



## SPECIAL REPORT

# Impaired IL-1 $\beta$ -induced neutrophil accumulation in tachykinin NK<sub>1</sub> receptor knockout mice

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Tachykinin NK<sub>1</sub> receptors play an important role in the development of neurogenic inflammatory responses. We have used the murine air-pouch model to investigate whether the neurogenic component of the cellular inflammatory response to interleukin-1 $\beta$  (IL-1 $\beta$ , 10 ng into the air-pouch) is altered in NK<sub>1</sub> receptor knockout mice compared to wild type controls. Air-pouches were washed following a 4 h IL-1 $\beta$  treatment, the wash collected and neutrophil number estimated using a Neubauer haemocytometer. The response to IL-1 $\beta$  was significantly attenuated in NK<sub>1</sub> receptor +/– (40% reduction) and –/– mice (62% reduction) compared to wild type controls (+/+), whilst the response to cytokine-induced neutrophil chemoattractant (CINC, 0.3  $\mu$ g) was unaffected. The response to substance P (7.5 nmol) was attenuated by approximately 50% in both NK<sub>1</sub> receptor +/– and –/– mice compared to wild type controls. In conclusion NK<sub>1</sub> receptors play a significant role in the cellular response to IL-1 $\beta$  in a model of inflammation.

**Keywords:** Substance P; inflammation; sensory C-fibre; chemokine

**Introduction** Inflammatory responses are complex processes involving several interrelated cellular and non-cellular components. In general most inflammatory responses possess a neurogenic component and this is often mediated by a particular subset of nerve fibres: sensory C-fibres (Holzer, 1991). The inflammation produced following sensory C-fibre activation has been proposed to be mediated by several distinct neuropeptides and non-peptidic substances. Particular mediators thought to play a prominent role in such inflammatory responses are the tachykinins, substance P (SP) and neurokinin A (NKA). SP produces its actions, in the large part, by interaction with the tachykinin NK<sub>1</sub> receptor (see Maggi *et al.*, 1993). Accordingly many of the inflammatory actions of endogenously released or exogenously applied SP are blocked by antagonists for this receptor (see Maggi *et al.*, 1993). There are three distinct tachykinin receptors NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub>. These receptors have been defined according to the rank order of potency of the natural tachykinins. As expected all the natural tachykinins interact with all of these receptors however they may be differentiated according to preferential potency i.e. SP is the most potent at NK<sub>1</sub> receptors, NKA at NK<sub>2</sub> and neurokinin B (NKB) at NK<sub>3</sub>. In the large part when tachykinins are implicated in inflammatory responses the effects are mediated via NK<sub>1</sub> receptors. Whilst this is suggestive of the involvement of SP one cannot firmly exclude the possible involvement of NKA or NKB.

A feature of the inflammatory response is the migration and accumulation of polymorphonuclear leukocytes (PMN) including neutrophils. Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a pro-inflammatory cytokine known to be involved in this process and causes neutrophil migration when injected into specific tissue sites (Rampart & Williams, 1988; Pettipher *et al.*, 1986). Using a murine air-pouch model the mechanisms of the neutrophil chemoattractant activity of IL-1 $\beta$  has been studied in depth. It has been shown that local administration of IL-1 $\beta$  into the air-

pouch causes a dose- and time-dependent PMN migration that is blocked by the recombinant IL-1 receptor antagonist and actinomycin D (Perretti & Flower, 1993), indicating activation of IL-1 receptors and *de novo* RNA synthesis. In addition we have demonstrated that this effect of IL-1 $\beta$  involves a neurogenic component since treatment with capsaicin, a selective neurotoxin for C-fibres, attenuated the neutrophil accumulation indicating activation of these fibres (Perretti *et al.*, 1993). Furthermore tachykinins are important mediators of this neurogenic component since blockade of NK<sub>1</sub> receptors, with selective antagonists, attenuated the response to IL-1 $\beta$  in a fashion similar to that achieved with capsaicin. We now provide evidence supporting the premise that NK<sub>1</sub> receptor activation is involved in mediation of the inflammatory cellular response to IL-1 $\beta$  using NK<sub>1</sub> receptor knockout mice.

**Methods** *Animals* Tachykinin NK<sub>1</sub> receptor knockout mice were generated as described by De Felipe and coworkers (De Felipe *et al.*, 1998). A genomic DNA clone containing exon 1 of the mouse NK<sub>1</sub> receptor gene was isolated by screening a 12001 mouse 129 library with a rat NK<sub>1</sub> cDNA probe, clones were developed and two targeted clones were injected into C57BL/6 blastocysts and chimaeric males were mated with C57BL/6 females. Finally, mice homozygous for the NK<sub>1</sub> mutation were produced by crossing heterozygotes. Experiments were carried out using both male and female NK<sub>1</sub> –/+ and –/– mice and compared to responses in the wild type controls (C57BL/6).

For estimation of responses to chemotactic agents air-pouches were created in the dorsal skin of mice (26–34 g), by s.c. injection of 2.5 ml of air on days 0 and 3 (Perretti & Flower, 1993). Pouches were ready for test on day 6.

*Stimulus-induced PMN migration* To determine whether NK<sub>1</sub> knockout mice show altered responses to inflammatory stimuli that induce neutrophil migration the following

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experiments were carried out: (i) IL-1 $\beta$  was used to induce cell migration (>90 % neutrophils) into the mouse air-pouch as previously described (Perretti & Flower, 1993). On day 6, 10 ng murine recombinant IL-1 $\beta$  was dissolved in 0.5 ml of 0.5 % carboxymethylcellulose (CMC) and injected into the pouches. Control mice received CMC alone. (ii) the cytokine-induced neutrophil chemoattractant (CINC) was used at the dose of 0.3  $\mu$ g in 0.5 ml of CMC per air-pouch as an alternative stimulus to IL-1 $\beta$ . The chemokine was dissolved in CMC and injected into pouches as previously reported (Harris *et al.*, 1996). (iii) SP (7.5 nmol) was dissolved in 0.5 ml of CMC and injected into air-pouches as previously reported. This dose of SP produces a neutrophil migration response comparable to IL-1 $\beta$  (10 ng) in normal mice (Perretti *et al.*, 1993).

In all cases air pouches were washed 4 h after administration of the stimulus with 2 ml of phosphate buffered saline (PBS) containing heparin (50 U ml<sup>-1</sup>) and EDTA (3 mM) and the samples collected. To establish the effects of the stimulus on cell migration the samples were centrifuged at 220 g for 15 min at 4°C and the pellets resuspended in 2 ml of PBS containing heparin and EDTA. PMN numbers were estimated by counting after specific staining with Turk's solution using a Neubauer haematocytometer.

**Drugs and solutions** Rat CINC (72 amino acids; molecular weight 8 kDa) was synthesized chemically in the liquid phase and generously supplied by Dr K Watanabe (Institute for Cytosignal Research, Tokyo, Japan). Murine recombinant IL-1 $\beta$  was a generous gift of Dr RC Newton (Du Pont-Merck, Glenolden, DE). SP was purchased from Sigma, Poole UK and CMC was obtained from BDH, UK.

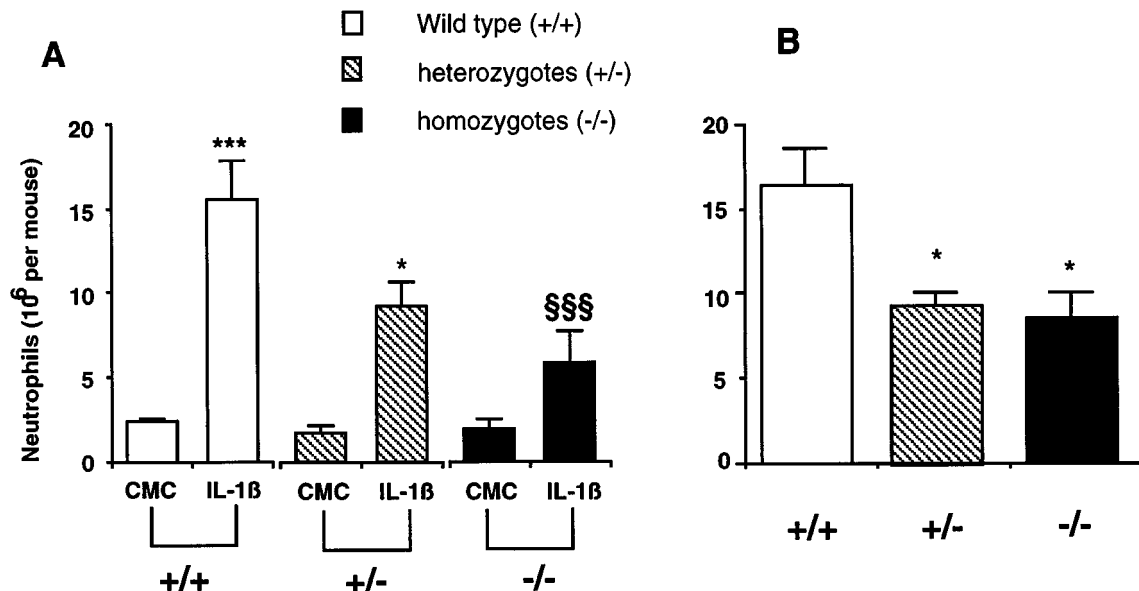
**Data and statistics** Neutrophil migration is expressed as the number of cells recovered from each animal. Data are

expressed as mean  $\pm$  s.e.mean of *n* mice per group. Statistical differences were assessed by ANOVA followed by Bonferroni for multiple comparisons. A *P* value <0.05 was considered significant.

**Results** Local injection of murine IL-1 $\beta$  into air-pouches of wild type (+/+) mice caused a significant (*P*<0.001) increase in the migration of neutrophils (*n*=9), measured at the 4 h time-point, above that achieved with the CMC alone (*n*=5) (Figure 1A). In heterozygous (+/-) mice, IL-1 $\beta$  (*n*=8) also induced a significant (*P*<0.05) increase in cell migration above that achieved by the vehicle alone (*n*=5). This response was approximately 60% of that achieved by IL-1 $\beta$  in wild type control mice, however this difference did not quite reach statistical significance. In contrast IL-1 $\beta$  (*n*=9) had no significant effect on cell number above vehicle controls (*n*=5) in homozygous mice (-/-). This response to IL-1 $\beta$  in NK<sub>1</sub> receptor -/- mice was significantly different to that achieved in the wild type controls (*P*<0.001). The responses to the vehicle alone in either the +/- or -/- mice were not significantly different from the response to vehicle in the wild type controls.

SP produced a profound neutrophil migration, to a similar degree as that achieved by IL-1 $\beta$  (10 ng), when injected locally into the air pouches of wild type mice (*n*=5) (Figure 1B). The response to SP in both heterozygous (*n*=5) and homozygous mice (*n*=5) was approximately 50% lower than and significantly different from (*P*<0.05 in each case) that achieved in wild type controls.

In a separate series of experiments CINC produced a similar significant (*P*<0.05) neutrophil migration above control in both wild type ( $1.8 \pm 0.44 \times 10^6$ , *n*=4) and -/- mice ( $1.4 \pm 0.13 \times 10^6$ , *n*=4), CMC control value being  $0.13 \pm 0.07 \times 10^6$  (*n*=3).



**Figure 1** IL-1 $\beta$  and substance P (SP) induced neutrophil migration is impaired in NK<sub>1</sub> receptor knockout mice. In (A) the bars represent the responses to the vehicle carboxymethylcellulose (CMC; 0.5%) and IL-1 $\beta$  (10 ng) and (B) the responses to substance P; in wild type mice (+/+; open bars), NK<sub>1</sub> receptor heterozygous mice (+/-; hatched bars) and NK<sub>1</sub> receptor homozygous mice (-/-; solid bars). The values shown are mean  $\pm$  s.e.mean of *n*=5–9 mice. In (A) statistical significance is shown by \*, \*\*\* (for *P*<0.05 and 0.001 respectively) for comparison of the response to IL-1 $\beta$  to the CMC control and §§§ (for *P*<0.001) for comparison of the response to IL-1 $\beta$  in -/- mice to the response in +/+ mice. In (B) statistical significance is shown by \* (for *P*<0.05) for comparison of the response to SP in +/+ mice in comparison to the response in either +/- or -/- mice. The CMC vehicle control in these experiments was  $2.4 \pm 0.2 \times 10^6$ ,  $1.7 \pm 0.4 \times 10^6$  and  $1.9 \pm 0.6 \times 10^6$  for +/+, +/- and -/- mice respectively.

**Discussion** This study demonstrates, for the first time, that neutrophil extravasation, one of the initial events in the host inflammatory response, is impaired in the absence of tachykinin NK<sub>1</sub> receptors. More specifically our data support the contention that tachykinins, and therefore activation of sensory C-fibres, plays an important role in IL-1 $\beta$ -induced neutrophil accumulation.

NK<sub>1</sub> knockout mice have recently become available (De Felipe *et al.*, 1998) as a new tool to test the role of tachykinin receptors in inflammatory conditions. It has already been demonstrated that neurogenic responses known to be associated with activation of sensory C-fibres and tachykinin activity at NK<sub>1</sub> receptors are significantly attenuated in the NK<sub>1</sub>  $-/-$  mice. For example, the nociceptive response associated with formalin injection and immune complex inflammation have been reported to be substantially reduced in NK<sub>1</sub>  $-/-$  mice over wild type controls (De Felipe *et al.*, 1998; Bozic *et al.*, 1996).

In agreement with previous studies local administration of the cytokine IL-1 $\beta$ , in wild type animals, produced a strong neutrophil influx into the murine air-pouch that was approximately 6.5-fold greater than the response to CMC vehicle alone (Perretti & Flower, 1993). This neutrophil migration was significantly impaired in the NK<sub>1</sub>  $-/-$  animals, thereby implicating tachykinins and NK<sub>1</sub> receptors in mediation of the cytokine response. These findings are in agreement with previous studies which showed that blockade of NK<sub>1</sub> receptors with selective antagonists reduced the cellular response to IL-1 $\beta$  by 50% (Perretti *et al.*, 1993) i.e. to a similar degree as that achieved in the NK<sub>1</sub>  $-/-$  mice. Thus whilst it is clear that NK receptors are involved in this process of neutrophil migration there are other mechanisms that also contribute to the overall cell accumulation to IL-1 $\beta$  in this model. Together these results support the hypothesis that IL-1 $\beta$ , through an as yet unknown mechanism, activates sensory C-fibres to cause the release of tachykinins, possibly SP, which acts via activation of NK<sub>1</sub> receptors to contribute to the cellular influx. How IL-1 $\beta$  produces its effect on these nerve types is unclear however studies using the same air-pouch model have demonstrated that the effect of the cytokine may

be through the induction of a kinin receptor, termed B<sub>1</sub> (Ahluwalia & Perretti, 1996). Interestingly, IL-1 $\beta$  superfusion of rat spinal cord slices produces a dose-dependent fast increase in electrically-evoked SP release that is blocked by IL-1 receptor antagonist (Malcangio *et al.*, 1996), suggesting that IL-1 $\beta$  may act directly on nerves to cause neuropeptide release. Whilst the exact mechanism of the effect of IL-1 $\beta$  is unknown it is clear that the response to local application of IL-1 $\beta$ , in this model, involves the release of sensory neuropeptides from nerves.

SP itself produced a significant neutrophil migration when injected locally into the air-pouch of wild type mice which was comparable to the magnitude of the response to IL-1 $\beta$ . SP-induced neutrophil influx was reduced by 50% in both NK<sub>1</sub>  $+/-$  and NK<sub>1</sub>  $-/-$  mice, indicating that the response to SP was mediated in part by activation of NK<sub>1</sub> receptors. It is very likely that the remainder of the response to the neuropeptide is consequent to activation of tissue mast cells *via* a non-receptor mechanism (Yano *et al.*, 1989).

This loss of responsiveness to IL-1 $\beta$  did not simply represent a decreased ability for these knockout mice to develop a cellular response since the response to the chemokine CINC was not significantly different from the response in the wild type controls. Again this is in agreement with previous work showing that neuropeptide receptor antagonists have no effect on the response to the chemokine interleukin-8 (Ahluwalia & Perretti, 1994). Thus knockout of NK<sub>1</sub> receptors in these mice does not result in a general defect in cellular migration but rather in a selective inhibition of the response to IL-1 $\beta$  and SP.

To conclude, whilst the mechanism of the relationship between IL-1 $\beta$  and presumably release of tachykinins, including SP, from nerve-endings is unknown it is clear that NK<sub>1</sub> receptors play an important role in the manifestation of the cellular component of an inflammatory response.

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