

# Mast Cells Control Neutrophil Recruitment during T Cell-mediated Delayed-type Hypersensitivity Reactions through Tumor Necrosis Factor and Macrophage Inflammatory Protein 2

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## Abstract

Polymorphonuclear leukocytes (PMNs) characterize the pathology of T cell-mediated autoimmune diseases and delayed-type hypersensitivity reactions (DTHR) in the skin, joints, and gut, but are absent in T cell-mediated autoimmune diseases of the brain or pancreas. All of these reactions are mediated by interferon  $\gamma$ -producing type 1 T cells and produce a similar pattern of cytokines. Thus, the cells and mediators responsible for the PMN recruitment into skin, joints, or gut during DTHR remain unknown. Analyzing hapten-induced DTHR of the skin, we found that mast cells determine the T cell-dependent PMN recruitment through two mediators, tumor necrosis factor (TNF) and the CXC chemokine macrophage inflammatory protein 2 (MIP-2), the functional analogue of human interleukin 8. Extractable MIP-2 protein was abundant during DTHR in and around mast cells of wild-type (WT) mice but absent in mast cell-deficient WBB6F<sub>1</sub>-*Kit*<sup>W</sup>/*Kit*<sup>W-v</sup> (*Kit*<sup>W</sup>/*Kit*<sup>W-v</sup>) mice. T cell-dependent PMN recruitment was reduced >60% by anti-MIP-2 antibodies and >80% in mast cell-deficient *Kit*<sup>W</sup>/*Kit*<sup>W-v</sup> mice. Mast cells from WT mice efficiently restored DTHR and MIP-2-dependent PMN recruitment in *Kit*<sup>W</sup>/*Kit*<sup>W-v</sup> mice, whereas mast cells from TNF<sup>-/-</sup> mice did not. Thus, mast cell-derived TNF and MIP-2 ultimately determine the pattern of infiltrating cells during T cell-mediated DTHR.

Key words: chemokines • inflammation • type 1 T cells • cytokines • autoimmune disease

## Introduction

Recruitment of polymorphonuclear leukocytes (PMNs) into sites of inflammation plays a central role in the pathogenesis of rheumatoid arthritis, Crohn's disease, inflammatory bowel disease, or psoriasis, whereas PMNs are virtually absent during other T cell-mediated diseases such as experimental allergic encephalomyelitis (EAE)<sup>1</sup> or autoimmune

pancreatitis (1–11). All of these diseases are considered to be the consequence of delayed-type hypersensitivity reactions (DTHR), which are induced by IFN- $\gamma$ -producing type 1 T cells. The data suggest not only that PMN recruitment is determined by the functional phenotype of the infiltrating T cells, but also that resident cells control PMN recruitment into the skin, joints, or gut when activated by infiltrating type 1 T cells.

Recruitment of PMNs into sites of inflammation requires two major events, attachment and migration (12, 13). TNF and IL-1 induce the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells, which are required for the attachment of lymphocytes and of PMNs (14). However, TNF induction alone is not sufficient for PMN recruitment. Thus, PMNs

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<sup>1</sup>Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; CHSR, contact hypersensitivity reaction; DTHR, delayed-type hypersensitivity reaction; EAE, experimental allergic encephalomyelitis; IP, IFN- $\gamma$  inducible protein; KC, cytokine-induced neutrophil chemoattractant; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; TNCB, trinitrochlorobenzene; WT, wild-type.

are absent during T cell-mediated destruction of islet cells in nonobese diabetes or EAE, two strictly TNF-dependent DTHRs (10, 15). T cell-mediated PMN recruitment into sites of inflammation therefore requires chemoattractants in addition to TNF.

In the mouse, the two best-defined chemokines capable of recruiting PMNs are cytokine-induced neutrophil chemoattractant (KC), the homologue of the human chemokine growth-related oncogene (GRO) (16), and macrophage inflammatory protein (MIP)-2, considered as the functional analogue of human IL-8 (17). Both CXC chemokines can be produced by a variety of hematopoietic, epithelial, and stromal cells, and bind to the chemokine receptor CXCR2. Studying PMN recruitment into the skin during T cell-independent, toxic reactions, Fairchild and coworkers found that a chemokine derived from keratinocytes, the CXC chemokine KC, is capable of recruiting PMNs into the epidermis (18).

Although innate and toxic reactions can induce the production of TNF and chemokines by numerous cells, including epithelia, endothelia, and stromal cells, the relative contribution of these different cell types to the organization of a strictly T cell-mediated inflammation remains unclear (19). An emerging concept suggests that hematopoietic cells provide the TNF required for T cell-mediated DTHRs (19), even though the cellular source providing this TNF remains open. T cells and macrophages are both needed for the appropriate development of DTHRs and are considered an important source of TNF (20, 21). However, B cell-deficient or mast cell-deficient mice also have profound defects in various DTHRs, suggesting an important role for B cell- and mast cell-derived mediators during the organization of DTHRs (22–24).

Mast cells are capable of producing large amounts of TNF (25) and chemokines, including MIP-2/IL-8 (26–28), and have been shown to provide the biologically relevant TNF during innate (29, 30) and IgE-mediated (31) immune reactions. Moreover, mast cells can enhance T cell-dependent DTHRs, such as EAE (32) and contact hypersensitivity reactions (CHSRs; reference 23), which are attenuated in mast cell-deficient WBB6F<sub>1</sub>-*Kit<sup>W</sup>/Kit<sup>W-v</sup>* (*Kit<sup>W</sup>/Kit<sup>W-v</sup>*) mice. More detailed analysis of CHSRs in mast cell-deficient *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice revealed that hapten-specific DTHRs seem to have a normal T cell infiltrate (33, 34) but are almost devoid of PMNs (24). As mast cells are an important source of cytokines and chemokines, including TNF and MIP-2/IL-8 (29, 30), they may provide the central mediators recruiting PMNs during T cell-dependent DTHRs.

To analyze the mechanisms responsible for PMN recruitment during type 1 T cell responses, we used trinitrochlorobenzene (TNCB)-induced CHSRs. TNCB-induced DTHRs are strictly dependent on hapten-specific, type 1 memory T cells and are associated with a strong infiltrate of PMNs. These memory T cells lead to CHSRs when the hapten is applied to the skin of sensitized mice (34) and to inflammatory bowel disease, an important model of Crohn's disease, when the hapten is applied intrarectally (35). Using TNCB-induced CHSRs as a model disease, we

found that mast cells are the central hematopoietic cells regulating PMN recruitment in response to infiltrating type 1 T cells by delivering both the TNF and the MIP-2 required for the migration of PMNs.

## Materials and Methods

**Animals.** Female BALB/c or C57BL/6 mice were from Charles River Laboratories; TNF<sup>-/-</sup> 129/Svx C57BL/6 mice (36) and TNF<sup>+/+</sup> 129/Svx C57BL/6 mice, genetically mast cell-deficient *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice, and congenic normal WBB6F<sub>1</sub><sup>+/+</sup> (*Kit<sup>+/+</sup>*) mice were bred at the GSF-National Research Center. *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice are derived from heterozygous breeder pairs originally obtained from The Jackson Laboratory. Adult *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice contain <1.0% of the number of dermal mast cells present in the skin of the congenic wild-type (WT) mice. All mice were between 6 and 10 wk of age.

**Reagents and Abs.** TNCB was a gift from Dr. S. Katz (National Institutes of Health, Bethesda, MD), anti-Thy 1.2 mAb (T23) was a gift from Dr. R. Mocikat (GSF, Munich, Germany), recombinant mouse IL-1 $\beta$  and MIP-2 were from R&D Systems, IL-2 was from Chiron Corp., and ionomycin was from Sigma-Aldrich. Anti-MIP-2 serum was as described (37).

**In Vivo Experiments.** BALB/c mice were sensitized with 7% TNCB (100  $\mu$ l of a 4:1 mixture of acetone/olive oil) at the shaved abdomen. As in pilot studies, the majority of *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice died when we used this strong sensitization protocol; sensitizations in experiments that involved *Kit<sup>W</sup>/Kit<sup>W-v</sup>* and congenic WT mice were carried out with 2% TNCB (20  $\mu$ l of a 4:1 mixture of acetone/olive oil) at both sides of one ear. This protocol induced similar ear swelling responses after elicitation in WT mice without causing mortality in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice after sensitization. 1 wk later, mice were challenged with 1% TNCB (20  $\mu$ l of a 1:9 mixture of acetone/olive oil) on both sides of the previously untreated ear. Where indicated, *Kit<sup>W</sup>/Kit<sup>W-v</sup>* and congenic WT mice were sensitized on both sides of one ear with 1.6% oxazolone (20  $\mu$ l of a 4:1 mixture of acetone/olive oil) and, 1 wk later, challenged with 0.8% oxazolone (20  $\mu$ l of a 1:9 mixture of acetone/olive oil). Specific ear swelling was determined by measuring ear thickness with a micrometer (Oditest<sup>®</sup>; Kroepelin) before and 24 h after TNCB challenge (34). Nonspecific swelling, caused by 1% TNCB in five naive animals (irritant reaction), was subtracted. Anti-MIP-2 (30  $\mu$ l) or preimmune serum was injected intracutaneously 1.5 h before the TNCB challenge. *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice were locally reconstituted with mast cells by injecting intracutaneously  $0.5 \times 10^6$  cultured bone marrow-derived mast cells (BMMCs) 5 wk before the sensitization, exclusively into the ear selected for the elicitation of CHSRs.

**Histology and Immunohistology.** Ear tissue was collected 24 h after TNCB challenge and sections were stained with hematoxylin and eosin. For immunoperoxidase staining, formalin-fixed and paraffin-embedded sections were dewaxed and rehydrated through graded concentrations of ethanol. Alternatively, we used frozen tissue. The thawed tissues were fixed with acetone. Tissue sections were incubated with either anti-MIP-2 antiserum (37) or preimmune serum, washed, incubated for 10 min in secondary goat anti-rabbit serum, washed, and incubated with peroxidase-antiperoxidase complex (Dako). After development, sections were counterstained with alcian blue.

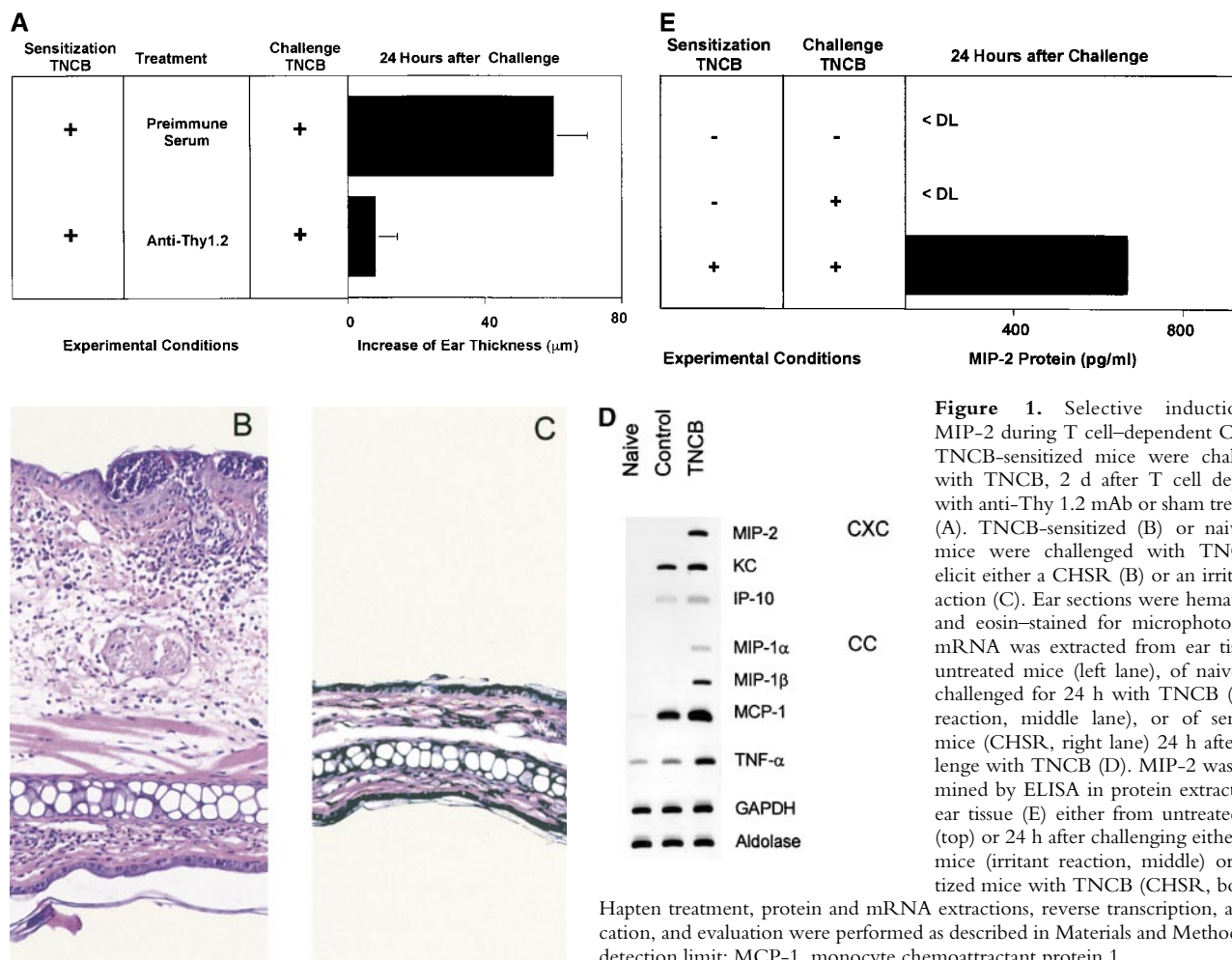
**Protein Analysis.** Protein was extracted from ear tissues and MIP-2 was determined by using rat anti-mouse MIP-2 mAb (MAB452; R&D Systems) for coating and anti-MIP-2 serum

(37) for detection, followed by horseradish peroxidase-conjugated goat anti-rabbit Ig and 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS; Boehringer Mannheim) as substrate (BD PharMingen). Myeloperoxidase (MPO) activity was determined in protein extracts from frozen ear tissue and MPO activity was expressed as units per gram (37). MPO activity of sensitized WT mice samples was set as 100%.

**PCR.** Mouse ears were directly frozen in liquid nitrogen 24 h after the TNCB challenge and homogenized in lysis buffer without thawing. Total RNA was purified (RNeasy kit; QIAGEN), reverse transcribed into cDNA, and analyzed by PCR as described (38). Equal amounts of RNA from individual mice were reverse transcribed into cDNA. 20 ng of cDNA was amplified with intron-spanning primer pairs for 25 cycles (1 min at 95°, 1 min at 65°, 1 min at 72°, 5 min at 72°C final) in 25  $\mu$ l with oil overlay. Equal volumes of cDNAs were amplified by PCR. 10  $\mu$ l of amplicates was visualized by ethidium bromide staining after agarose gel electrophoresis and quantified with an electronic video system and analysis software (WinCam 2.1; Cybertec). The following primers were used (the sequences are given in the 5' to 3' direction): KC (530-bp amplicate), 5'-AACGGAGAAAGAAGACAGACTGCT and 3'-GACGAGACCAGGAGAAACAGGG; IFN- $\gamma$  inducible protein of 10 kD (IP-10; 256 bp), 5'-TCCCTCTCGCAAGGACGGTC and 3'-GTGTGTGCGTGGCTTCACTC; MIP-1 $\alpha$  (275 bp), 5'-CCTGCTCAACATCATGAAGGTCTC and 3'-GGTCAGTGAT-

GTATTCTTGGACC; MIP-1 $\beta$  (540 bp), 5'-CCACAATAGCAGAGAAACAGCAAT and 3'-AACCCCGAGCAACACC-ATGAAG; MIP-2 (466 bp), 5'-AGTTTGCCTTGACCCTGAAGCC and 3'-TGGGTGGGATGTAGCTAGTTCC; TNF (353 bp), 5'-CTCTTCTGTCTACTGAACTTCGG and 3'-AGATAGCAAATCGGCTGACGG; monocyte chemoattractant protein 1 (345 bp), 5'-ACCTGCTGCTACTCATTACCC and 3'-CACTGTCACACTGGTCACTCC; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 669 bp), 5'-CTCACTCAAGATTGTCAGCAATG and 3'-GAGGGAGATGCTCA-GTGTGG; and aldolase (571 bp), 5'-AGCTGTCTGACATCGCTCACCG and 3'-CACATACTGGCAGCGCT-TCAAG.

**Cell Cultures.** T cells were isolated from draining lymph nodes and  $125 \times 10^3$  or  $250 \times 10^3$  T cells were stimulated with either unmodified or hapten-modified T cell-depleted spleen cells ( $5 \times 10^5$ ) in a total volume of 250  $\mu$ l of medium. After 72 h of culture in DMEM containing 10% FCS, 2-mercaptoethanol, and 2 mM glutamine (all from Biochrom) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, cells were pulsed with [<sup>3</sup>H]thymidine for the final 6–8 h. Femoral Kit<sup>+/+</sup> bone marrow cells were cultured in the presence of IL-3 and c-kit ligand. After 4 wk of culture, BMMCs were either used in vivo or stimulated in vitro with ionomycin (2  $\mu$ M) and recombinant mouse IL-1 $\beta$  (2 ng/ml; reference 39). Cells were harvested for mRNA analysis and supernatants for MIP-2 determination after 24 h.



**Figure 1.** Selective induction of MIP-2 during T cell-dependent CHSRs. TNCB-sensitized mice were challenged with TNCB, 2 d after T cell depletion with anti-Thy 1.2 mAb or sham treatment (A). TNCB-sensitized (B) or naive (C) mice were challenged with TNCB to elicit either a CHSR (B) or an irritant reaction (C). Ear sections were hematoxylin and eosin-stained for microphotographs. mRNA was extracted from ear tissue of untreated mice (left lane), of naive mice challenged for 24 h with TNCB (irritant reaction, middle lane), or of sensitized mice (CHSR, right lane) 24 h after challenge with TNCB (D). MIP-2 was determined by ELISA in protein extracts from ear tissue (E) either from untreated mice (top) or 24 h after challenging either naive mice (irritant reaction, middle) or sensitized mice with TNCB (CHSR, bottom).

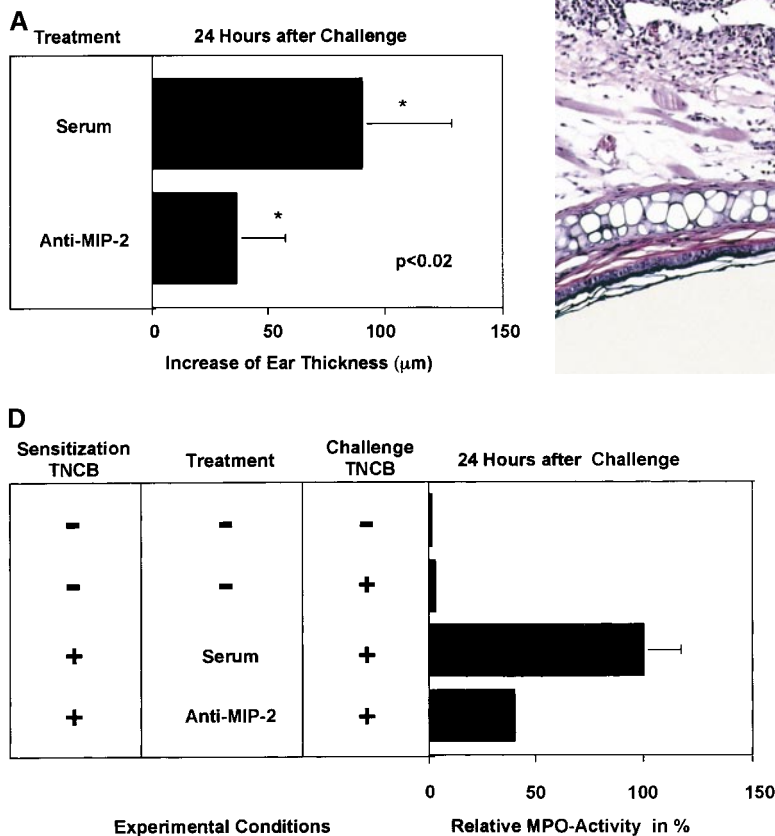
Hapten treatment, protein and mRNA extractions, reverse transcription, amplification, and evaluation were performed as described in Materials and Methods. DL, detection limit; MCP-1, monocyte chemoattractant protein 1.

**Statistical Analysis.** Differences of specific ear swelling ( $\pm$  SD) were determined using the two-tailed Student's *t* test and considered significant at  $P < 0.05$ .

## Results

**Selective Induction of MIP-2 during T Cell-dependent DTHR.** To analyze the mechanisms that control PMN recruitment during T cell-mediated DTHR, we selected hapten-induced CHSRs, a strictly T cell-dependent type of DTHR (Fig. 1 A) that is associated with strong PMN recruitment (Fig. 1, B and C). Comparing the chemokine mRNA expression pattern of T cell-dependent, hapten-induced CHSRs (Fig. 1 B) with that of T cell-independent, hapten-induced irritant reactions (Fig. 1 C), we found two strikingly different profiles. Some CXC and CC chemokines, such as KC or monocyte chemoattractant protein 1, were already induced during T cell-independent, TNCB-induced irritant reactions in nonsensitized control mice (Fig. 1 D). In sharp contrast, others, such as the CXC chemokines MIP-2 and IP-10 and the CC chemokines MIP-1 $\alpha$  and MIP-1 $\beta$ , were selectively in-

creased during the T cell-dependent DTHR in sensitized mice (Fig. 1 D). TNF was already induced in irritant reactions, as described by others (40). However, the expression increased during CHSRs (Fig. 1 D). MIP-1 $\alpha$ , MIP-1 $\beta$ , and IP-10 regulate T lymphocyte recruitment during DTHR and exhibit no chemotactic activity on PMNs (8, 41, 42). MIP-2 and KC bind to the chemokine receptor CXCR2 and have important chemotactic activities on mouse PMNs (18, 43). TNCB-induced irritant reactions in nonsensitized control mice are almost devoid of PMNs (Fig. 1 C) despite strong expression of KC mRNA (Fig. 1 D), whereas PMNs are prominent during T cell-dependent DTHR (Fig. 1 B). Thus, in WT mice, MIP-2 mRNA and not KC mRNA expression correlated with TNCB-specific PMN recruitment during this adaptive immune response. MIP-2 protein production closely correlated with MIP-2 mRNA expression and was exclusively increased in protein extracts of ear tissues undergoing hapten-specific DTHR (Fig. 1 E). Production of MIP-2 protein was strictly dependent on T cells, as T cell depletion not only suppressed ear swelling but also PMN recruitment and MIP-2 expression (Fig. 1 A and data not shown).



**Figure 2.** A critical role of MIP-2 for PMN recruitment during CHSRs. TNCB-sensitized mice were challenged with hapten 1.5 h after intracutaneous injection of either preimmune serum or anti-MIP-2 serum (A). Representative microphotographs of hematoxylin and eosin-stained CHSR reactions in mice pretreated with preimmune serum (B) or anti-MIP-2 serum (C). MPO activity in protein extracts from ear tissues (D) challenged with TNCB after pretreatment with either preimmune serum or anti-MIP-2 serum



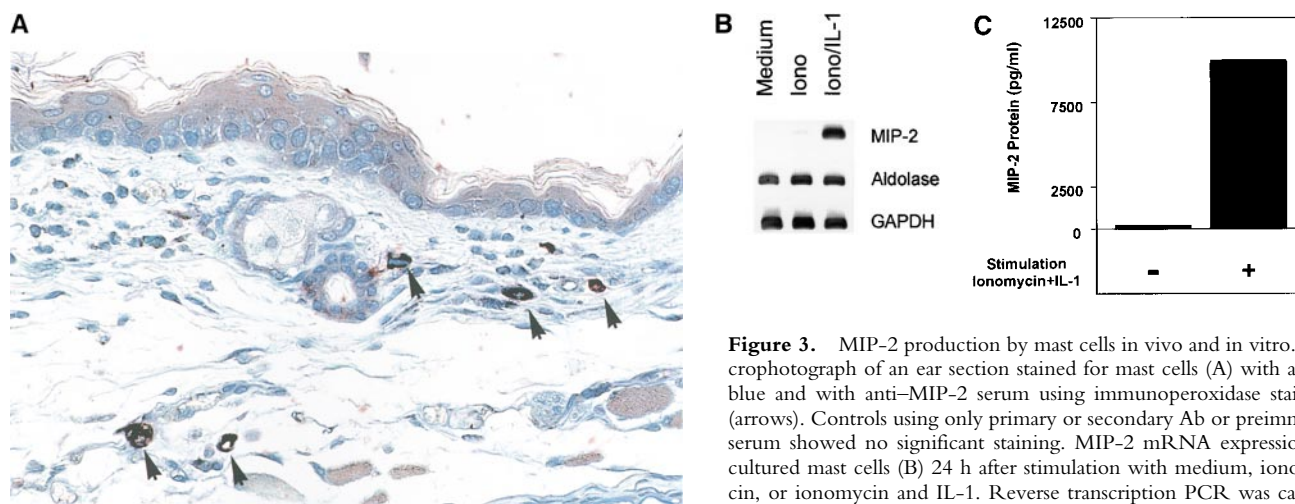
**Control of PMN Recruitment by MIP-2.** To determine whether MIP-2 production was of functional relevance for the recruitment of PMNs, we neutralized MIP-2 with a specific Ab at the site of TNCB application. Local injection of anti-MIP-2 Ab reduced hapten-specific ear swelling by ~60% (Fig. 2 A). More importantly, it also reduced PMN recruitment  $\geq 60\%$ , as determined by histology (Fig. 2, B and C) and determination of MPO activity (Fig. 2 D).

**MIP-2 Production by Mast Cells In Vivo and In Vitro.** Several cells have been suggested as putative source for the cytokines regulating PMN attraction in DTHRs. In the skin, keratinocytes, fibroblasts, endothelial cells, APCs, or T lymphocytes (22, 44, 45) count among the most important candidates. Surprisingly, immunohistology of ears undergoing CHSRs revealed that MIP-2 reactivity localized almost exclusively to mast cells, whereas keratinocytes and endothelia showed only very weak immunoreactivity (Fig. 3 A). Especially during early phases of the CHSR, at 7 h, at least 80% of mast cells were strongly MIP-2 positive (data not shown). No MIP-2 protein was visible in fibroblasts and keratinocytes and, only at 24 h, relatively little around infiltrating PMNs. Although keratinocytes largely exceed mast cells by number (Fig. 3 A), they are separated from dermal vessels by space and a basal membrane, and the influence of keratinocyte-derived cytokines on the underlying tissues is still not clear (46). Mast cells are abundant in the dermal tissue (7,000/mm<sup>3</sup>) and in close proximity to the small vessels that nurture the skin (Fig. 3 A).

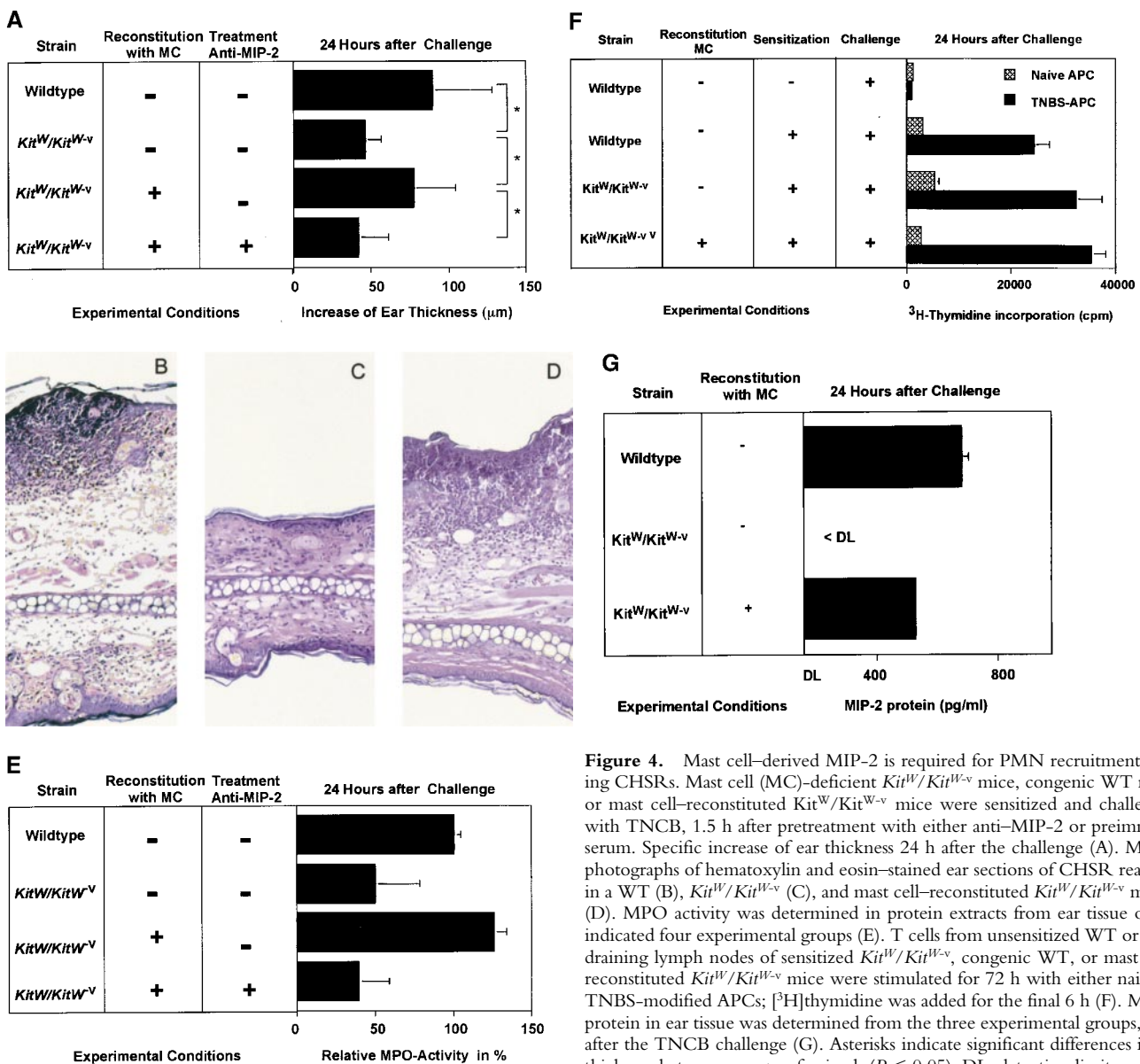
Mast cells strongly increased MIP-2 mRNA expression (Fig. 3 B) and produced large amounts of MIP-2 protein when stimulated with ionomycin and IL-1 in vitro (39; Fig. 3 C). As MIP-2 was also induced by coculturing mast cells with IFN- $\gamma$ -producing type 1 T cells (data not shown), mast cell-T cell interactions may also induce the production of the MIP-2 protein required for PMN recruitment in vivo.

**Mast Cell-dependent PMN Recruitment during Hapten-specific DTHRs.** Thus, keratinocytes and mast cells seem to be the two potential sources capable of providing the MIP-2 required for PMN recruitment. To determine the role of mast cells and mast cell-derived MIP-2 during T cell-dependent PMN recruitment, we first analyzed TNCB-induced CHSRs in mast cell-deficient *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice. As reported by others (24), mast cell-deficient *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice had 50–70% reduced tissue swelling compared with WT mice (Fig. 4 A). Reduced ear swelling was associated with severely impaired PMN recruitment during hapten-specific CHSRs, as demonstrated by the strongly reduced number of abscesses ( $\leq 10\%$ ; Fig. 4, B and C) and the low MPO activity (Fig. 4 E). Using oxazolone as an alternative hapten, we also found 60% reduction of the ear swelling in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice ( $P = 0.018$ ), and the number of PMN abscesses was again reduced to ~10% in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice. Importantly, T cell responses were not impaired in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice. Hapten-specific T cell proliferation (Fig. 4 F) and the precursor frequency of hapten-specific IFN- $\gamma$ -producing T cells (not shown) was identical in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* and congenic WT mice. The mean number of small mononuclear cells per 0.5-mm ear section was similar in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* and congenic WT mice ( $96 \pm 15$  and  $111 \pm 13$  cells, respectively, mean  $\pm$  SEM,  $n = 4$ ; see also Fig. 4, B and C), as reported previously (33, 34).

**PMN Recruitment Requires Mast Cell-derived MIP-2.** Extractable MIP-2 protein was absent in mast cell-deficient *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice during TNCB-induced CHSRs (Fig. 4 G). These data suggest that the biologically active MIP-2 required for PMN recruitment during T cell-dependent CHSRs was dependent on the presence of mast cells and that the T cell-dependent MIP-2 mRNA expression in ear tissue of *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice was of minor relevance for PMN recruitment. To address this hypothesis, we reconstituted *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice with in vitro-cultured BMMCs



**Figure 3.** MIP-2 production by mast cells in vivo and in vitro. Microphotograph of an ear section stained for mast cells (A) with alcian blue and with anti-MIP-2 serum using immunoperoxidase staining (arrows). Controls using only primary or secondary Ab or preimmune serum showed no significant staining. MIP-2 mRNA expression of cultured mast cells (B) 24 h after stimulation with medium, ionomycin, or ionomycin and IL-1. Reverse transcription PCR was carried out with primers for MIP-2 mRNA (30 cycles). IL-1 alone did not induce MIP-2 mRNA expression. MIP-2 content in the supernatants from mast cells stimulated for 24 h with ionomycin and IL-1 was determined by ELISA (C). No or only very minor MIP-2 mRNA or MIP-2 protein was detected when mast cells were stimulated with either ionomycin or IL-1 alone.

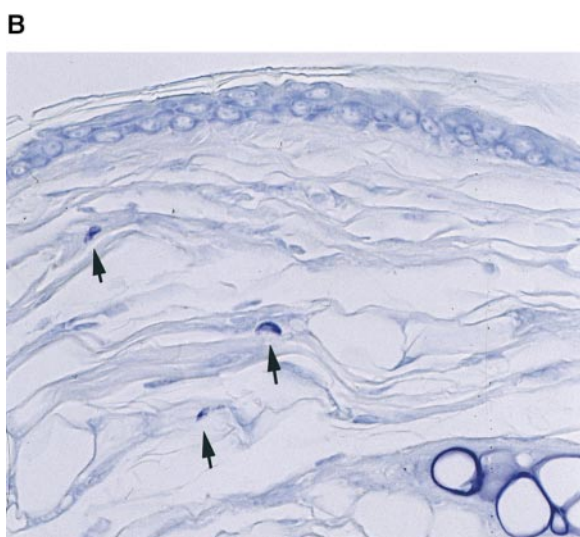
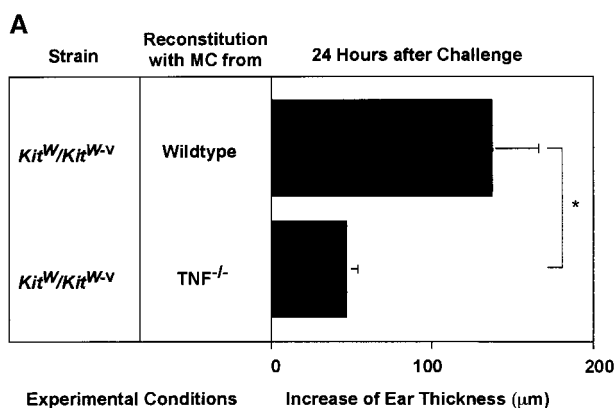


**Figure 4.** Mast cell-derived MIP-2 is required for PMN recruitment during CHSRs. Mast cell (MC)-deficient *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice, congenic WT mice, or mast cell-reconstituted *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice were sensitized and challenged with TNBC, 1.5 h after pretreatment with either anti-MIP-2 or preimmune serum. Specific increase of ear thickness 24 h after the challenge (A). Microphotographs of hematoxylin and eosin-stained ear sections of CHSR reaction in a WT (B), *Kit<sup>W</sup>/Kit<sup>W-v</sup>* (C), and mast cell-reconstituted *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mouse (D). MPO activity was determined in protein extracts from ear tissue of the indicated four experimental groups (E). T cells from unsensitized WT or from draining lymph nodes of sensitized *Kit<sup>W</sup>/Kit<sup>W-v</sup>*, congenic WT, or mast cell-reconstituted *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice were stimulated for 72 h with either naive or TNBS-modified APCs; [<sup>3</sup>H]thymidine was added for the final 6 h (F). MIP-2 protein in ear tissue was determined from the three experimental groups, 24 h after the TNBC challenge (G). Asterisks indicate significant differences in ear thickness between groups of animals ( $P < 0.05$ ). DL, detection limit.

exclusively at the site selected for DTHR elicitation (29, 31). After local reconstitution with WT BMDCs, the ear tissues again contained large amounts of extractable MIP-2 protein during CHSRs (Fig. 4 G). Similarly, hapten-specific ear swelling (Fig. 4 A), PMN recruitment (Fig. 4 D), and MPO activity (Fig. 4 E) were restored. These effects of mast cell reconstitution were again entirely antagonized by anti-MIP-2 Ab (Fig. 4, A and E).

**PMN Recruitment Requires Mast Cell-derived TNF.** As mast cell-derived TNF is needed for PMN recruitment during innate (29, 30) and IgE-mediated immune responses (47), and as TNF is also needed for the expression of adhesion molecules involved in the induction of CHSRs (14), we analyzed the role of mast cell-derived TNF in T cell-dependent CHSRs. We locally reconstituted ears of mast cell-deficient *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice with BMDCs generated from either TNF<sup>+/+</sup> or TNF<sup>-/-</sup> mice. As shown in Fig. 5

A, adoptively transferred BMDCs from TNF<sup>-/-</sup> mice restored neither ear swelling (Fig. 5 A) nor PMN recruitment in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice. In sharp contrast, BMDCs from WT mice entirely restored CHSRs and PMN recruitment in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice (Fig. 5 A). The number of mast cells in the ears of *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice reconstituted with mast cells derived from WT mice was similar to that found in ears reconstituted with mast cells from TNF<sup>-/-</sup> mice (~16 mast cells per 2-mm ear section). Thus, the reduced ear swelling and PMN recruitment were directly related to the missing TNF production by mast cells. During CHSRs, both TNF<sup>-/-</sup> and TNF<sup>+/+</sup> mast cells stained strongly positive for MIP-2 (Fig. 6, A–C), suggesting that MIP-2 production by these mast cells was TNF independent. To analyze MIP-2 production in a completely TNF-free environment, we induced CHSRs in TNF<sup>-/-</sup> mice and determined MIP-2 reactivity in frozen ear sections. Again, strong MIP-2



**Figure 5.** CHSRs in *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice depend on mast cell–derived TNF. *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice were reconstituted with mast cells (MC) from either congenic WT or TNF<sup>-/-</sup> mice, sensitized, and challenged with TNCB as described in Materials and Methods. The increase of ear thickness was determined 24 h after the challenge (A). The asterisk indicates significant difference in ear thickness ( $P < 0.05$ ). (B) Mast cells from TNF<sup>-/-</sup> mice in ear tissue from *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice stained with toluidine blue (arrowheads).

reactivity was found and was almost entirely restricted to mast cells (Fig. 6 D). Thus, mast cell–derived TNF is essential for T cell–mediated PMN recruitment during this CHSR, even in the presence of the most potent neutrophil-attracting chemokines, such as MIP-2/IL-8. Even though TNF and MIP-2 can be produced by a variety of cells, and even though TNF mRNA and MIP-2 mRNA expression increased in the ear tissue of TNCB-challenged *Kit<sup>W</sup>/Kit<sup>W-v</sup>* mice (data not shown), the adoptive transfer experiments clearly demonstrated that the TNF and MIP-2 relevant for PMN recruitment were dependent on the presence of mast cells.

## Discussion

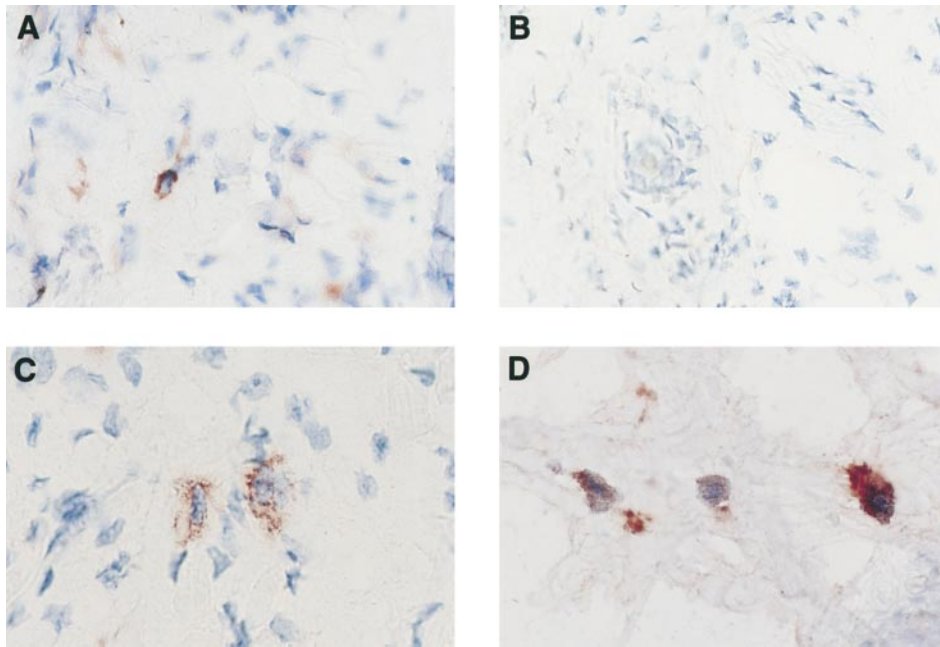
These data demonstrated a novel, unexpected role for hematopoietic cells by showing that tissue mast cells not

only are involved in the initiation and amplification of DTHRs (27, 32), but also determine the pattern of cells infiltrating into sites of inflammation through the chemokines they produce. Both TNF and MIP-2 were essential for appropriate PMN recruitment during TNCB-induced CHSRs, and both were dependent on the presence of mast cells. The combination of these two mediators is of special importance for PMN recruitment, as TNF and MIP-2 provide two qualitatively different but synergistic signals. The major biological significance of TNF seems to be the induction of adhesion molecules required for PMN attachment to endothelial cells (14), whereas MIP-2 establishes a chemotactic gradient required for diapedesis and directed migration of PMNs (48–50). These data support the emerging concept postulating that TNF of predominantly hematopoietic origin regulates leukocyte movement (19). More importantly, however, they showed that hematopoietic cells may even provide the chemokines regulating PMN recruitment into sites of inflammation. TNF was not required for MIP-2 induction during CHSRs, as TNCB application induced equivalent levels of MIP-2 in TNF<sup>-/-</sup> and WT mice (Figs. 3 A and 6 D, and data not shown).

In vivo induction of MIP-2 during CHSRs was strictly dependent on the presence of mast cells and on local activation of memory-type T cells (Fig. 1), which shows that the infiltrating type 1 T cells deliver signals that induced both TNF and MIP-2 production by mast cells. Our in vivo data do not allow for the determination of whether T cell–mast cell interactions can directly result in TNF and MIP-2 production or whether intermediate cells, such as APCs or fibroblasts, provided the signals required for mast cell activation and MIP-2 production in vivo. Preliminary in vitro data suggest that T cells can directly induce MIP-2 production by mast cells. Thus, culture of mast cells in the presence of hapten-specific type 1 T cells resulted in significant MIP-2 production (Kneilling, M, T. Biedermann, L. Hultner, and M. Rocken, unpublished data), suggesting that T cell–mast cell interactions lead not only to mast cell degranulation (51), but also to cytokine and chemokine production (52).

Various groups that have examined CHSRs in normal versus mast cell–deficient mice reported divergent results. Some found equivalent ear swelling responses in mast cell–deficient and WT mice (33, 34), whereas others found strongly reduced ear swelling responses in mast cell–deficient animals (23, 24). Similarly, in another T cell–dependent inflammatory immune response, allergic asthma, mast cells contributed to the tissue inflammation under some but not all experimental conditions. These different requirements for mast cells during T cell–mediated immune responses suggest that the requirement for mast cells depends on the strength of these immune responses. This strength is influenced by various additional factors such as concentration of the allergen/hapten, the adjuvant/solvent used for sensitization and elicitation of the immune response, and the housing conditions, which determine the activation status of memory cells and especially of APCs (53).





**Figure 6.** MIP-2 immunoreactivity in tissue mast cells from  $TNF^{-/-}$  mice.  $Kit^W/Kit^{W-v}$  mice were reconstituted with mast cells from either  $TNF^{-/-}$  mice (A and B) or congenic WT mice (C). These animals and, in addition,  $TNF^{-/-}$  mice (D), were sensitized and challenged with TNCB. MIP-2 (A, C, and D) immunoreactivity was analyzed in fixed cryosections as described in Materials and Methods. (B) Analysis of immunoreactivity in the absence of the primary Ab. Similarly, no staining was found in the corresponding controls for C and D.

Importantly, the deficiency in ear swelling responses and PMN recruitment in  $Kit^W/Kit^{W-v}$  mice can be due to factors that are independent of mast cells (23, 24). Thus, Kit-deficient mice have multiple defects in their hematopoietic system (54), and profound defects in T cell responses or altered migration through vascular walls would be alternative explanations for defective DTHR in  $Kit^W/Kit^{W-v}$  mice. This seems not to apply in this system, as hapten-specific proliferation and  $IFN-\gamma$  production were equivalent in T cells of WT and  $Kit^W/Kit^{W-v}$  mice (Fig. 4). Moreover, elicitation of CHSRs strictly required mast cell reconstitution at the site of TNCB challenge, whereas mast cell reconstitution at the site of sensitization with TNCB did not affect hapten-specific T cell responses, determined in vitro, or ear swelling responses, or PMN recruitment (data not shown).

This exclusive role of mast cells in providing the TNF and, most likely, also the MIP-2 required for the recruitment of PMN during the effector phase of DTHR was unexpected, as stromal cells such as fibroblasts, endothelia, or keratinocytes are other potential sources for these two mediators. Moreover, others have shown that mast cell-derived TNF can recruit inflammatory cells by paracrine induction of chemokines in cells such as fibroblasts (55, 56). However, immunohistology clearly showed that most of the MIP-2 protein localized to mast cells and no extractable MIP-2 was detectable in ear tissues from mast cell-deficient mice, and mast cells from  $TNF^{-/-}$  mice did not restore PMN recruitment in  $Kit^W/Kit^{W-v}$  mice. As in  $Kit^W/Kit^{W-v}$  mice, the endothelia, fibroblasts, or keratinocytes surrounding the transplanted  $TNF^{-/-}$  mast cells are in principle capable of producing TNF; this experiment clearly demonstrated that mast cell-derived TNF cannot be replaced by TNF from any other source during TNCB-induced DTHR. Moreover, the data underline the need

for a coordinated action of MIP-2 and TNF for the recruitment of PMNs.

The finding that tissue mast cells regulate PMN recruitment in response to infiltrating type 1 T cells through TNF and MIP-2 may be of general importance for the understanding of PMN recruitment during T cell-mediated autoimmune diseases. Activated mast cells and increased levels of MIP-2/IL-8 characterize all T cell-mediated autoimmune diseases associated with a predominant PMN accumulation, such as psoriasis (6, 57, 58), rheumatoid- or collagen-induced arthritis (1, 2, 59), Crohn's disease, and inflammatory bowel disease (4, 60, 61). Importantly, activated mast cells and increased levels of TNF are also found during T cell-mediated autoimmune diseases devoid of PMNs, such as EAE. In contrast, MIP-2/IL-8 is not induced during EAE and even the mRNA levels for this cytokine remain low (8, 9, 11, 32). One possibility is that EAE-inducing type 1 T cells express a unique pattern of surface molecules or cytokines that activate mast cells without inducing MIP-2/IL-8. Alternatively, mast cells of the central nervous system may have a reduced capacity in producing MIP-2/IL-8 in response to infiltrating type 1 T cells. We favor this second hypothesis, as the type 1 T cells infiltrating the central nervous system, the skin, the joints, or the intestinal wall, seem to produce a very similar pattern of cytokines. In contrast, in the different anatomic sites mast cells represent heterogeneous populations that vary in many aspects of phenotype, including morphology, histochemistry, mediator content, and response to stimuli of activation (for a review, see reference 27). In the context of our findings it is intriguing that Perrin and coworkers found MIP-2 induction and PMN recruitment during experimental autoimmune meningitis (62), where mast cells reside in a loose connective tissue, whereas MIP-2 and PMN were absent during EAE, as reported previously by



others (8, 9, 11, 32). In line with these experiments, we found that the capacity of mast cells to produce MIP-2 is critically influenced by the culture condition (our unpublished data).

It is long recognized that rheumatoid arthritis, Crohn's disease, inflammatory bowel disease, and psoriasis are associated with type 1 T cells, activated mast cells, MIP-2/IL-8, and TNF, but the link between these phenomena was missing (1, 4, 6, 63). Thus, recognizing that mast cells are capable of regulating the recruitment of PMNs during DTHR<sub>s</sub> by producing both TNF and the MIP-2/IL-8 required for PMN recruitment may be not only relevant for CHSRs but of general importance for understanding the mechanisms leading to clinically relevant autoimmune diseases.

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