

# A Novel Inflammatory Pathway Involved in Leukocyte Recruitment: Role for the Kinin B<sub>1</sub> Receptor and the Chemokine CXCL5<sup>1</sup>

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The kinin B<sub>1</sub> 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 IL-1 $\beta$ . 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 $\beta$ -induced leukocyte rolling, adherence, and emigration in mesenteric venules of wild-type (WT) mice, associated with an increase in B<sub>1</sub> receptor mRNA expression, were substantially attenuated (>80%) in B<sub>1</sub> receptor knockout mice (B1KO). This effect in B1KO mice was correlated with a selective down-regulation of IL-1 $\beta$ -induced CXCL5 mRNA and protein expression compared with WT mice. Furthermore a selective neutralizing CXCL5 Ab caused profound suppression of leukocyte emigration in IL-1 $\beta$ -treated WT mice. Finally, treatment of human endothelial cells with IL-1 $\beta$  enhanced mRNA expression of the B<sub>1</sub> receptor and the human (h) CXCL5 homologues (hCXCL5 and hCXCL6). This response was suppressed by ~50% when cells were pretreated with the B<sub>1</sub> receptor antagonist des-Arg<sup>9</sup>-[Leu<sup>8</sup>]-bradykinin while treatment with des-Arg<sup>9</sup>-bradykinin, the B<sub>1</sub> receptor agonist, caused a concentration-dependent increase in hCXCL5 and hCXCL6 mRNA expression. This study unveils a proinflammatory pathway centered on kinin B<sub>1</sub> receptor activation of CXCL5 leading to leukocyte trafficking and highlights the B<sub>1</sub> receptor as a potential target in the therapeutics of inflammatory disease. *The Journal of Immunology*, 2007, 179: 4849–4856.

Leukocyte recruitment at sites of tissue injury is an important facet of an inflammatory response (1). Polymorphonuclear neutrophils (PMNs)<sup>3</sup> are the first cells recruited to the inflammatory site and their uncontrolled accumulation is thought to contribute to organ dysfunction (2). One of the integral cytokines involved in cell recruitment is IL-1 $\beta$  (3). IL-1 $\beta$  brings about its effects by activating a number of proinflammatory pathways. In particular, this cytokine induces the expression of several acute response proteins, one of which is the kinin B<sub>1</sub> receptor. Our previous work demonstrates that this receptor plays an important role in mediating IL-1 $\beta$ -induced leukocyte recruitment (4, 5).

The kinins are commonly recognized 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<sup>9</sup>-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: B<sub>1</sub> and B<sub>2</sub>. The B<sub>2</sub> receptor, which is activated by BK, is constitutively expressed, suffers rapid desensitization following activation, and mediates many of the acute actions of the kinins including edema, increased blood flow, and pain (6–8). In contrast the B<sub>1</sub> 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 B<sub>1</sub> agonist, DABK, and does not undergo desensitization upon activation (6–8). This receptor is induced during inflammation by certain immunostimulants, the optimal inducer being IL-1 $\beta$  (5, 7–9). In addition to a number of the inflammatory functions exhibited by B<sub>2</sub> receptor activation (6–8), B<sub>1</sub> 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 B<sub>1</sub> receptors in vivo attenuates IL-1 $\beta$ -induced leukocyte accumulation (5), and inflammatory responses dependent upon leukocyte recruitment are attenuated in kinin B<sub>1</sub> 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 because 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

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Received for publication January 5, 2007. Accepted for publication July 19, 2007.

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<sup>1</sup> This work was supported by the Fondation pour la Recherche Medicale and the British Heart Foundation (to J.D.). F.L. was supported by The Wellcome Trust.

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<sup>3</sup> Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; BK, bradykinin; B1KO, B<sub>1</sub> receptor knockout; DABK, des-Arg<sup>9</sup>-BK; h, human; MPO, myeloperoxidase; WT, wild type.

chemokines have been identified to date 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 LPS-inducible CXC chemokine or LIX), and CXCL7 (also called neutrophil activating peptide-2 or NAP-2). Although good evidence supports a role for the kinin B<sub>1</sub> receptor in neutrophil recruitment (4, 5, 10), whether this is dependent upon ELR-CXCL chemokine expression and activity is unknown.

In this report, we demonstrate that the kinin B<sub>1</sub> receptor plays an essential role in IL-1 $\beta$ -induced neutrophil recruitment by using kinin B<sub>1</sub> 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 are likely produced by endothelial cells following direct activation of endothelial kinin B<sub>1</sub> receptor. Because kinin B<sub>1</sub> 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 B<sub>1</sub> receptor-CXCL5 pathway as a novel therapeutic target.

## Materials and Methods

### Animals

All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Male C57BL/6J wild-type (WT) or B1KO (10) mice (C57BL/6 background) at 5 wk of age (10–15 g) were used in all experiments.

### Intravital microscopy

Male WT and B1KO mice received either murine IL-1 $\beta$  (5 ng/mouse, i.p.; PeproTech) or a saline vehicle. After 4 h mice were anesthetized with diazepam (60 mg/kg, s.c.) and Hypnorm (0.7 mg/kg fentanyl citrate and 20 mg/kg fluanisone, i.m.), and the mesenteric vascular bed was prepared for viewing by intravital microscopy. Mesenteries were superfused with bicarbonate-buffered solution (132 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 17.9 mM NaHCO<sub>3</sub>, and 2.0 mM CaCl<sub>2</sub> (pH 7.4), gassed with 5% CO<sub>2</sub> and 95% N<sub>2</sub>) at 37°C at a rate of 2 ml/min. The temperature of the stage was maintained at 37°C. The extent of the inflammatory response elicited by IL-1 $\beta$  was analyzed by counting the number of white blood cells rolling per minute. Cell adhesion was quantified by counting, for each vessel, the number of adherent neutrophils in a 100- $\mu$ m length, and leukocyte emigration from the microcirculation into the tissue was quantified by counting the number of cells that had emigrated up to 50  $\mu$ m away from the wall of 100- $\mu$ m vessel segments. Venular blood flow was calculated from the product of mean RBC velocity ( $V_{\text{mean}}$  = centerline velocity/1.6) and microvascular cross-sectional area, assuming a cylindrical geometry. Wall shear rate was calculated by the Newtonian definition: shear rate =  $8,000 \times (V_{\text{mean}}/\text{diameter})$ . A minimum of three postcapillary venules (diameter between 20 and 40  $\mu$ m; length of at least 100  $\mu$ m) were observed for each mouse. To evaluate the role of CXCL5 in regulating leukocyte recruitment, a selective neutralizing mAb or control IgG (20  $\mu$ g per animal, i.p.) was injected into the tail vein 30 min before IL-1 $\beta$  treatment. After 4 h, 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 4 h after IL-1 $\beta$  treatment, was homogenized in 1 ml of a 0.5% hexadecyltrimethylammonium bromide in MOPS buffer (10 mM, pH 7). After homogenization, samples were centrifuged at  $4000 \times g$  for 20 min at 4°C and the supernatant was collected for

determination of MPO levels as previously described (15). Data are expressed as units per gram of total protein content in the tissue as 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-1 $\beta$ -treated (for 2 h) mice as described above, snap frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until use. Samples were homogenized and total RNA was isolated using a NucleoSpin RNA II purification kit (Macherey-Nagel) and then stored at  $-80^{\circ}\text{C}$  until use. cDNA was synthesized from 1  $\mu$ g of total RNA with Moloney murine leukemia virus reverse transcriptase (Promega) using oligo(dT) nucleotides. The following primers were used for mouse: CXCL1, 5'-TGAGCTGCGCTGTCAGTGCCCT-3' and 5'-AGAA GCCAGCGTTCACCAGA-3'; CXCL2, 5'-GAGCTTGAGTGTGACGCC CCCAGG-3' and 5'-GTTAGCCTTGCCCTTTGTTTCAGTATC-3'; CXCL5, 5'-GCATTTCTGTTGCTGTTACAGCTG-3' and 5'-CCTCTTCTGTTT TTAGAGTTAGC-3'; CXCL7, 5'-TGGGCTGATCCTTGTGCGC-3' and 5'-GCACCGTTTTTGTCCATTCTTCAG-3'; B<sub>1</sub> receptor, 5'-TGGA GTTGAACGTTTTGGGTTT-3' and 5'-GTGAGGATCAGCCCCATT GT-3'; and  $\beta$ -actin, 5'-GAAATCGTGCCTGACATCAAAG-3' and 5'-TG TAGTTTCATGGATGCCACAG-3'. Standard curves for these molecules were generated to determine the amplification efficiencies of target and reference genes. Quantitative PCR was performed on an ABI Prism 7900 sequence detection system with 100 nM primers and 20 ng of cDNA. Chemokine/receptor expression was normalized to  $\beta$ -actin and expressed as a relative value using the comparative threshold cycle (Ct) method ( $2^{-\Delta\Delta C_t}$ ) according to the manufacturer's instructions. The levels of mRNA expression of genes of interest were normalized to saline control.

### Measurement of CXCL5 protein expression

Mesenteric tissue of saline and IL-1 $\beta$ -treated (for 4 h) WT and B1KO mice was collected and homogenized and supernatants were 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.

### HUVEC culture

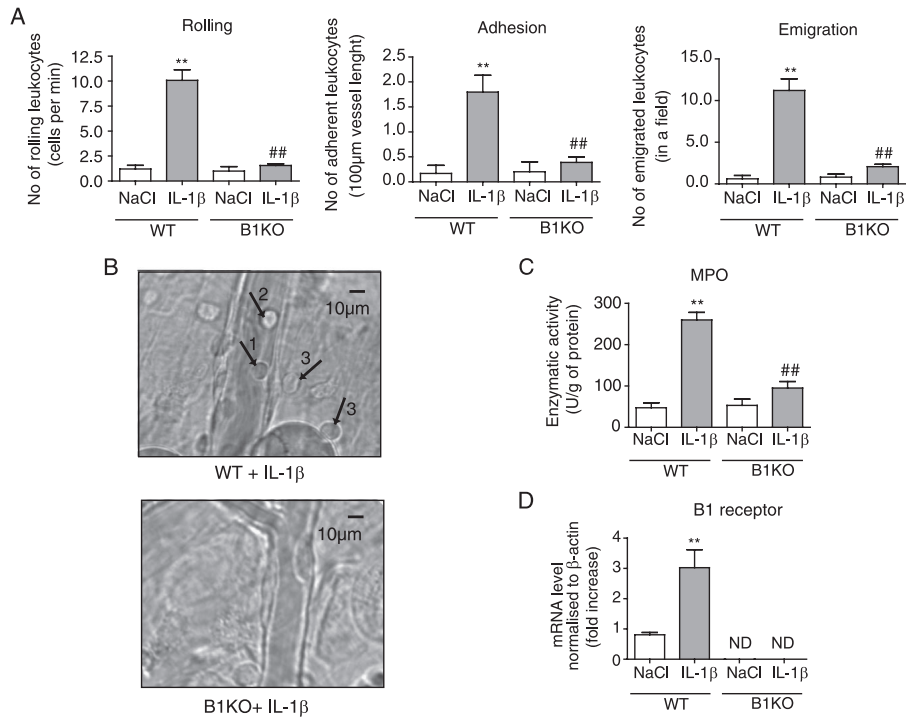
HUVECs were cultured to passage 3 in EGM-2 endothelial cell medium (Cambrex/Lonza). Confluent cells were treated with vehicle (saline) or IL-1 $\beta$  (1 ng/ml for 0–24 h). In some experiments, the B<sub>1</sub> antagonist Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-BK (10  $\mu$ M) was added to the medium 15 min before IL-1 $\beta$  treatment and the reaction was stopped after 8 h. In a further series of experiments, cells were incubated with the B<sub>1</sub> agonist Lys-des-Arg<sup>9</sup>-BK (1–10,000 nM) for 4 h either directly or following a 24-h pretreatment with IL-1 $\beta$ .

### Real-time quantitative RT-PCR of endothelial cells

HUVECs from the above experiments were washed with sterile PBS and collected by scraping, and samples were kept at  $-80^{\circ}\text{C}$  until mRNA extraction. The human (h) CXCL5 homologues hCXCL5 and hCXCL6 and kinin B<sub>1</sub> receptor mRNA expression were determined as described above. The following primers were used: hCXCL5, 5'-GAGAGCTGCGTTGCGT TTG-3' and 5'-TTTCCTTGTTCACCCGTTCCA-3'; hCXCL6 5'-GGTC CTGTCTCTGTGTGC-3' and 5'-GGGAGGCTACCACTTCCA-3'; hB<sub>1</sub> receptor, 5'-ACGCCTTCATTTTCTGCCTG-3' and 5'-GCTGGCTCTG GTTGGAGGAT-3'; kininogen, 5'-AGACACGGCATTACAGTACTTTAA CA-3' and 5'-TGGGCCCGTTTTACTTCATT-3'; kallikrein, 5'-GGGTCC CACAACCTGTTG-3' and 5'-GCTGTAGTCCCTGCTGCTT-3'; and GAPDH, 5'-CATGTTTCGTCATGGGTGTGAA-3' and 5'-ATGGACTG TGGTCATGAGTCCTT-3'.

### Western blotting of endothelial cells

Following the treatment outlined above, cells were washed with ice-cold PBS, scraped, and lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 5 mM EDTA, and 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>·10H<sub>2</sub>O). Supernatants were collected and protein concentration was determined by Bradford assay. Samples (20  $\mu$ g) were subjected to electrophoresis using an 8% polyacrylamide gel followed by electrotransfer to a nitrocellulose membrane. To detect B<sub>1</sub> receptor, nitrocellulose were incubated with the polyclonal B<sub>1</sub> receptor Ab (A15C 16), a gift of Dr. J.-L. Bascands; dilution 1/5000) overnight at 4°C and then with a secondary peroxidase-coupled goat anti-rabbit Ab (dilution 1/2000; DakoCytomation). Visualization of bands was achieved by chemiluminescence (ECL kit; Amersham Biosciences). Selectivity of the Ab was determined by preadsorption of the Ab to its corresponding peptide at a concentration of 10  $\mu$ g/ml at 4°C overnight. The autoradiographic bands were semiquantified and normalized to  $\alpha$ -tubulin levels.



**FIGURE 1.** IL-1 $\beta$ -induced PMN recruitment is abolished in B1KO mice. *A*, Leukocyte-endothelial cell interactions in mouse mesenteric postcapillary venules in vivo in response to IL-1 $\beta$  (5 ng, 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 4 h after treatment with saline (i.p.) or IL-1 $\beta$ . *B*, The arrows show rolling (point 1), adherent (point 2), and emigrated (points 3) cells. *C*, PMN accumulation was determined by measurement of MPO activity. Mesenteric tissue were removed from WT and B1KO mice 4 h following treatment with saline or IL-1 $\beta$  (5 ng, i.p.), and 25 ng of protein extract from each animal was used to measure MPO activity. The data are expressed as the unit of MPO per gram of total protein. *D*, The inducibility of B<sub>1</sub> receptor mRNA expression in mesenteric tissue in response to IL-1 $\beta$  (5 ng, i.p.) was assessed in WT and B1KO mice by quantitative real-time RT-PCR. The data are expressed as fold increase compared with control (WT NaCl) normalized to  $\beta$ -actin. Data are mean  $\pm$  SEM for  $n = 6$  animals per group. \*\*,  $p < 0.01$ , saline vs treated values; \*\*\*,  $p < 0.01$ , WT vs B1KO values.

*Statistical analysis*

Values are given as means  $\pm$  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 two groups or one-way ANOVA for more than two groups. Differences were considered significant when  $p < 0.05$ .

**Results**

*IL-1 $\beta$ -induced PMN recruitment is absent in B1KO mice*

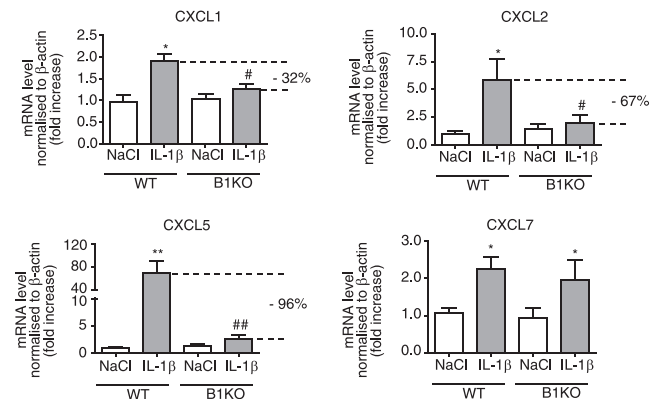
IL-1 $\beta$  caused a significant increase of mesenteric B<sub>1</sub> receptor mRNA expression (Fig. 1D) that was associated with a pronounced cellular recruitment in WT mice as indicated by the augmentation of leukocyte rolling, adhesion, and emigration (Fig. 1, A and B). All parameters of IL-1 $\beta$ -induced leukocyte recruitment were profoundly suppressed in B1KO mice, and this was likewise associated with a complete absence of B<sub>1</sub> receptor mRNA expres-

sion (Fig. 1D). These differences were not due to changes in venular hemodynamics, because there were no significant differences in venule diameter or blood flow between WT and B1KO animals (see Table I). IL-1 $\beta$ -induced cellular recruitment in WT animals was associated with a  $\sim$ 3-fold increase in MPO activity that was markedly attenuated in B1KO mice (Fig. 1C).

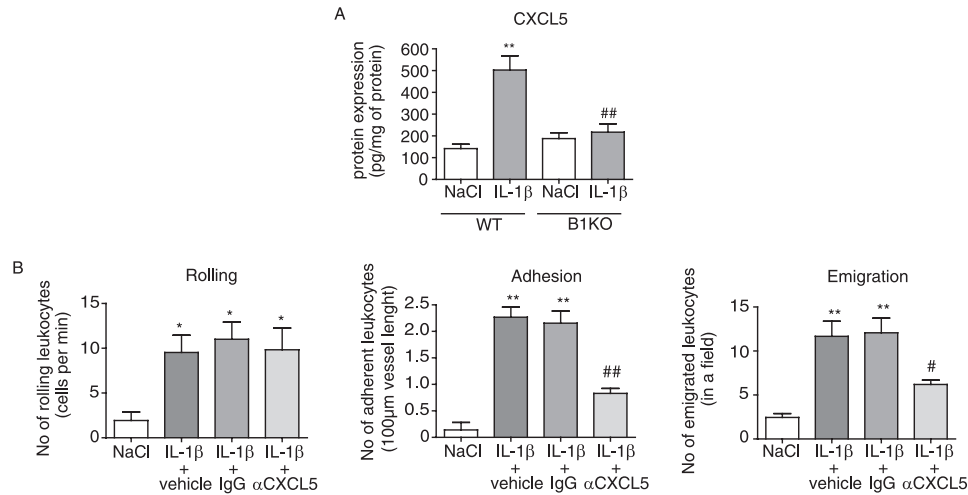
**Table I.** Hemodynamic parameters in animals used for intravital microscopy studies<sup>a</sup>

Genotype	Treatment	No. of Venules	Venule Diameter ( $\mu$ m)	Shear Rate (s <sup>-1</sup> )
WT	Saline (4 h)	12	32.2 $\pm$ 3.3	254 $\pm$ 31
WT	IL-1 $\beta$ (4 h)	18	31.1 $\pm$ 2.9	276 $\pm$ 27
B1KO	Saline (4 h)	10	29.7 $\pm$ 4.2	235 $\pm$ 34
B1KO	IL-1 $\beta$ (4 h)	16	31.8 $\pm$ 3.7	287 $\pm$ 25

<sup>a</sup> Mice received either saline (100  $\mu$ l, i.p.) or IL-1 $\beta$  (5 ng, i.p.). Data are mean  $\pm$  SEM.



**FIGURE 2.** IL-1 $\beta$ -induced ELR-CXCL chemokine mRNA expression is attenuated in B1KO mice. mRNA expression of CXCL1, CXCL7, CXCL2, and CXCL5 was assessed using quantitative real-time RT-PCR of mesenteric tissue from WT and B1KO mice treated with saline or IL-1 $\beta$  (5 ng, i.p.; 2 h). The data are expressed as fold increase above control (WT NaCl) normalized to  $\beta$ -actin. Data shown are mean  $\pm$  SEM for  $n = 6$  animals per group. \*\*,  $p < 0.01$ , saline vs treated values; \*\*\*,  $p < 0.01$ , WT vs B1KO values; \*,  $p < 0.05$ , saline vs treated values; #,  $p < 0.05$  WT vs B1KO values.



**FIGURE 3.** CXCL5 plays a major role in IL-1 $\beta$ -induced leukocyte recruitment. *A*, CXCL5 protein was measured by ELISA in mesenteric tissue removed from WT and B1KO mice 4 h after treatment with saline or IL-1 $\beta$  (5 ng, i.p.). The data are expressed as CXCL5 per milligram of total protein (pg/mg protein). Data shown are mean  $\pm$  SEM for  $n = 6$  animals per group. \*\*,  $p < 0.01$ , saline vs treated values; ##,  $p < 0.01$  WT vs B1KO values. *B*, Leukocyte-endothelial cell interactions in WT mouse mesenteric postcapillary venules were measured by intravital microscopy in vivo 4 h following treatment with IL-1 $\beta$  (5 ng, i.p.) in mice preinjected (30 min before IL-1 $\beta$ ) with saline, control IgG, or anti-CXCL5 Ab (20  $\mu$ g/mice, i.p.). Rolling, adhesion, and emigration of leukocytes were measured. Data shown are mean  $\pm$  SEM for  $n = 6$  animals per group. \*\*,  $p < 0.01$ , saline vs treated values; ##,  $p < 0.01$ , WT vs treated values; \*,  $p < 0.05$ , saline vs treated values; #,  $p < 0.05$ , WT vs treated values.

#### CXCL5 expression is abolished in B1KO mice

Quantitative PCR of the mesenteric tissue of IL-1 $\beta$ -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-1 $\beta$ -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 up-regulated in WT mice, was not changed in B1KO mice.

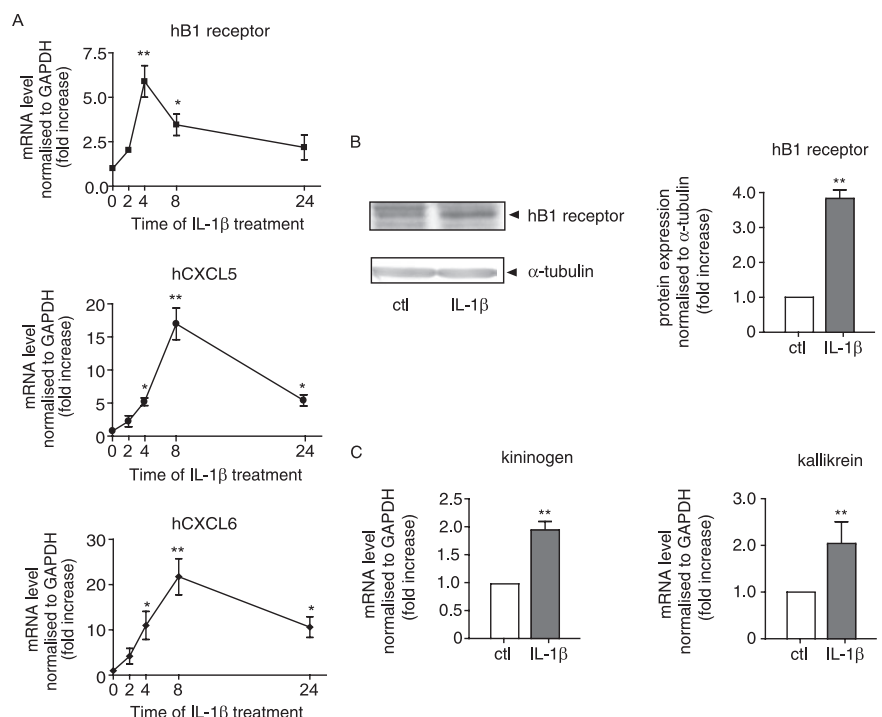
Because CXCL5 appeared to be the most profoundly suppressed ELR-CXCL chemokine in B1KO mice (Fig. 2), we sought to as-

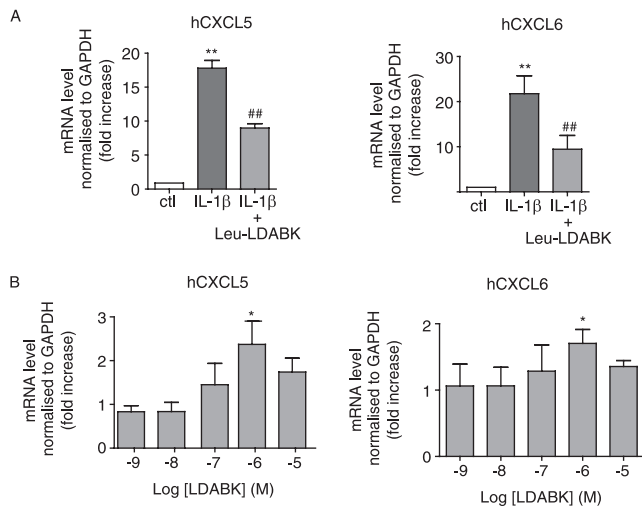
certain whether the changes in mRNA were reflected in protein expression. CXCL5 protein expression in the mesenteries of IL-1 $\beta$ -treated WT mice was substantially elevated compared with saline control ( $503 \pm 66$  pg/mg protein,  $n = 6$  vs  $142 \pm 20$  pg/mg protein,  $n = 5$ ;  $p < 0.01$  respectively). In contrast, this response to IL-1 $\beta$  was entirely absent in B1KO mice ( $218 \pm 38$  pg/mg protein,  $n = 5$  vs  $188 \pm 27$  pg/mg protein,  $n = 6$ ; non significant) (Fig. 3A).

#### Neutralization of CXCL5 reduces IL-1 $\beta$ -induced leukocyte adhesion and emigration

Treatment of WT mice with a neutralizing anti-CXCL5 Ab inhibited IL-1 $\beta$ -induced cell adhesion and emigration by ~50%, but no

**FIGURE 4.** Temporal relationship between endothelial kinin B<sub>1</sub> receptor, hCXCL5, hCXCL6, and kallikrein/kininogen expression in IL- $\beta$ -treated endothelial cells. *A*, Time course (0, 4, 8, and 24 h) of B<sub>1</sub> receptor, hCXCL5, and hCXCL6 mRNA expression in response to IL-1 $\beta$  (1 ng/ml) in HUVECs. Expression of B<sub>1</sub> receptor, hCXCL5, and hCXCL6 were measured by quantitative real-time RT-PCR. *B*, Protein expression of B<sub>1</sub> receptor in HUVECs after 8 h of IL-1 $\beta$  treatment by Western blotting. *C*, mRNA expression of kininogen and kallikrein in HUVECs after 4 h of IL-1 $\beta$  treatment was measured by quantitative real-time RT-PCR. The data are expressed as the fold increase above control (ctl; nontreated cells) normalized to GAPDH for mRNA and to tubulin for protein. Data shown are mean  $\pm$  SEM for  $n = 4$ . \*,  $p < 0.05$ , control vs treated values; \*\*,  $p < 0.01$ , control vs treated values.





**FIGURE 5.** Kinin B<sub>1</sub> receptor-induced hCXCL5 and hCXCL6 expression in HUVEC. *A*, Effect of B<sub>1</sub> antagonist (Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-BK at 10 μM; 15-min pretreatment at *t* = 0) on IL-1β (1 ng/ml, 8 h)-induced hCXCL5 and hCXCL6 mRNA expression in HUVECs. *B*, Effect of B<sub>1</sub> agonist (Lys-des-Arg<sup>9</sup>-BK, 4 h; 1–10,000 nM) on hCXCL5 and hCXCL6 mRNA expression. The data are expressed as the fold increase above control (ctl; nontreated cells) normalized to GAPDH for mRNA. Data are mean ± SEM for *n* = 4. \*\*, *p* < 0.01, saline vs treated values; \*, *p* < 0.05, saline vs treated values; ##, *p* < 0.01, IL-1β vs IL-1β plus Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-BK values.

significant effect on cell rolling was observed at the 4-h time point (Fig. 3*B*). In contrast, control rabbit IgG had no significant effect on leukocyte recruitment.

#### Human CXCL5 homologues are regulated by B<sub>1</sub> receptor in human endothelial cells

Because previous evidence suggests that endothelial cells express B<sub>1</sub> receptor following exposure to inflammatory stimuli (5, 17–19) and because the endothelial cell is an important cellular source of chemoattractant (20–22), we investigated whether the endothelial cell might be an *in vivo* source of B<sub>1</sub> receptor-induced chemokine production. In HUVEC, kinin B<sub>1</sub> receptor mRNA was induced within 2 h of IL-1β treatment, peaked at 4 h (~5-fold increase), and remained significantly elevated at 8 h, returning to near basal levels by 24 h (Fig. 4*A*). This IL-1β-induced change in mRNA was associated with increases in kinin B<sub>1</sub> receptor protein expression (Fig. 4*B*). 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. 4*C*). Basal levels of chemokines were low in unstimulated HUVECs; however, treatment with IL-1β caused a time-dependent increase in hCXCL5 homologue (hCXCL5 and hCXCL6) mRNA expression, peaking at 8 h but with a lag time of 2–4 h relative to kinin B<sub>1</sub> receptor expression (Fig. 4*A*). B<sub>1</sub> receptor blockade significantly suppressed IL-1β-induced hCXCL5 and hCXCL6 expression at the 8 h time point by ~50% (Fig. 5*A*). Treatment of cells with a B<sub>1</sub> agonist produced concentration-dependent increases in hCXCL5 and hCXCL6 expression (Fig. 5*B*) in control cells. In addition, in cells pretreated with IL-1β for 24 h the application of a B<sub>1</sub> agonist produced a further 1.3 ± 0.07-fold increase (*n* = 4; *p* < 0.05, *t* test compared with IL-1β alone) in hCXCL5 expression above that induced by IL-1β alone.

## Discussion

We have previously demonstrated that the kinin B<sub>1</sub> receptor plays an important role in mediating the recruitment of PMNs to a site of

inflammation (4, 5), a finding that was supported by the recent observation that cell-dependent inflammation is reduced in B1KO mice (10). However, the exact mechanisms involved in this response were unclear. Our findings, in this report, support the thesis that B<sub>1</sub> 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 the induction and subsequent endogenous activation of kinin B<sub>1</sub> receptors on endothelial cells is likely to play a major role in B<sub>1</sub> receptor-induced CXCL5 expression.

Treatment of WT mice with IL-1β induced a substantial elevation of mesenteric B<sub>1</sub> receptor mRNA expression. These observations are in accordance with our previous findings demonstrating low levels of B<sub>1</sub> receptor mRNA expression in control untreated murine mesenteric tissue but profound induction of expression following exposure to inflammatory stimuli (5). In contrast, no B<sub>1</sub> receptor mRNA expression was evident in the mesenteries of B1KO mice, either in the controls or following cytokine treatment. This absence of B<sub>1</sub> receptor expression in B1KO mice had a major impact on the magnitude of the inflammatory response to IL-1β treatment. Indeed, whereas 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 because all hemodynamic parameters were similar between the two genotypes. We chose to use the mesenteric preparation with a 4-h IL-1β treatment because we have previously established that the B<sub>1</sub> receptor plays a major role in mediating leukocyte recruitment in the mouse mesentery at this time point and that this is associated with B<sub>1</sub> receptor mRNA expression (5). That the B<sub>1</sub> receptor is essential in this response is, of itself, an important observation because 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 estimate of the magnitude of the kinin B<sub>1</sub> receptor-mediated component (using B<sub>1</sub> receptor antagonists) of the cell recruitment response to IL-1β, in the order of 50%, was a substantial underestimate (4, 5). This may have been due to the fact that the antagonists, des-Arg<sup>9</sup>-[Leu<sup>8</sup>]-BK and des-Arg<sup>10</sup>-Hoe140, 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 B<sub>1</sub> 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 B<sub>1</sub> receptor in experimental models of inflammatory disease in B1KO mice, including diabetes (28), neuropathic pain (29), inflammatory hyperalgesia (10), and intestinal ischemia/reperfusion injury (30).

The innate immune response is a tightly orchestrated sequence of events; each stage is associated with the recruitment of a specific inflammatory cell type 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, CXCL2, CXCL5, and CXCL7 (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, although the human chemokine hCXCL8 (also known as IL-8) binds this receptor, no specific endogenous murine chemokine ligand has been identified. IL-1 $\beta$  treatment of WT mice was associated with enhanced expression of all four murine of the CXCL chemokines measured. Moreover, our studies exposed a temporal and sequential relationship between kinin B<sub>1</sub> 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-1 $\beta$ -induced transcription of CXCL5 and substantial inhibition of CXCL1 and CXCL2 expression with no effect on CXCL7 expression. The association between B<sub>1</sub> receptor activation and CXCL chemokine production is supported by previous work, albeit in different cells and with a different chemokine, where B<sub>1</sub> receptor activation of human fibroblasts stimulated hCXCL8 production (35).

Of the chemokines linked to B<sub>1</sub> receptor activation, CXCL5 appears to be the most closely associated because its production in B1KO mice was almost abolished, suggesting that perhaps it is this chemokine that predominantly mediates the effects of B<sub>1</sub> 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 although basal levels of CXCL5 mRNA are very low in WT animals, IL-1 $\beta$  treatment causes a ~70-fold increase in expression, a response inhibited by >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 the measurement of CXCL5 by ELISA. The observation that the neutralizing Ab to CXCL5 significantly attenuated leukocyte recruitment to IL-1 $\beta$  supports the thesis that CXCL5 plays a major role in mediating the cellular response. However, the lack of effect of the Ab on leukocyte rolling suggests that perhaps the role of CXCL5 is centered on the adhesion and emigration steps of leukocyte recruitment. It is important to note, however, that although the CXCL5 Ab did not affect leukocyte rolling, IL-1 $\beta$ -induced rolling was abolished in B1KO mice. These findings suggest that B<sub>1</sub> receptor activation results in the stimulation of other pathways involved in cell rolling that are unrelated to chemokine synthesis. An obvious pathway that is likely to be implicated is the adhesion molecule pathway, specifically either at the level of the 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-1 $\beta$  (20, 36). Because our previous studies excluded the possibility that B<sub>1</sub> receptor-induced cell recruitment is due to the direct activation of B<sub>1</sub> receptors on neutrophils (4), we hypothesized that the endothelial cell might be an important cellular source of B<sub>1</sub> receptor-induced CXCL5 production. However, in contrast to this thesis a recent publication has demonstrated that the direct activation of neutrophil B<sub>1</sub> receptors does cause neutrophil migration (41). This response was only ev-

ident in IL-1 $\beta$  pretreated cells in vitro and required a 24-h exposure to the B<sub>1</sub> agonist. Because we have shown that the B<sub>1</sub> receptor-dependent IL-1 $\beta$ -induced leukocyte recruitment response in vivo is evident after 2 h and peaks at 4 h (4, 5), it is unlikely that the slowly developing direct activation of neutrophil B<sub>1</sub> receptors contributes to the response evident in the current study. It is possible, however, that at later stages of the inflammatory response the direct activation of neutrophilic B<sub>1</sub> 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 B<sub>1</sub> receptor, as has been demonstrated previously (17, 19). In addition, like others (18, 43) we have shown that the elements of the kallikrein-kinin system necessary for endogenous B<sub>1</sub> 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 the elevation in expression of the B<sub>1</sub> receptor. These findings intimate that endogenous endothelial B<sub>1</sub> receptor activation might support the chemokine production evidenced in vivo.

Indeed, the treatment of human endothelial cells with IL-1 $\beta$  stimulated a time-dependent increase in B<sub>1</sub> receptor expression that was subsequently followed by a pronounced stimulation of hCXCL5 and hCXCL6 expression. This chemokine synthesis was likely to be a consequence of B<sub>1</sub> receptor activation, because the treatment of cells with the B<sub>1</sub> antagonist, Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-BK, significantly reduced this response. Moreover, this finding is supported by the observation that the treatment of endothelial cells with a B<sub>1</sub> agonist stimulated a concentration-dependent increase in hCXCL5 and hCXCL6 expression. The level of this enhanced expression, while significant, was at least 10-fold lower than that evident in vivo. This lower level of induction may have been related to the level of basal B<sub>1</sub> receptor expression in cells not stimulated with cytokine. Therefore, we investigated the activity of the B<sub>1</sub> agonist in IL-1 $\beta$ -treated cells. The relative increase in chemokine expression in B<sub>1</sub> agonist-treated cells vs cytokine-only treated cells, surprisingly, appeared similar to that in unstimulated cells. However, it is important to note that the 24 h following IL-1 $\beta$ -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 B<sub>1</sub> receptor activity synergizes/interacts with a blood-borne factor not present in these in vitro experiments. Finally, although it is clear that the endothelial cell is a major source of B<sub>1</sub>-induced CXCL5/CXCL6, we cannot exclude the possibility that other cell types within the vasculature might also be sources of this B<sub>1</sub>-induced chemokine production. In particular, both the fibroblast (44) and the mast cell (45) are cellular sources of CXCL5 and are cells that also express the B<sub>1</sub> receptor (7, 46).

The molecular mechanisms involved in this B<sub>1</sub> receptor-induced chemokine expression are uncertain; however, the transcription factor NF- $\kappa$ B, a pivotal transcriptional factor regulating inflammatory gene expression (47), has been identified as playing an essential role in IL-1 $\beta$ -induced hCXCL5 (48) or hCXCL6 (49) expression in human nonvascular cell types. Similarly, NF $\kappa$ B plays an essential role in mediating IL-1 $\beta$ -induced CXCL5 expression in mice (20). This regulation by NF- $\kappa$ B is of interest because B<sub>1</sub> receptor expression itself is also tightly regulated by a NF- $\kappa$ B-dependent pathway (16). This group demonstrated that

the application of a B<sub>1</sub> agonist to human fibroblasts enhanced B<sub>1</sub> receptor expression as a consequence of NF- $\kappa$ B activation, auto-regulation that is more pronounced in the presence of IL-1 $\beta$  (35). In addition, B<sub>1</sub> receptor activation itself also stimulates further IL-1 $\beta$  synthesis (50), suggesting a complex facilitatory interaction between the B<sub>1</sub> receptor and IL-1 $\beta$  that may play an important role in amplification of the inflammatory response especially because the kallikrein-kinin pathway is up-regulated at inflammatory sites, increasing endogenous B<sub>1</sub> agonist production (51). Together, these studies suggest that, during an inflammation following the initial induction of the kinin B<sub>1</sub> receptor by the appropriate inflammatory stimulus, the pathway may be continuously self-amplified to sustain the inflammatory response.

In summary, although the B<sub>1</sub> receptor has been proposed to play a role in inflammatory pathologies (7, 8, 10), its exact contribution to the inflammatory process has been uncertain. The findings from this study have allowed clarification of the key role of the kinin B<sub>1</sub> 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 B<sub>1</sub> receptor-CXCL5 pathway. These results, taken together with the observations that B<sub>1</sub> receptor expression is induced by inflammation in different diseases, endogenous B<sub>1</sub> agonist concentration increases at sites of inflammation, and B<sub>1</sub> receptor activation causes a range of cellular proinflammatory effects, highlights the B<sub>1</sub> receptor and, in particular, this novel B<sub>1</sub> receptor-CXCL5 pathway as potential therapeutic targets for inflammatory disease.

## Disclosures

The authors have no financial conflict of interest.

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