The role of CXCR2 activity in the contact hypersensitivity response in mice

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ABSTRACT. The recruitment of polymorphonuclear neutrophil leukocytes (PMN) into a challenge site, and their subsequent activation, are thought to play a role in the elicitation of the contact hypersensitivity (CHS) response. The present study investigated the role played by CXCR2 activity in tissue PMN infiltration and subsequent triggering of CHS. Our results show that the cutaneous infiltration by PMN, induced by hapten challenge was dramatically inhibited in sensitized, CXCR2-deficient ($CXCR2^{-/-}$) mice. Inhibition of PMN recruitment into the hapten-challenged ears of $CXCR2^{-/-}$ mice was associated with a consistent reduction of the CHS response (ear swelling) in $CXCR2^{-/-}$ mice as compared with that observed in neutropenic, wild-type ($CXCR2^{+/+}$) mice. Prevention of skin PMN infiltration and the ear swelling response by the absence of functional CXCR2 was observed regardless of the hapten used. These data clearly suggest that CXCR2 activity plays an essential role in mediating cutaneous recruitment and activation of PMN, and thus indirectly regulates recruitment of hapten-primed T cells into challenge sites, with the subsequent elicitation of the CHS response. The role played by CXCR2 activity in the CHS response provides the rationale for testing CXCR2 inhibitors as a new therapeutic approach to skin diseases.

Keywords: contact hypersensitivity, CXCR2, PMN recruitment

Contact hypersensitivity (CHS) is a T cell-mediated inflammatory response of the epidermis following skin challenge in hapten-sensitized animals [1]. Hapten-specific $CD8^+$ T cells are the primary effector cells that mediate the CHS response to different haptens, including 2,4-dinitrofluorobenzene (DNFB) and oxazolone (OX). Several studies have shown that sensitization with DNFB or OX induces polarized populations of hapten-specific T cells. Recruitment and activation of $CD8^+$ T cells into the challenge site results in proinflammatory cytokine production, including interferon (IFN)-γ and tumor necrosis factor (TNF)-α which cause the characteristic oedema/spongiosis associated with the CHS response [2-4].

Although recruitment of sensitized T cells and their production of cytokines are required to generate the inflammatory phase of CHS, considerable interest has been expressed concerning the ability of polymorphonuclear neutrophil leukocyte (PMN) infiltration to regulate elicitation of CHS. Indeed, selective depletion of PMN has been demonstrated to be effective in inhibiting the effector phase of CHS [5], and chemotactic factors produced by PMN are thought to mediate hapten-primed T cell infiltration into the hapten challenge site [6].

Interleukin-8 (IL-8/CXCL8), a member of the CXC chemokine family, is a chemoattractant of PMN. Two, high-affinity, human CXCL8 receptors are known, CXC chemokine receptor 1 (CXCR1) and CXC chemokine receptor 2 (CXCR2). Only one corresponding receptor has been identified in the mouse, CXCR2, and this is recognized by ligands including keratinocyte-derived chemokine (KC/CXCL1) and macrophage inflammatory protein 2 (MIP-2/CXCL2) that act as PMN attractants. However, a mouse orthologue of CXCL8 has not been identified [7-9]. Among the chemotactic factors, CXC chemokines are thought to be involved in directing PMN recruitment to hapten challenge sites during elicitation of CHS [5, 10, 11]. On the other hand, a possible link between C5a chemotactic activity and PMN infiltration in the elicitation of CHS has also been hypothesized [12].

The aim of our study was to investigate the mechanism of PMN recruitment and the subsequent regulation of the CHS response in the hapten challenge site. In particular, we focused our attention on the role played by CXCR2 activity in PMN recruitment and in the elicitation of the CHS response. To this end, we induced a CHS response in CXCR2-deficient ($CXCR2^{-/-}$) mice. $CXCR2^{-/-}$ mice are

reported to develop normally but their PMN are unable to respond chemotactically to CXCL1 and CXCL2 as compared with their wild-type ($CXCR2^{+/+}$) littermates [13].
Our results indicate that the cutaneous infiltration by PMN induced by hapten challenge was dramatically less in $CXCR2^{-/-}$ mice. In addition, the CHS response was strongly inhibited in $CXCR2^{-/-}$ mice, suggesting that CXCR2 activity plays an essential role in PMN recruitment and in the regulation of CHS elicitation.

MATERIALS AND METHODS

Animals

Male, 8-12 week-old BALB/c mice were obtained from Charles River Laboratories (Calco, LC, Italy). Animals were housed and acclimatised for 1 week under conditions of controlled temperature (20 °C ± 2), humidity (55 % ± 10 %) and lighting (7:00 am-7:00 pm); standard sterilised food and water were supplied *ad libitum* during acclimatisation and experiments. Male $CXCR2^{-/-}$ mice with a BALB/c background (strain C129S2 (B6)-IL8RB) were derived from founders provided by Jackson Laboratories (Bar Harbor, ME, USA) and were genotyped using the polymerase chain reaction (PCR) [14].
Procedures involving animals and their care conformed to institutional guidelines that comply with national (D.L. n. 116, G.U. suppl. 40; February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1; December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

Hapten sensitization and elicitation of CHS

Mice were sensitized and challenged to elicit a CHS response to DNFB or OX. For the induction of CHS to DNFB (Sigma-Aldrich, St. Louis, MO, USA), mice were anaesthetized with a mixture of ketamine/xylazine (Sigma-Aldrich) 5:1 (w/w), and were sensitized by applying 25 µL of 0.25 % DNFB (v/v in a mixture 4:1 acetone/olive oil) to the previously shaved abdomen, and 5 µL on each subplantar side of the paws, for two consecutive days (day 0 and day 1). Five days later (day 5), sensitized mice and non-sensitized mice (control groups) were challenged by painting of 10 µL of 0.2% DNFB onto each side of the right ear [15].
For the induction of CHS to OX (Sigma-Aldrich), mice were sensitized by applying 25 µL of 3 % OX (w/v in a mixture 4:1 acetone/olive oil) to the previously shaved abdomen, and 5 µL on each subplantar side of the paws (day 0). Five days later (day 5), sensitized and non-sensitized mice (control groups) were challenged by applying 10 µL of 0.5 % OX onto each side of the right ear [2, 16]. Mice were sacrificed at different times after challenge (0, 24, 48 and 72 h) for evaluation of ear PMN recruitment and the swelling response.

Anti-PMN antibody treatment

Anti-PMN antibody (RB6-8C5) was a kind gift from Prof. A. Mantovani (Mario Negri Institute, Milan, Italy). The anti-PMN antibody was dissolved in saline containing 0.1 % bovine serum albumin (BSA) (Sigma-Aldrich) and administered i.p., 24 h before challenge (250 µg/mice; 250 µL/mice) [17]. The extent of PMN depletion was assessed at the time of sacrifice (24 h after challenge) by differential cell count.

Evaluation of PMN recruitment

PMN recruitment into the ear was determined as myeloperoxidase (MPO) activity, a well known specific marker of PMN [18]. MPO activity was determined in protein extracts from frozen (-80 °C) ear tissue [19]. Briefly, ear tissues were suspended in 0.75 mL of 50 mM phosphate buffer pH 6.0, containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB) (Sigma-Aldrich), and homogenized on ice with an Ultra-Turrax homogenizer (Ultra-Turrax, Ika-Werk, Staufen, Germany) (three times for 30 sec each). Next, homogenized tissues were sonicated (Ultrasonic processor XL, New Highway, Farmingdale, NY, USA) on ice three times for 10 sec each. After three freeze (-80 °C)-thaw (30 °C) cycles, samples were ultracentrifuged (Sorvall centrifuge, Wilmington, Delaware, USA) for 15 min at 40,000 x *g* at 4 °C. MPO activity was determined in the supernatants by spectrophotometer analysis and expressed as mU/ear. One unit of enzyme is the amount that consumes 1 µmol of H_2O_2 per minute [20].

Evaluation of ear swelling

The ear swelling response, evaluated as increase of vascular permeability, was determined as described previously [21]. Briefly, mice were injected (i.v.; 250 µL/30 g bw) with a 1 % solution of Evans blue dye (Sigma-Aldrich) at different times after hapten challenge and were sacrificed 30 min later. Next, the ears were removed, and a 9 mm diameter punch of each ear was made with a cork borer. Ear punches were transferred into a tube containing 1 mL of extraction buffer (600 µL acetone and 400 µL 0.5 % Na_2SO_4) and cut in smaller pieces inside the tube using scissors. After overnight storage at room temperature, the samples were centrifuged for 15 min at 1,500 x *g* (Centrifuge 4236, ALC, Milan, Italy) and the optical density (OD) of the supernatant fluids was read at 620 nm using a Victor^2 -1420 multi-label counter (Wallac Oy, Turku, Finland) against extraction buffer (blank). Ear swelling was expressed as follows:

$$\text{Ear swelling } (OD_{620}/mm^2) = \frac{OD_{620} \text{ right ear} - OD_{620} \text{ left ear}}{63.5 \text{ mm}^2}$$

where 63.5 mm^2 is the area of the ear punches [21, 22].

Histology

After sacrifice, ears were removed and sections (6 µm) were stained with hematoxylin and eosin (Autostainer XL, Leica Microsystem GmbH, Heidelberg, Germany).

Statistical analysis

Data are expressed as mean ± SEM. All the analyses were conducted by using SAS 9,1 TS Level 1M3 in Windows XP professional environment. ANOVA followed by Dunnett's multiple comparison test were performed for testing treatment differences [23]. Statistical significance was set at $p < 0.05$ and $p < 0.01$.

RESULTS

The CHS response was induced by sensitization of CXCR2$^{+/+}$ mice with an optimal dose of DNFB (0.25 %) on two consecutive days. Five days after the first DNFB treatment, mice were challenged with 0.2 % DNFB [15]. As shown in *figure 1A*, PMN infiltration into the challenged ears, evaluated as MPO activity, strongly increased 24h after DNFB challenge (15.75 ± 0.66 *versus* 1.89 ± 0.02) in DNFB-sensitized and challenged CXCR2$^{+/+}$ mice *versus* only DNFB-challenged CXCR2$^{+/+}$ mice, respectively, peaking at 48 h (46.51 ± 2.66 *versus* 5.91 ± 0.06, respectively). On the other hand, ear swelling peaked at 24 h after DNFB challenge (8.2 ± 0.82 *versus* 0.35 ± 0.04) in DNFB-sensitized and challenged CXCR2$^{+/+}$ mice *versus* only DNFB-challenged CXCR2$^{+/+}$ mice, respectively, and gradually decreased thereafter *(figure 1B)*.

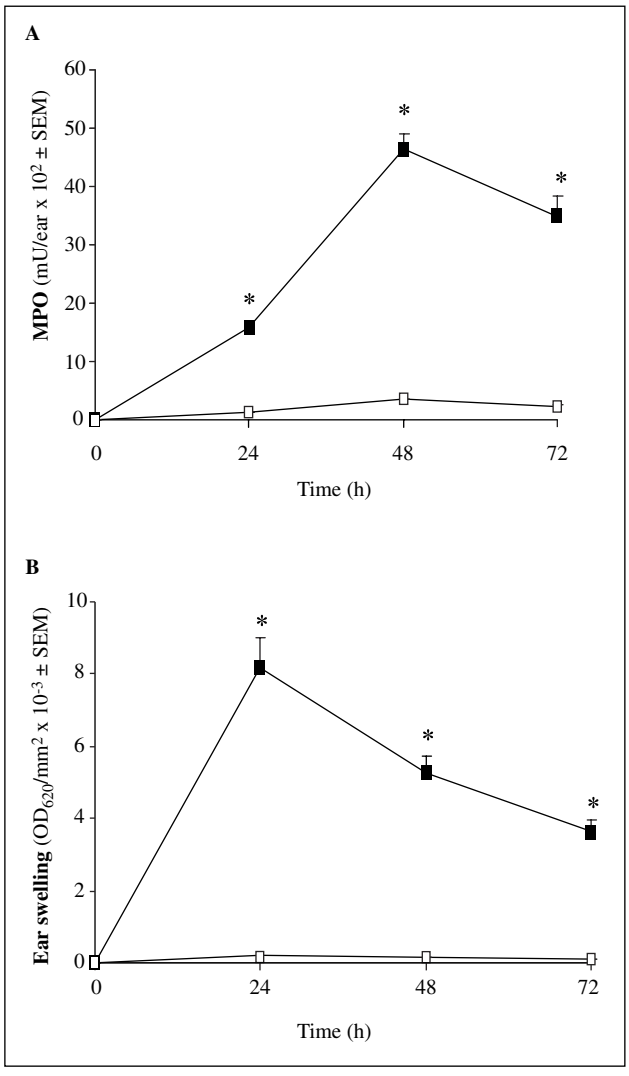

Figure 1

DNFB-mediated PMN recruitment and elicitation of the CHS response in CXCR2$^{+/+}$ mice. Animals were sensitized with 0.25 % DNFB for two consecutive days. Five days later mice were challenged with 0.2 % DNFB onto each side of the right ear. Control groups were treated only with DNFB challenge dose. Mice were sacrificed at different times (0, 24, 48 and 72 h) after hapten challenge. Ear MPO activity (**A**) and swelling response (**B**) were determined in DNFB-sensitized and challenged (■) or only challenged (□) groups as described in Materials and Methods. Data are expressed as mean ± SEM (seven animals per group for each time point from one experiment of three).

*$p < 0.01$ *versus* corresponding control (only DNFB-challenged) groups by Dunnett's t test.

In order to evaluate the role played by CXCR2 activity in PMN recruitment into DNFB-challenged ears, MPO activity was determined 24 h and 48 h after challenge in CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice. As shown in *figure 2*, PMN recruitment induced by DNFB challenge was dramatically less in CXCR2$^{-/-}$ mice, the MPO activity detected in hapten-challenged ears of sensitized CXCR2$^{-/-}$ mice being almost comparable with that observed in only DNFB-challenged CXCR2$^{-/-}$ mice *(figure 2A* and *2B)*. Blocking of PMN infiltration in CXCR2$^{-/-}$ mice was observed both at 24 h *(figure 2A)* and 48 h *(figure 2B)* after DNFB challenge (87 % and 91 % inhibition of MPO activity *versus* corresponding CXCR2$^{+/+}$ experimental groups at 24 h and 48 h after DNFB challenge, respectively). Inhibition of PMN infiltration observed in CXCR2$^{-/-}$ mice was also confirmed by histological evaluation of the DNFB-challenged ears. As shown in *figure 3B*, DNFB challenge induced a dramatic infiltration of PMN into the ears of sensitized CXCR2$^{+/+}$ mice causing microabscess formation *(figure 3C)*. Conversely, only sporadic, infiltrated PMN were observed in the ears of DNFB-sensitized and challenged CXCR2$^{-/-}$ mice *(figure 3E)*.

Next, we investigated the role played by CXCR2 activity in the CHS elicitation induced by DNFB challenge. As shown in *figure 4*, the ear swelling response induced by DNFB challenge was significantly reduced in neutropenic CXCR2$^{+/+}$ mice (59 % inhibition of ear swelling at 24 h after hapten challenge). Elicitation of the CHS response in sensitized CXCR2$^{-/-}$ mice was significantly decreased at 24 h after hapten challenge the ear swelling response being equivalent to that observed in neutropenic CXCR2$^{+/+}$ mice. Similarly, a consistent reduction of ear swelling was also observed in sensitized CXCR2$^{-/-}$ mice 48 h after hapten challenge (56 % inhibition as compared to corresponding CXCR2$^{+/+}$ experimental group).

In order to further investigate the role played by CXCR2 activity in CHS regulation, PMN recruitment and ear swelling was induced using a different hapten. CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice were sensitized with 3 % OX and subsequently challenged by applying 0.5 % OX [2, 16]. As shown in *figure 5*A, PMN infiltration induced by OX challenge was robustly reduced (88 % inhibition) in OX-sensitized CXCR2$^{-/-}$ mice 24 h after hapten challenge. Again, elicitation of the CHS response induced by hapten challenge was significantly reduced in sensitized CXCR2$^{-/-}$ mice, the ear swelling in CXCR2$^{-/-}$ mice being comparable to that observed in neutropenic CXCR2$^{+/+}$ mice (*figure 5B*; 3.77 ± 0.54 *versus* 3.31 ± 0.64 in neutropenic CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice, respectively).

DISCUSSION

PMN infiltration is believed to be involved in the CHS response. Although chemokines and unrelated chemotactic factors are thought to mediate leukocyte recruitment into a hapten challenge site, information about the mechanism involved in the regulation of PMN recruitment and the subsequent CHS response is scanty. We demonstrate here that PMN infiltration in the CHS response elicited by two different haptens is mainly mediated by CXCR2 activity. CXCR2$^{-/-}$ mice have limited, if any, PMN infiltration

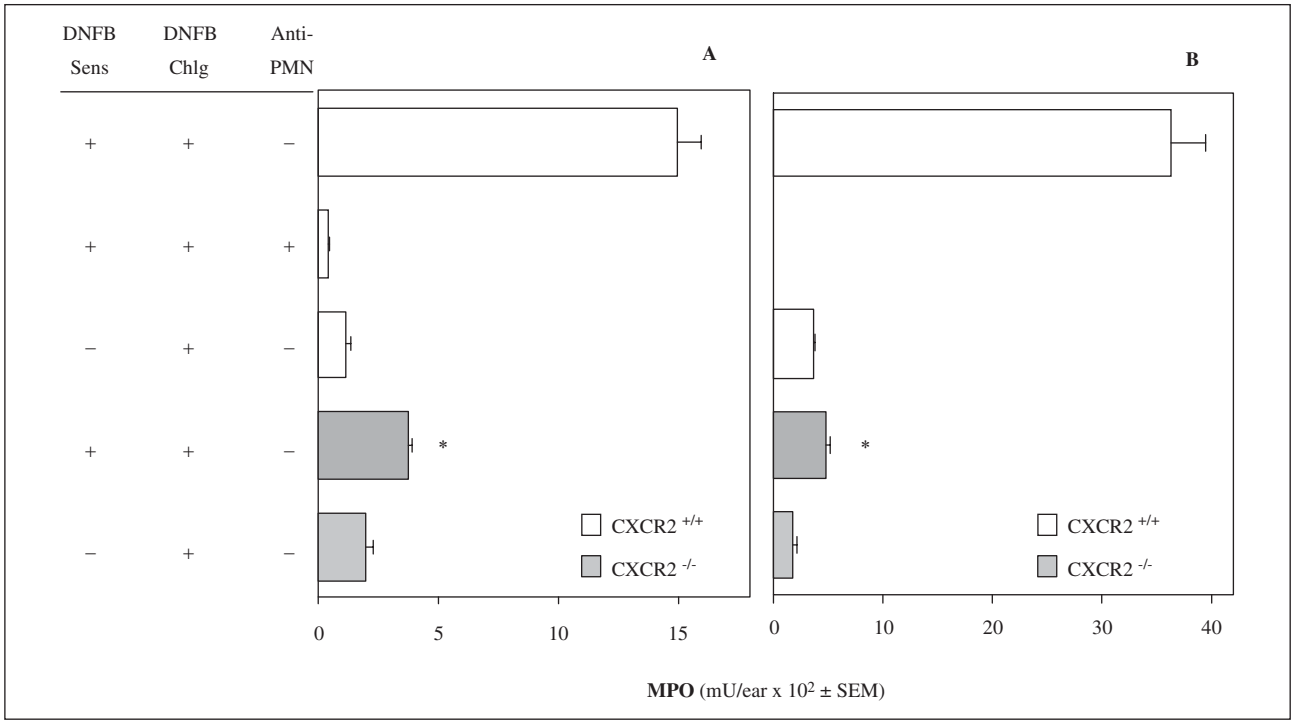

Figure 2
PMN recruitment into DNFB-challenged ears of CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice. Mice were sensitized with 0.25 % DNFB and subsequently challenged with 0.2 % DNFB. Animals were sacrificed 24 h (**A**) or 48 h (**B**) after hapten challenge. Ear MPO activity was evaluated in DNFB-sensitized and challenged or only challenged CXCR2$^{+/+}$ (■) groups as described in *Materials and Methods*. Ear MPO activity in sensitized CXCR2$^{+/+}$ mice depleted of PMN was determined only 24 h after DNFB challenge. Data are expressed as mean ± SEM (seven animals per group from one experiment of three).
*$p < 0.01$ *versus* DNFB-sensitized and challenged CXCR2$^{+/+}$ experimental group by Dunnett's t test.

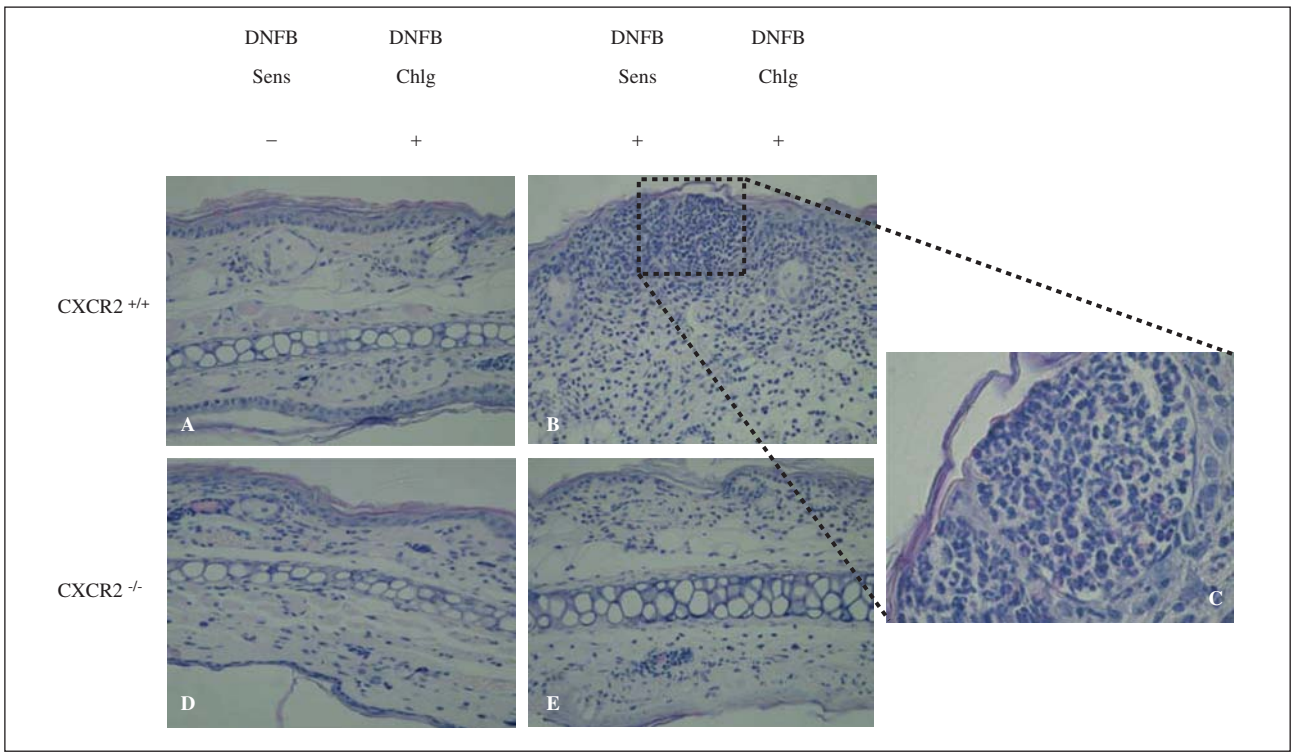

Figure 3
Histological analysis of DNFB-challenged ears of CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice. Mice were sensitized with 0.25 % DNFB and subsequently challenged with 0.2 % DNFB. Control groups were treated only with a DNFB challenge dose. Animals were sacrificed 24 h after challenge. Ears were removed and sections were stained with hematoxylin and eosin; representative pictures are shown. Panel **A** and **D** are from control (only DNFB-challenged) CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice respectively. Panels **B** and **E** are from DNFB-sensitized and challenged CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice, respectively. DNFB challenge induced a dramatic infiltration of PMN (panel **B**) causing microabscess formation (panel **C**) into ear of sensitized CXCR2$^{+/+}$ mice. On the contrary, only sporadic infiltrated PMN were observed into ear tissue of DNFB-sensitized and challenged CXCR2$^{-/-}$ mice (panel **E**). Original magnification, x 400 (panels **A, B, D** and **E**), x 1000 (panel **C**).

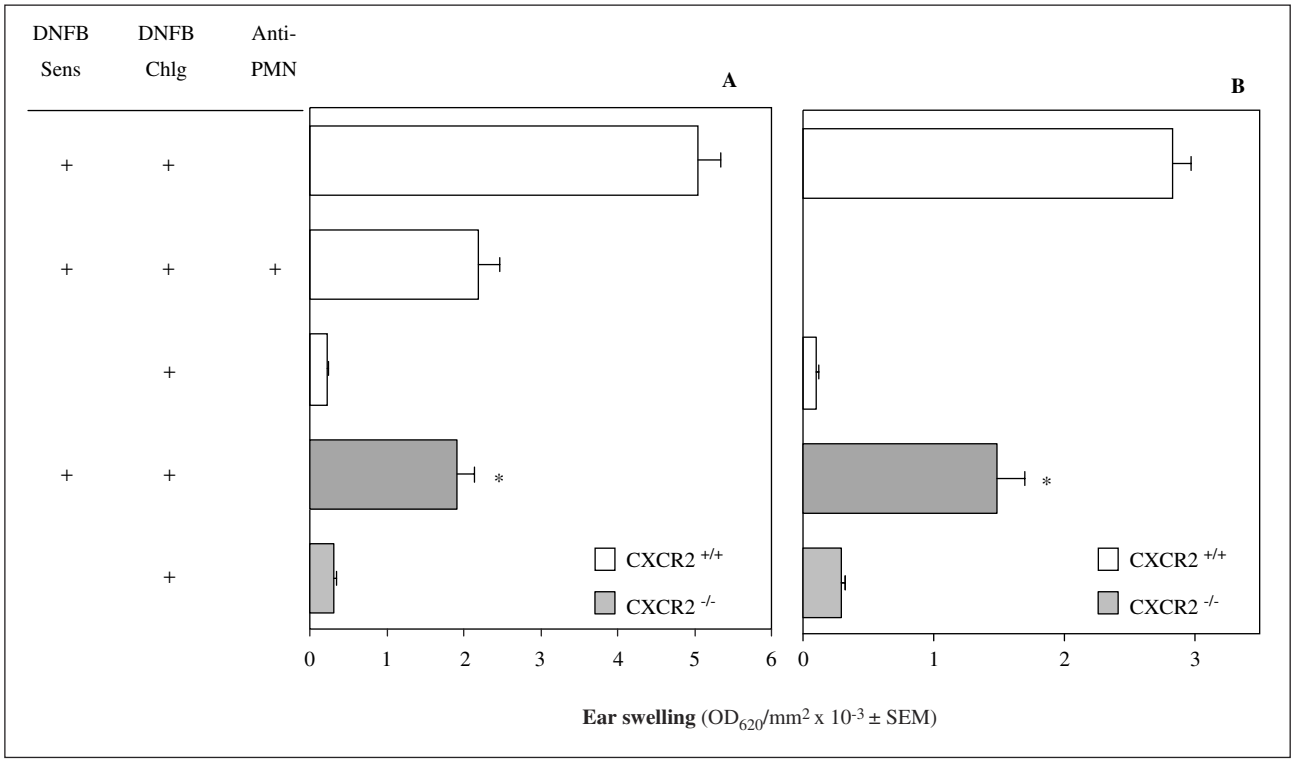

Figure 4

Ear swelling response into DNFB-challenged ears of CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice. Animals were sensitized with 0.25 % DNFB and subsequently challenged with 0.2 % DNFB. Mice were sacrificed 24 h (**A**) or 48 h (**B**) after DNFB-challenge. Ear swelling was determined in DNFB-sensitized and challenged or only challenged CXCR2$^{+/+}$ (■) mice as described in *Materials and Methods*. Ear swelling response in sensitized CXCR2$^{+/+}$ mice depleted of PMN was determined only 24h after DNFB challenge. Data are expressed as mean ± SEM (seven animals per group from one experiment of three).

*$p < 0.01$ *versus* DNFB-sensitized and challenged CXCR2$^{+/+}$ experimental group by Dunnett's t test.

into a cutaneous hapten challenge site. Accordingly, with the role thought to be played by PMN infiltration in mediating elicitation of the CHS response, the concomitant characteristic oedema formation was greatly reduced in mice lacking a functional CXCR2.

To assess the pathophysiological role of CXCR2 in the elicitation of the CHS response we used a mouse model of the hapten-mediated CHS response. In this model, cutaneous infiltration by PMN into a hapten challenge site was dramatically reduced in sensitized CXCR2$^{-/-}$ mice, ear MPO activity being almost comparable with that observed in non-sensitized CXCR2$^{-/-}$ mice. Inhibition of cutaneous PMN recruitment in hapten-challenged CXCR2$^{-/-}$ mice was further confirmed by histological evaluation. Prevention of cutaneous PMN infiltration by the absence of functional CXCR2 was observed independently of the hapten used, indicating that the role played by CXCR2 activity in PMN recruitment is not dependent on susceptibility to a specific antigen. Taken together, these data are in keeping with the hypothesis that CXCR2 activity is a key element in inducing PMN recruitment into a hapten challenge site during elicitation of the CHS response.

Inhibition of PMN recruitment into challenged ears of CXCR2$^{-/-}$ mice was associated with a consistent reduction in the CHS response. Tissue oedema, the main feature of the CHS response, was considerably inhibited in CXCR2$^{-/-}$ mice, being strictly comparable with that observed in neutropenic, CXCR2$^{+/+}$ mice. Again, inhibition of the CHS response was independent of the type of allergen used. These data are further in keeping with the concept that PMN recruitment is required for elicitation of CHS and they strongly suggest that CXCR2 activity could play an essential role in a PMN-mediated CHS response. In agreement with our findings, studies in a rat model of delayed-type hypersensitivity (DTH) have indicated the requirement of PMN in the elicitation of the response [24] and that depletion of PMN reduced the DTH response to viral antigen challenge in sensitized mice [25]. Moreover, in a rabbit model, treatment of tuberculin-sensitized animals with a neutralizing anti-CXCL8 antibody decreased PMN infiltration into the challenge site and the subsequent CHS response [10]. Finally, production of CXCL1 and CXCL2 was induced by cutaneous contact with reactive antigen, and selective neutralization of CXCR2 ligands significantly reduced the CHS response in sensitized mice [5, 11].

Whereas cutaneous infiltration by PMN seems to be mediated mainly by CXCR2, the role of CXCR2 activity in the elicitation of the CHS response should be, at least in part, mediated by recruitment into a skin challenge site of hapten-primed T cells. Nevertheless, murine CXCR2 ligands are not chemoattractants for murine T cells [5], suggesting that the role of CXCR2 in the elicitation of the CHS response may be essentially mediated through cutaneous PMN recruitment and activation into the challenge site. In keeping with this hypothesis, PMN produce many chemokines and other chemoattractants, including those for antigen-activated T cells [26, 27]. Furthermore, PMN infiltration into cutaneous antigen challenge sites has been reported to regulate the number of antigen-primed T cells recruited into the site and to the magnitude of the immune response [28].

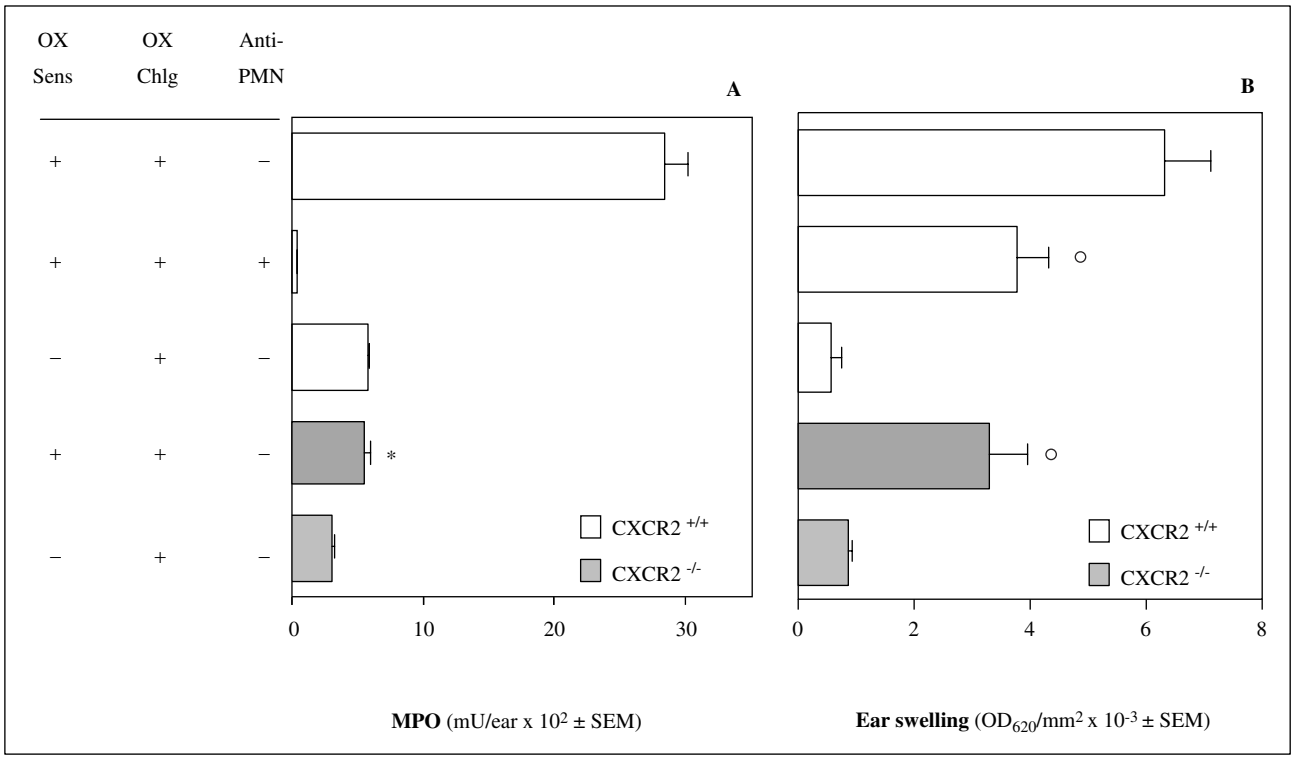

Figure 5
OX-mediated PMN recruitment and elicitation of CHS response in CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice. Animals were sensitized with 0.3 % OX and subsequently challenged by painting 0.5 % OX. Control groups were treated only with OX challenge dose. Mice were sacrificed 24 h after OX challenge. Ear MPO activity (**A**) and swelling response (**B**) were determined in OX-sensitized and challenged or only challenged CXCR2$^{+/+}$ (■) mice as described in *Materials and Methods*. Data are expressed as mean ± SEM (seven animals per group from one experiment of three). °$p < 0.05$ and *$p < 0.01$ *versus* OX-sensitized and challenged CXCR2$^{+/+}$ experimental group by Dunnett's t test.

Taken together, these findings demonstrate that CXCR2 plays a key role in mediating cutaneous infiltration by PMN during elicitation of the CHS response. Prevention of PMN recruitment has been associated with a strong reduction in tissue oedema, the main feature of the CHS response, thus suggesting that CXCR2 activity may indirectly regulate, through the modulation of PMN infiltration, recruitment of hapten-primed effector T cells into a challenge site. These data may provide the rationale for the testing of CXCR2 inhibitors as a new therapeutic approach to skin diseases. Potent and specific, low molecular weight inhibitors of both CXCL8 receptors have been recently described and are being tested in clinical studies on ischemia/reperfusion injury [29, 30].

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