelial cells with leukocytes (1). More than 50 chemokines have been identified so far and have been classified into four groups according to the location of the conserved cysteine residues: CXCL, CCL, CL and CX3CL (12). These chemokines play differential roles in specifically recruiting different cell types to an inflammatory site. With respect to neutrophil recruitment the presence of a trio of amino acids, glutamate-leucine-arginine (ELR), before the CXCL motif appears to confer selectivity for promoting neutrophil migration (13). The most well described ELR-CXCL chemokines in mice include CXCL1 (also called keratinocyte-derived chemokine or KC), CXCL2 (also called macrophage inflammation protein-2 or MIP-2), CXCL5 (also called lipopolysaccharide-inducible CXC chemokine or LIX) and CXCL7 (also called neutrophil activating peptide-2 or NAP-2). Whilst good evidence supports a role for the kinin B1 receptor in neutrophil recruitment (4, 5, 10), whether this is dependent upon ELR-CXCL chemokine expression and activity is unknown.

Herein, we demonstrate that the kinin B1 receptor plays an essential role in IL-1β-induced neutrophil recruitment using kinin B1 receptor knockout (B1KO) mice (10). Moreover, we show that this effect is associated with the expression of a number of chemokines in particular CXCL5, that is likely produced by endothelial cells following direct activation of endothelial kinin B1 receptor. Since kinin B1 receptor expression is raised in inflammatory disease (6-8) and leukocyte recruitment is proposed to play an important role in the innate immune response associated with a wide range of inflammatory diseases, from traditional inflammatory conditions such as rheumatoid arthritis or sepsis to the more recently

appreciated inflammatory disease of atherosclerosis (1), our findings highlight the B1 receptor-CXCL5 pathway as a novel therapeutic target.

## MATERIELS and METHODS

### Animals

All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Male C57BL6J wild type (WT) or B1KO (10) mice (C57BL6 background) at 5 weeks of age (10-15g) were used in all experiments.

### Intravital Microscopy

Male WT and B1KO mice received either murine IL-1β (5ng/mouse, i.p., PeproTech, UK) or saline vehicle. After 4h mice were anesthetized with diazepam (60mg/kg subcutaneously) and Hypnorm® (0.7mg/kg fentanyl citrate and 20mg/kg fluanisone intramuscularly) and the mesenteric vascular bed prepared for viewing by intra-vital microscopy. Mesenteries were superfused with bicarbonate-buffered solution at 37°C (132mM NaCl, 4.7mM KCl, 1.2mM $MgSO_,$ 17.9mM $NaHCO_{3,}$ 2.0mM $CaCl_2$, pH 7.4, gassed with 5% $CO_2$, 95% $N_2$) at a rate of 2ml/min. The temperature of the stage was maintained at 37°C. The extent of the inflammatory response elicited by IL-1β was analyzed by counting the number of white blood cells rolling per min. Cell adhesion was quantified by counting, for each vessel, the number of adherent neutrophils in a 100μm length, and leukocyte emigration from the microcirculation into the tissue was quantified by counting the number of cells that had emigrated up to 50μm away from the wall of 100μm vessel segments. Venular blood flow was calculated from the product of mean RBC velocity ($V_{mean}$ = centerline velocity/1.6) and microvascular cross-sectional area, assuming a cylindrical geometry. Wall shear rate was calculated by the Newtonian definition: shear rate = 8,000× ($V_{mean}$/diameter). A minimum of 3 postcapillary venules (diameter between 20-40μm; length of at least 100μm) were observed for each mouse. To evaluate the role of CXCL5 in regulating leukocyte recruitment, a selective neutralizing monoclonal antibody or control IgG (20μg per animal, i.p., PeproTech, UK) was injected into the tail vein 30min prior to IL-1β treatment. After 4h, leukocyte rolling, adhesion and emigration were measured as described above.

### Myeloperoxidase (MPO) assay

MPO activity was determined in mesenteric tissue as an index of neutrophil accumulation (14). Mesenteric tissue, collected 4h after IL-1β treatment, was homogenized in 1mL of a 0.5% hexa-decyl-trimethyl ammonium bromide (HTAB) in MOPS buffer (10mM, pH 7). After homogenization, samples were centrifuged at 4000g for 20min at 4°C and the supernatant collected for determination of MPO levels as previously described (15). Data are expressed as U/g of total protein content in the tissue determined by Bradford assay.

### Real-Time Quantitative RT-PCR of murine mesenteric tissue

Chemokine mRNA expression was determined by real-time quantitative RT-PCR. Briefly mesenteric tissue was removed from saline or IL-1β (2h)-treated mice as above, snap frozen in liquid nitrogen and stored at −80°C until use. Samples were homogenised and total RNA isolated using a NucleoSpin RNA II purification kit (Macherey-Nagel) and then stored at −80°C until use. cDNA was synthesized from 1μg of total RNA with M-MLV reverse transcriptase (Promega, UK) using oligo-dt nucleotides. The following primers were used for mouse: **CXCL1** (TGA GCT GCG CTG TCA GTG CCT and AGA AGC CAG CGT TCA CCA GA), **CXCL2** (GAG CTT GAG TGT GAC GCC CCC AGG and GTT AGC CTT GCC TTT GTT CAG TAT C), **CXCL5** (GCA TTT CTG TTG CTG TTC ACG CTG and CCT CCT TCT GGT TTT TCA GTT TAG C), **CXCL7** (TGG GCC TGA TCC TTG

TTG CGC AND GCA CCG TTT TTT GTC CAT TCT TCA G), **B1 recepto**r (TGG AGT TGA ACG TTT TGG GTT T and GTG AGG ATC AGC CCC ATT GT) and **βactin** (GAA ATC GTG CGT GAC ATC AAA G and TGT AGT TTC ATG GAT GCC ACA G). Standard curves for these molecules were generated to determine amplification efficiencies of target and reference genes. Quantitative PCR was performed on ABI Prism 7900 with 100nM of primers and 20ng of cDNA. Chemokine/receptor expression was normalized to ß-actin and expressed as relative value using the comparative Ct method (2-ΔΔCt) according to the manufacturer's instructions. The levels of mRNA expression of genes of interest were normalised to saline control.

### Measurement of CXCL5 protein expression

Mesenteric tissue of saline and IL-1β-treated (4h) WT and B1KO mice were collected, homogenised and supernatants collected. Mouse CXCL5 protein levels were determined by ELISA (Duoset R&D Systems) according to the manufacturer's protocol. CXCL5 levels were expressed relative to total protein concentration of the supernatant samples.

### Human Umbilical Vein Endothelial Cell (HUVEC) Culture

HUVEC were cultured to passage 3 in EGMII medium (Cambrex, UK). Confluent cells were treated with vehicle (saline) or IL-1β (1ng/ml, 0-24h). In some experiments, the B1 antagonist Lys-[Leu$^8$]des-Arg$^9$-BK (10μM) was added to the medium 15 min prior to IL-1β treatment and the reaction stopped after 8h. In a further series of experiments cells were incubated with the B1 agonist Lys-des-Arg$^9$-BK (1–10,000nM) for 4h either directly or following a 24h pre-treatment with IL-1β.

### Real-Time Quantitative RT-PCR of endothelial cells

HUVEC from the above experiments were washed with sterile PBS, collected by scraping and samples kept at −80°C until mRNA extraction. The human CXCL5 homologues hCXCL5 and hCXCL6, and kinin B1 receptor mRNA expression were determined as described above. The following primers were used: **hCXCL5** (GAG AGC TGC GTT GCG TTT G and TTT CCT TGT TTC CAC CGT CCA) **hCXCL6** (GGT CCT GTC TCT GCT GTG C and GGG AGG CTA CCA CTT CCA) **hB1 receptor** (ACG CCT TCA TTT TCT GCC TG and GCT GGC TCT GGT TGG AGG AT) **kininogen** (AGA CAC GGC ATT CAG TAC TTT AAC A and TGG GCC CGT TTT ACT TCA TT) **kallikrein (**GGG TCG CCA CAA CTT GTT TG and GCT GTA GTC CTC GTC TGC TT) and **GAPDH** (CAT GTT CGT CAT GGG TGT GAA and ATG GAC TGT GGT CAT GAG TCC TT).

### Western blotting of endothelial cells

Following treatment cells outlined above cells were washed with ice-cold PBS, scraped and lysed in ice-cold lysis buffer (20mM Tris-HCl [pH 7.4], 50mM NaCl, 50mM NaF, 5mM EDTA, and 20mM $Na_4P_2O_7{\cdot}10H_2O$). Supernatants were collected and protein concentration determined by Bradford assay. Samples (20μg) were subjected to eletrophoresis using an 8% polyacrylamide gel followed by electrotransfer to nitrocellulose membrane. To detect B1 receptor, nitrocellulose were incubated with the polyclonal B1 receptor antibody (A15C (16); kind gift of Dr Jean-Loup Bascands, dilution 1:5000) overnight at 4°C and then a secondary peroxidase-coupled goat anti–rabbit antibody (1/2000, Dako). Visualization of bands was achieved by chemiluminescence (ECL kit; Amersham Pharmacia Biotech). Selectivity of the antibody was determined by preadsorption of the antibody to its corresponding peptide at a concentration of 10μg/ml at 4°C overnight. The autoradiogaphic bands were semiquantified and normalised to α-tubulin levels.

### Statistical analysis

Values are given as means ± SE where n represents the number of animals or the number of experiments conducted for cells. Statistical comparisons were conducted using paired or unpaired Student's t test for 2 groups or one way ANOVA for more than 2 groups. Differences were considered significant when $p < 0.05$.

## RESULTS

### IL-1β-induced PMN recruitment is absent in B1KO mice

IL-1β caused a significantly increases of mesenteric B1 receptor mRNA expression (Fig.1D) that was associated with a pronounced cellular recruitment in WT mice as indicated by augmentation of leukocyte rolling, adhesion and emigration (Fig.1A and B). All parameters of IL-1β-induced leukocyte recruitment were profoundly suppressed in B1KO mice and this was likewise associated with a complete absence of B1 receptor mRNA expression (Fig. 1D). These differences were not due to changes in venular haemodynamics since there were no significant differences in venule diameter or blood flow between WT and B1KO animals (see Table 1). IL-1β-induced cellular recruitment in WT animals was associated with a ~3-fold increase in MPO activity that was markedly attenuated in B1KO mice (Fig.1C).

### CXCL5 expression is abolished in B1KO mice

Quantitative PCR of mesenteric tissue of IL-1β-treated WT animals revealed significant mRNA expression of the CXCL1, CXCL2, CXCL5 and CXCL7 chemokines above that measured in saline treated controls (Fig.2). However, this IL-1β-induced chemokine elevation was profoundly suppressed in tissues of B1KO mice with respect specifically to CXCL1 (~32% inhibition), CXCL2 (~67% inhibition) and CXCL5 (>95% inhibition). In contrast, CXCL7 which was upregulated in WT mice was not changed in B1KO mice.

Since CXCL5 appeared to be the most profoundly suppressed ELR-CXCL chemokine in B1KO mice (Fig.2), we sought to ascertain whether the changes in mRNA were reflected in protein expression. CXCL5 protein expression in mesenteries of IL-1β–treated WT mice was substantially elevated compared to saline control (503 ±66pg/mg of protein n=6 vs 142±20pg/mg of protein, n=5, $P<0.01$ respectively). In contrast this response to IL-1β was entirely absent in B1KO mice (218±38pg/mg of protein n=5 vs 188±27pg/mg of protein n=6, non significant) (Fig.3A).

### Neutralization of CXCL5 reduces IL-1β-induced leukocyte adhesion and emigration

Treatment of WT mice with a neutralizing anti-CXCL5 antibody inhibited IL-1β-induced cell adhesion and emigration by ~50%, but no significant effect on cell rolling was observed at the 4h time point (Fig.3B). In contrast control rabbit IgG had no significant effect on leukocyte recruitment.

### Human CXCL5 homologues are regulated by B1 receptor in human endothelial cells.

Since previous evidence suggests endothelial cells express B1 receptor following exposure to inflammatory stimuli ((5, 17-19) and since the endothelial cell is an important cellular sources of chemoattractant (20-22), we investigated whether the endothelial cell might be an *in vivo* source of B1 receptor-induced chemokine production. In HUVEC, kinin B1 receptor mRNA was induced within 2h of IL1β treatment, peaked at 4h (~5 fold increase) and remained significantly elevated at 8h returning to near basal levels by 24h (Fig 4A). This IL-1β-induced change in mRNA was associated with increases in kinin B1 receptor protein expression (Fig. 4B). We also observed that IL-1β treatment induced an increase in the expression of the components of the kallikrein-kinin system with an elevation in the levels

of both kallikrein and kininogen (Fig. 4C). Basal levels of chemokines were low in unstimulated HUVECs, however treatment with IL-1β caused a time-dependent increase in human CXCL5 homologues (hCXCL5 and hCXCL6) mRNA expression, peaking at 8h, but with a lag time of 2-4h relative to kinin B1 receptor expression (Fig.4A). B1 receptor blockade significantly suppressed IL-1β-induced hCXCL5 and hCXCL6 expression at the 8h time point by ~50% (Fig.5A). Treatment of cells with B1 agonist produced concentration-dependent increases in hCXCL5 and hCXCL6 expression (Fig.5B) in control cells. In addition, in cells pretreated with IL-1β for 24h, application of B1 agonist produced a further 1.3 ± 0.07 fold increase (n=4, $p<0.05$, t-test compared to IL-1β alone) in hCXCL5 expression above that induced by IL-1β alone.

## DISCUSSION

We have previously demonstrated that the kinin B1 receptor plays an important role in mediating recruitment of PMNs to a site of inflammation (4, 5), a finding that was supported by the recent observations that cell-dependent inflammation is reduced in B1KO mice (10). However, the exact mechanisms involved in this response were unclear. Our findings, herein, support the thesis that B1 receptor activation is essential for IL-1β driven cell recruitment and, moreover, that neutrophil chemoattractant chemokines belonging to the ELR-CXCL family, most notably CXCL5, mediate this effect. In addition, we have established that induction and subsequent endogenous activation of kinin B1 receptors on endothelial cells is likely to play a major role in B1 receptor-induced CXCL5 expression.

Treatment of WT mice with IL-1β induced a substantial elevation of mesenteric B1 receptor mRNA expression. These observations are in accordance with our previous findings demonstrating low levels of B1 receptor mRNA expression in control untreated murine mesenteric tissue, but profound induction of expression following exposure to inflammatory stimuli (5). In contrast, no B1 receptor mRNA expression was evident in the mesenteries of B1KO mice, either in the controls or following cytokine treatment. This absence of B1 receptor expression, in B1KO mice, had a major impact on the magnitude of the inflammatory response to IL-1β treatment. Indeed, whilst in WT animals IL-1β produced a characteristic increase in leukocyte recruitment, in B1KO mice this response was abolished; an effect that was not due to inherent differences in venular hemodynamics, since all hemodynamic parameters were similar between the two genotypes. We chose to use the mesenteric preparation, with a 4h IL-1β treatment, since we have previously established that the B1 receptor plays a major role in mediating leukocyte recruitment in the mouse mesentery at this time-point, and that this is associated with B1 receptor mRNA expression (5). That the B1 receptor is essential in this response is, of itself, an important observation since IL-1β is a pivotal regulator of cell activation in acute inflammation (23-25). It would be of interest to determine whether this phenomenon extends to other preparations of intravital microscopy, including the cremaster microcirculation.

The near abolition of cell recruitment, in B1KO mice, also suggests that our previous estimates of the magnitude of the kinin B1 receptor-mediated component (using B1 receptor antagonists) of the cell recruitment response to IL-1β, of the order of 50%, were a substantial underestimate (4, 5). This may have been due to the fact that the antagonists, des $Arg^{9}[Leu]^{8}$BK and des-$arg^{10}$Hoe 140 are peptidic in nature, and therefore prone to degradation, and express partial agonist activity (7, 8). Antagonists displaying no partial agonist activity and resistance to degradation, such as SSR240612 (26) or compound 11 (27), are likely to prove highly effective at inhibiting PMN recruitment. The essential role of the kinin B1 receptor in leukocyte recruitment is highlighted by the observation that no compensatory mechanisms are activated in B1KO mice to maintain the inflammatory response to IL-1β. Our data also suggests that inhibition of leukocyte recruitment is likely to

play a major role in the apparent protection, afforded by the absence of the B1 receptor, in experimental models of inflammatory disease in B1KO mice, including diabetes (28), neuropathic pain (29), inflammatory hyperalgesia (10) and intestinal ischaemia/reperfusion injury (30).

The innate immune response is a tightly orchestrated sequence of events; each stage associated with the recruitment of specific inflammatory cell types to the site of inflammation. The exact cell type recruited at each stage is determined by the sensitivity to, and activity of, distinct chemotactic factors (13). PMN recruitment is specifically dependent on the activity of ELR-CXCL chemokines. To date, four ELR-CXCL chemokines have been described in mice: CXCL1, −2, −5 and −7 (also called KC, MIP2, LIX and NAP2 respectively) (31, 32). In the mouse, the ELR-CXCL chemokines bind to the chemokine receptor CXCR2, present on the neutrophil cell surface, to mediate cell migration (33). More recently, the mouse CXCR1 has been cloned (34), however, whilst the human chemokine, hCXCL8 (also known as IL-8), binds this receptor no specific murine chemokine has been identified to bind. IL-1β treatment of WT mice was associated with enhanced expression of all four murine CXCL chemokines measured. Moreover, our studies exposed a temporal and sequential relationship between kinin B1 receptor expression, chemokine production and cell recruitment. In contrast, the absence of cell recruitment in B1KO mice was associated with almost complete abrogation of IL-1β–induced transcription of CXCL5 and, substantial inhibition of CXCL1 and CXCL2 expression with no effect on CXCL7 expression. The association between B1 receptor activation and CXCL chemokine production is supported by previous work, albeit in different cells and with a different chemokine, where B1 receptor activation of human fibroblasts stimulated hCXCL8 production (35).

Of the chemokines linked to B1 receptor activation, CXCL5 appears to be the most closely associated, since its production in B1KO mice was almost abolished, suggesting that perhaps it is this chemokine that predominantly mediates the effects of B1 receptor activation. This is in accordance with previous publications identifying CXCL5 as the primary chemotactic agent underlying neutrophil recruitment in models of inflammatory disease, including myocardial ischemia-reperfusion injury (36), sepsis (37) and colitis (38). Indeed, in the present study we demonstrated that, whilst basal levels of CXCL5 mRNA are very low in WT animals, IL-1β treatment causes a ~70-fold increase in expression; a response inhibited by more than 95% in B1KO mice. This elevation in mRNA was associated with increases in protein expression that were likewise abolished in B1KO mice as evidenced by measurement of CXCL5 by ELISA. That the neutralising antibody to CXCL5 significantly attenuated leukocyte recruitment to IL-1β, supports the thesis that CXCL5 plays a major role in mediating the cellular response. However, the lack of effect of the antibody on leukocyte rolling suggests that perhaps the role of CXCL5 is centred on the adhesion and emigration steps of leukocyte recruitment. It is important to note, however, that whilst the CXCL5 antibody did not affect leukocyte rolling, IL-1β-induced rolling was abolished in B1KO mice. These findings suggest that B1 receptor activation results in the stimulation of other pathways, involved in cell rolling, unrelated to chemokine synthesis. An obvious pathway that is likely to be implicated is the adhesion molecule pathway, specifically either at the level of endothelial cell (such as P-selectin) or on the neutrophil itself (such as L-selectin). Further studies investigating this possibility are warranted to clarify this issue.

CXCL5, also called LIX in mice (39), is expressed in humans as ENA-78 (hCXCL5) and is also closely related to another human chemokine GCP-2 (hCXCL6) (40). Indeed, it has been proposed that the human hCXCL5 and hCXCL6 genes are the result of an evolutionary gene duplication (40). CXCL5 was first cloned in mouse fibroblasts (39) and was subsequently shown to be expressed in a number of different tissues (31) in response to inflammatory

cytokines, particularly IL-1β (20, 36). Since our previous studies excluded the possibility that B1 receptor-induced cell recruitment is due to direct activation of B1 receptors on neutrophils (4), we hypothesised that the endothelial cell might be an important cellular source of B1-receptor induced CXCL5 production. Although, in contrast to this thesis, a recent publication has demonstrated that direct activation of neutrophil B1 receptors does cause neutrophil migration (41). This response was only evident in IL-1β pre-treated cells, *in vitro*, and required a 24h exposure to B1 agonist. Since we have shown that the B1 receptor-dependent IL-1β-induced leukocyte recruitment response *in vivo* is evident after 2h and peaks at 4h (4, 5) it is unlikely that the slowly-developing direct activation of neutrophil B1 receptors contributes to the response evident in the current study. It is possible, however, that at later stages of the inflammatory response direct activation of neutrophilic B1 receptor may have a role to play in the ensuing activation of these cells.

The endothelial cell plays a major role in all steps of the neutrophil recruitment process (42) and endothelial cells are a major source of chemoattractant CXCL5 in mice (20), a characteristic also shared by human endothelial cells (21, 22). Analysis of HUVECs in the present study demonstrate that human endothelial cells express the B1 receptor, as has been demonsrated previously (17, 19). In addition, as others (18, 43), we have shown that the elements of the kallikrein-kinin system necessary fort endogenous B1 agonist production are also present in these cells basally i.e. kallikrein and kininogen. However, we now also demonstrate that following cytokine treatment, the expression of these factors is elevated in parallel with elevation in the expression of the B1 receptor. These findings intimate that endogenous endothelial B1 receptor activation might support the chemokine production evidenced *in vivo*.

Indeed, treatment of human endothelial cells with IL-1β stimulated a time-dependent increase in B1 receptor expression, subsequently followed by a pronounced stimulation of hCXCL5 and hCXCL6 expression. This chemokine synthesis was likely to be a consequence of B1 receptor activation since treatment of cells with the B1 antagonist, Lys-[Leu$^8$]des-Arg$^9$-BK, significantly reduced this response. Moreover, this finding is supported by the observation that treatment of endothelial cells with B1 agonist stimulated a concentration-dependent increase in hCXCL5 and hCXCL6 expression. The level of this enhanced expression, whilst significant, was at least 10-fold lower than that evident *in vivo*. This lower level of induction may have related to the level of basal B1 receptor expression in cells not stimulated with cytokine. Therefore, we investigated the activity of the B1 agonist in IL-1β-treated cells. The relative increase in chemokine expression in B1 agonist-treated-cells *vs* cytokine-only treated cells, surprisingly, appeared similar to that in unstimulated cells. However, it is important to note that at 24h following IL-1β-treatment chemokine expression is still significantly elevated, and therefore, the absolute potential for enhancement likely to be reduced. An alternative explanation for this apparent decreased potency in inducing chemokine expression *in vitro,* over *in vivo,* is simply that endothelial B1 receptor activity synergises/interacts with a blood-borne factor not present in these *in vitro* experiments. Finally, whilst it is clear that the endothelial cell is a major source of B1-induced CXCL5/CXCL6 we cannot exclude the possibility that other cell types, within the vasculature, might also be a source of this B1-induced chemokine production. In particular, both the fibroblast (44) and the mast cell (45) are cellular sources of CXCL5; cells that also express the B1 receptor (7, 46)

The molecular mechanisms involved in this B1 receptor-induced chemokine expression are uncertain, however, the transcription factor nuclear factor kappa B (NFκB), a pivotal transcriptional factor regulating inflammatory gene expression (47), has been identified as playing an essential role in IL-1β-induced hCXCL5 (48) or hCXCL6 (49) expression, in human non-vascular cell types. Similarly, NFκB plays an essential role in mediating IL-1β-

induced CXCL5 expression in mice (20). This regulation by NFκB is of interest since B1 receptor expression, itself, is also tightly regulated by an NFκB-dependent pathway (16). This group demonstrated that application of B1 agonist to human fibroblasts enhanced B1 receptor expression as a consequence of NFκB activation; autoregulation that is more pronounced in the presence of IL-1β (35). In addition, B1 receptor activation itself also stimulates further IL-1β synthesis (50) suggesting a complex facilitatory interaction between the B1 receptor and IL-1β that may play an important role in amplification of the inflammatory response; especially since the kallikrein-kinin pathway is upregulated at inflammatory sites increasing endogenous B1 agonist production (51). Together, these studies suggest that during an inflammation following the initial induction of the kinin B1 receptor, by the appropriate inflammatory stimulus, that the pathway may be continuously self-amplified to sustain the inflammatory response.

In summary, although the B1 receptor has been proposed to play a role in inflammatory pathologies (7, 8, 10), its exact contribution in the inflammatory process has been uncertain. The findings from this study have allowed clarification of the key role of the kinin B1 receptor in neutrophil recruitment at sites of inflammation, and have determined that CXCL5 production plays a major role in this response. Moreover, endothelial cells have been described as a potential source for this novel B1 receptor-CXCL5 pathway. These results, taken together with the observations that B1 receptor expression is induced by inflammation in different diseases, endogenous B1 agonist concentration increases at sites of inflammation and B1 receptor activation causes a range of cellular pro-inflammatory effects, highlights the B1 receptor and, in particular this novel B1 receptor-CXCL5 pathway, as a potential therapeutic target for inflammatory disease.

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## Abbreviations

| | |
|---|---|
| **BK** | bradykinin |
| **DABK** | des-Arg$^9$-BK |
| **PMN** | Polymorphonuclear neutrophils |
| **B1KO** | B1 receptor knockout |

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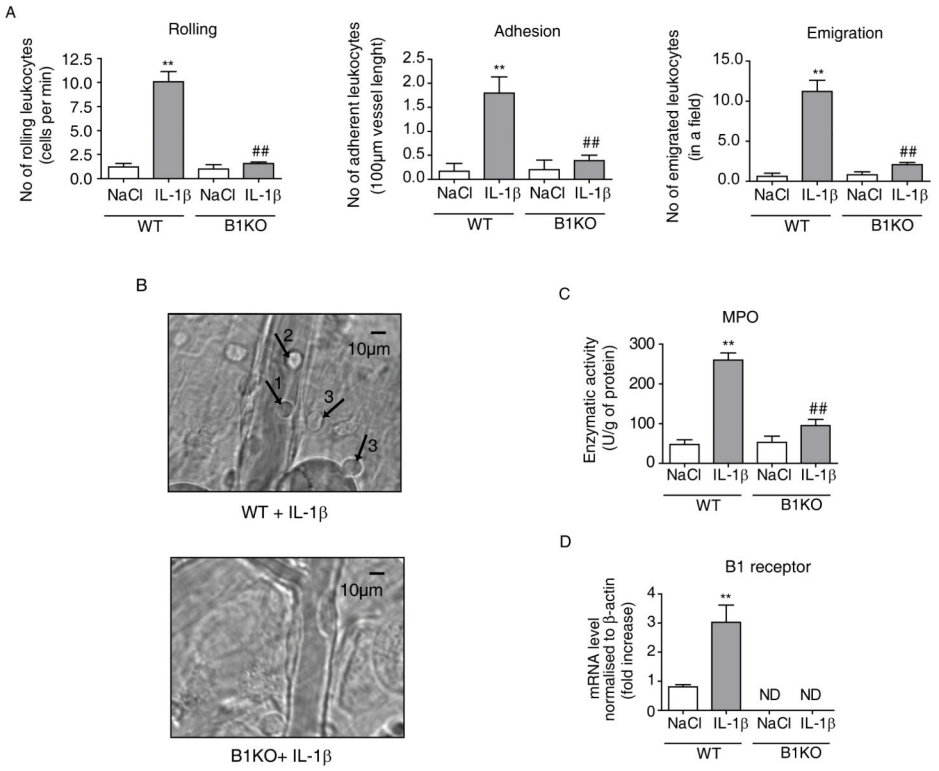


**Fig 1. IL-1β-induced PMN recruitment is abolished in B1KO mice**

(A) Leukocyte-endothelial cell interactions in mouse mesenteric postcapillary venules *in vivo* in response to IL-1β (5ng, i.p.) in WT and B1KO mice were measured by intravital microscopy. The different parameters of rolling, adhesion and emigration of leukocytes were measured in WT and B1KO mice 4h after treatment with saline (i.p.) or IL-1β. (B) The arrows show rolling (1), adherent (2) and emigrated (3) cells. (C) PMN accumulation was determined by measurement of MPO activity. Mesenteric tissue were removed from WT and B1KO mice 4h following treatment with saline or IL-1β (5ng, i.p.) and 25ng of protein extract used from each animal used to measure MPO activity. The data are expressed as the unit of MPO per g of total protein. (D) The inducibility of B1 receptor mRNA expression in mesenteric tissue in response to IL-1β (5ng, i.p.) was assessed in WT and B1KO mice by quantitative real-time RT-PCR. The data are expressed as fold increase compared to control (WT NaCl) normalised to β–actin. Data are mean ± SEM for n=6 animals per group. ** P<0.01 saline versus treated values. ## P<0.01 WT versus B1KO values.

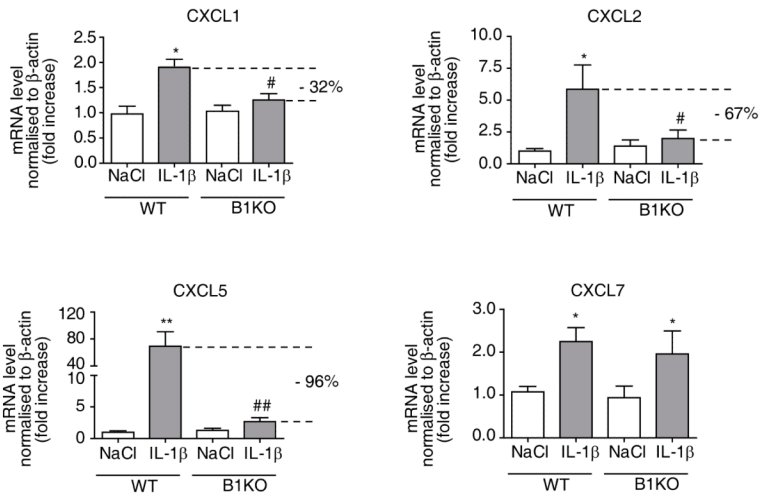


**Fig 2. IL-1β-induced ELR-CXCL chemokine mRNA expression is attenuated in B1KO mice**

mRNA expression of CXCL1, CXCL7, CXCL2 and CXCL5 assessed using quantitative real-time RT-PCR of mesenteric tissue from WT and B1KO mice treated with saline or IL-1β (5ng, i.p., 2h).The data are expressed as fold increase above control (WT NaCl) normalised to β–actin. Data shown are mean ± SEM for n=6 animals per group. ** P<0.01 saline versus treated values. ## P<0.01 WT versus B1KO values. * P<0.05 saline versus treated values. # P<0.05 WT versus B1KO values.

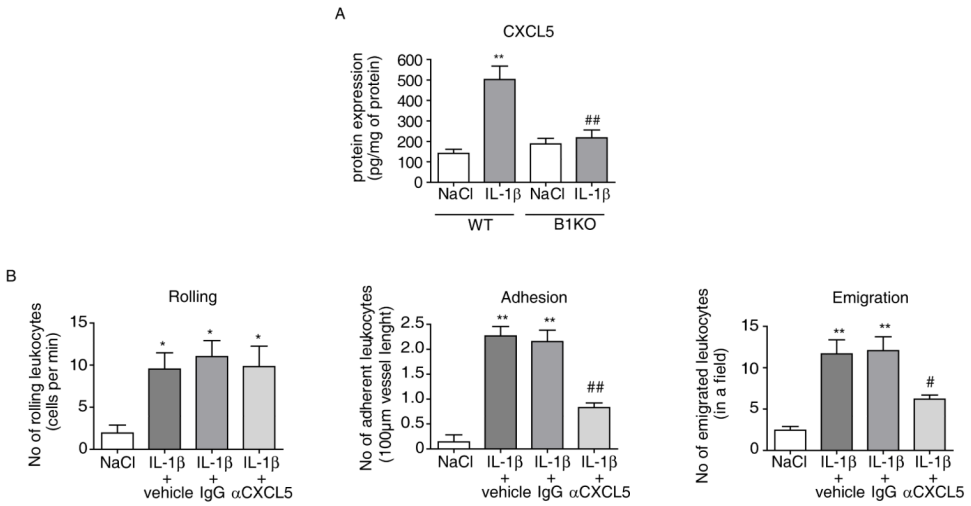


**Fig 3. CXCL5 plays a major role in IL-1β-induced leukocyte recruitment**

(A) CXCL5 protein was measured by ELISA in mesenteric tissue removed from WT and B1KO mice 4h after treatment with saline or with IL-1β (5ng, i.p.). The data are expressed as CXCL5 per mg of total protein (pg/mg of protein). Data shown are mean ± SEM for n=6 animals per group ** P<0.01 saline versus treated values. ## P<0.01 WT versus B1KO values. (B) Leukocyte-endothelial cell interactions in WT mouse mesenteric postcapillary venules were measured by intra-vital microscopy *in vivo* 4h following treatment with IL-1β (5ng, i.p.) in mice pre-injected (30min prior to IL-1β) with saline, control IgG or anti-CXCL5 antibody (20μg/mice, i.p.). Rolling, adhesion and emigration of leukocytes were measured. Data shown are mean ± SEM for n=6 animals per group ** P<0.01 saline versus treated values. ## P<0.01 WT versus treated values. * P<0.05 saline versus treated values. # P<0.05 WT versus treated values.




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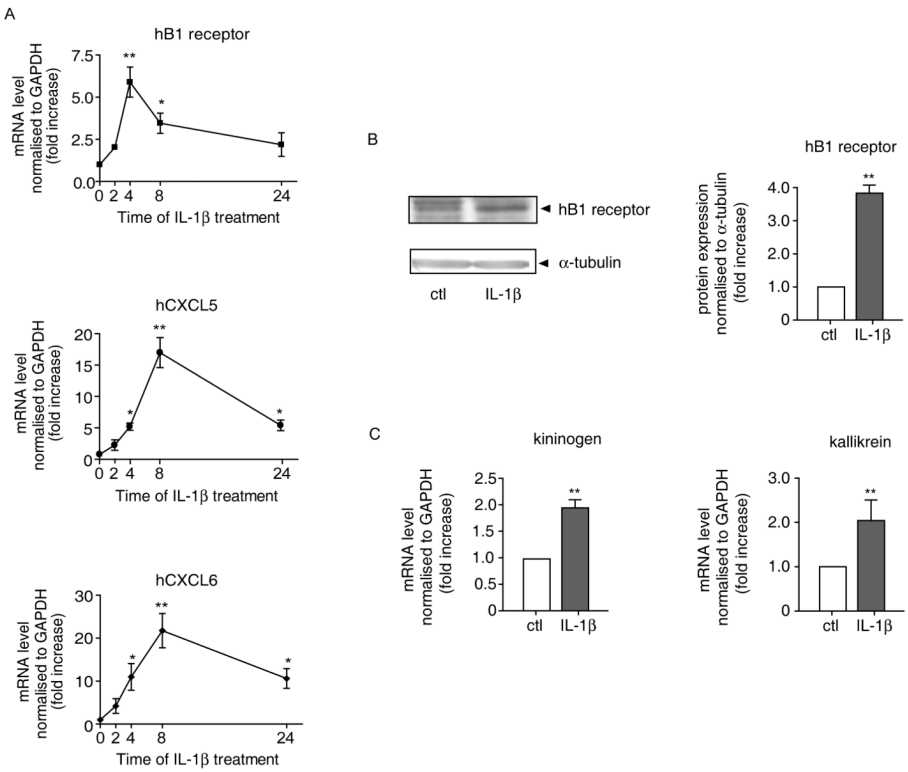


**Fig 4. Temporal relationship between endothelial kinin B1 receptor, hCXCL5, hCXCL6 and kallikrein/kininogen expression in IL-β-treated endothelial cells**

(A) Time course (0, 4h, 8h and 24h) of B1 receptor, hCXCL5 and hCXCL6 mRNA expression in response to IL-1β (1ng/mL) in HUVEC. Expression of B1 receptor, hCXCL5 and hCXCL6 were measured by quantitative real-time RT-PCR. (B) Protein expression of B1 receptor in HUVEC after 8h of IL-1β treatment by western blotting. (C) mRNA expression of kininogen and kallikrein in HUVEC after 4h of IL-1β treatment was measured by quantitative real-time RT-PCR. The data are expressed as the fold increase above control (non-treated cells) normalised to GAPDH for mRNA and to tubulin for protein. Data shown are mean ± SEM for n = 4. * P<0.05 control versus treated values. ** P<0.01 control versus treated values.




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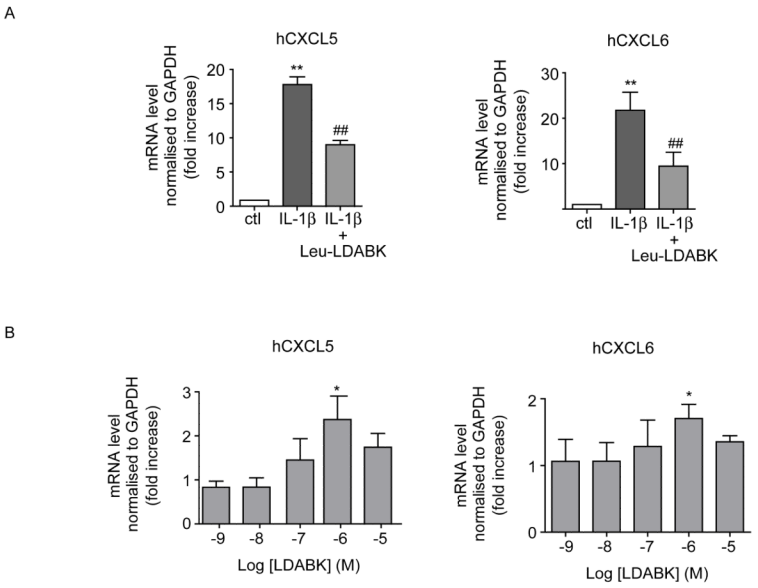


**Fig 5. Kinin B1 receptor-induced hCXCL5 and hCXCL6 expression in HUVEC**

(A) Effect of B1 antagonist (Lys-[Leu$^8$]des-Arg$^9$-BK at 10μM, 15min pre-treatment at t=0) on IL-1β (1ng/mL, 8h)-induced hCXCL5 and hCXCL6 mRNA expression in HUVEC. (B) Effect of B1 agonist (Lys-des-Arg$^9$-BK, 4h, 1-10,000 nM) on hCXCL5 and hCXCL6 mRNA expression. The data are expressed as the fold increase above control (non-treated cells) normalised to GAPDH for mRNA. Data are mean ± SEM for n = 4. ** P<0.01 saline versus treated values. * P<0.05 saline versus treated values. ## P<0.01 IL-1β versus IL-1β + Leu-LDBK values.




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**Table 1**

**Hemodynamics Parameters in animals used for intravital microscopy studies**

Mice received either saline (100μL, i.p.) or IL-1β (5 ng, i.p). Data are mean ± SEM.

| Genotype | Treatment | No. of Venules | Venule diameter (μm) | Shear rate ($s^{-1}$) |
|---|---|---|---|---|
| WT | Saline (4 h) | 12 | 32.2 ± 3.3 | 254 ± 31 |
| WT | IL-1β (4 h) | 18 | 31.1 ± 2.9 | 276 ± 27 |
| B1KO | Saline (4 h) | 10 | 29.7 ± 4.2 | 235 ± 34 |
| B1KO | IL-1β (4 h) | 16 | 31.8 ± 3.7 | 287 ± 25 |