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Mast Cell-Associated TNF Promotes Dendritic Cell Migration

Hajime Suto,2*‡ Susumu Nakae,2* Maki Kakurai,* Jonathon D. Sedgwick,3‡ Mindy Tsai,* and Stephen J. Galli4*

Mast cells represent a potential source of TNF, a mediator which can enhance dendritic cell (DC) migration. Although the importance of mast cell-associated TNF in regulating DC migration in vivo is not clear, mast cells and mast cell-derived TNF can contribute to the expression of certain models of contact hypersensitivity (CHS). We found that CHS to FITC was significantly impaired in mast cell-deficient KitW-sh/W-sh or TNF−/− mice. The reduced expression of CHS in KitW-sh/W-sh mice was fully repaired by local transfer of wild-type bone marrow-derived cultured mast cells (BMCMCs), but was only partially repaired by transfer of TNF−/− BMCMCs. Thus, mast cells, and mast cell-derived TNF, were required for optimal expression of CHS to FITC. We found that the migration of FITC-bearing skin DCs into draining lymph nodes (LNs) 24 h after epicutaneous administration of FITC in naive mice was significantly reduced in mast cell-deficient or TNF−/− mice, but levels of DC migration in these mutant mice increased to greater than wild-type levels by 48 h after FITC sensitization. Mast cell-deficient or TNF−/− mice also exhibited significantly reduced migration of airway DCs to local LNs at 24 h after intranasal challenge with FITC-OVA. Migration of FITC-bearing DCs to LNs draining the skin or airways 24 h after sensitization was repaired in KitW-sh/W-sh mice which had been engrafted with wild-type but not TNF−/− BMCMCs. Our findings indicate that mast cell-associated TNF can contribute significantly to the initial stages of FITC-induced migration of cutaneous or airway DCs. The Journal of Immunology, 2006, 176: 4102–4112.
of T cell sensitization, as reflected in T cell proliferation induced in response to Ag challenge in vitro, were detected in either mast cell-deficient or TNF-deficient mice at 6 days after epicutaneous sensitization to FITC.

Materials and Methods

Mice

Completely TNF-deficient mice (C57BL/6-TNF−/− mice), as well as mice expressing membrane TNF but lacking the ability to secrete a secreted TNF form (C57BL/6-mtmTNF−/− mice), were generated from C57BL/6 embryonic stem cells (16, 17). Fe−/− mice on the C57BL/6 background were obtained from Taconic Farms. WBB6F1-KitW−/− and KitW30/30 mice and C57BL/6-TNF−/− mice and C57BL/6-TNF−/− mice were obtained from The Jackson Laboratory. Mast-cell-deficient mice (KitW−/− mice) on the C57BL/6 background were generously provided by Dr. P. Besmer (Molecular Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, NY) (18); the KitW−/+−/− mice used herein were backcrossed in our laboratory for two to three generations onto the C57BL/6 background. Female 6- to 10-wk-old mice were used in all experiments. All mice were housed at the Animal Care Facilities at Stanford University Medical Center (Stanford, CA) kept under standard temperature, humidity, and timed lighting conditions, provided mouse chow and water ad libitum, and sacrificed by CO2 inhalation; all experiments were performed in compliance with the “Guidelines for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996) and the Stanford University Committee on Animal Welfare.

FITC-induced CHS

To analyze both DC migration in response to a hapten and the subsequent development and expression of CHS to that hapten at the same anatomical site, we developed in preliminary experiments a protocol in which both sensitization and challenge for CHS could be performed using the ear pinna. Mice were sensitized with a total of 80 µl of 2% FITC isomer-I (FITC, Sigma-Aldrich) in a vehicle consisting of acetone-dibutylphthalate (1:1) administered to both ears (20 µl to each side of each ear). Five days after sensitization with FITC, mice were challenged with a total of 40 µl of vehicle alone to the right ear (20 µl to each side) and 0.5% FITC to the left ear (20 µl to each side). Each mouse was housed in a separate cage to prevent contact with other mice after FITC challenge. Ear thickness was measured before and at multiple intervals after FITC challenge with an engineer’s micrometer (Ozaki). Some mice from each group were killed at 24 h after FITC or vehicle challenge for histological analysis to quantify numbers of mast cells or polymorphonuclear leukocytes (PMNs) in toulidine blue-stained or H&E-stained, respectively, 4-µm sections of ear skin. Cells were quantified by light microscopy at ×400, by observers not aware of the identity of the individual specimens. Mast cells and PMNs were counted in the entire area of dermis present in a strip of skin extending 6.6 mm in length from the base to the tip of the ear pinna (representing 1.25 ± 0.07 mm² of dermis); mast cells also were counted separately in 10 randomly selected areas of the dermis (−0.625 mm² in total area) in the central part of the ear pinna (representing the area in which bone marrow-derived cultured mast cells (BMCMCs) had been injected intradermally (i.d.) in the BMCMC-engrafted KitW−/+−/−− mice). All data are expressed as mast cells per mm² of dermis.

Preparation of BMCMCs and local mast cell engraftment of mast cell-deficient mice

BMCMCs were obtained by culturing bone marrow cells in WEHI-3-conditioned medium (containing IL-3) for 4–6 wk, at which time >98% of the cells were identified as mast cells by toluidine blue staining and by flow cytometry analysis. For mast cell reconstitution studies, BMCMCs were injected i.d. (1.3 × 10⁶ cells in 40 µl for studies of LC/DC migration and CHS) or i.v. (1.0 × 10⁷ cells for studies of lung DC migration) in 4- to 6-wk-old KitW−/+−/−− mice or KitW−/+−/−− mice. The mice were used for DC migration experiments 6 wk (in the setting of CHS) or 8 wk (in airway studies) after transfer of BMCMCs.

LC/DC migration: LC/DC release from epidermis

Naïve mice were treated with 2% FITC (left ear) or vehicle alone (right ear) as described above. Twenty-four, 48, or 72 hours after FITC treatment, mice were killed and ear skin was harvested and incubated in PBS containing 20 mM EDTA at 37°C for 2 h. Epidermal sheets then were collected on glass slides and fixed in cold acetone for 20 min. Specimens were washed in PBS for 20 min three times and incubated in PBS containing 3% BSA (blocking solution) at room temperature for 1 h. After blocking, the specimens were incubated with PE anti-mouse I-A/E (M5/114.15.2; eBioscience) at 4°C overnight, then washed with PBS containing 0.2% Tween 20 (Sigma-Aldrich) at room temperature for 1 h. The number of epidermal LCs were counted by fluorescence microscopy in 10 randomly selected areas of epidermal sheets (×400).

Skin LC/DC migration to draining LNs

Naïve mice were treated with 2% FITC (left ear) or vehicle alone (right ear) as described above. Twenty-four, 48, or 72 hours after FITC treatment, mice were killed for harvesting of submaxillary and brachial LNs on both the left (FITC-treated) and right (vehicle alone) sides, and a single-cell suspension was prepared as described (19). After incubation with anti-CD16/CD32 mAb (2.4G2; BD Pharmingen), cells were incubated with PE anti-mouse I-A/E (M5/114.15.2; eBioscience) and allophycocyanin anti-mouse CD11c (N418; eBioscience). The proportion (percentage) of FITC+ cells among 5000 7-aminoactinomycin D-negative, I-A/E+, CD11c+ cells was determined on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

Lung DC migration

Mice were treated with 20 µl × 5 (total 100 µl) of 10 mg/ml FITC-conjugated OVA (FITC-OVA) (20) intranasally (i.n.) (21). At 24 h after FITC-OVA inhalation, submaxillary or thoracic LNs were collected and single-cell suspensions were prepared (19). Cells were incubated with biotin anti-3D1 and allophycocyanin-anti-mouse CD11c mAbs after FC blocking and then incubated with PE-streptavidin (BD Pharmingen). The proportion of FITC+ cells among 5000 7-aminoactinomycin D-negative, CD11c+3D1+ cells was determined by FACS as described above.

Depletion of skin Lcs/LCs

Depletion of skin Lcs/LCs were performed as described elsewhere (22). Briefly, 0.2 g of corticosteroid cream (0.05% clobetasol-17-propionate; Glaxo) per mouse (or vehicle as a control) was applied topically to both sides each ear, daily for up to 5 days. After 1–5 days (see Fig. 3A) or 4 days (see Fig. 3B), epidermal sheets or draining LNs were harvested, respectively, for quantification of LC number after anti-I-A/E mAb staining in the epidermal sheets and for assessment of FITC+MHCII+CD11c+ DCs in the LNs.

FITC-specific LN cell proliferation

Mice were sensitized with 2.0% FITC on both left and right ears as described above. Six days after FITC treatment, submaxillary LNs were collected and pooled, single-cell suspensions were prepared, and the LN cells were cultured in the presence or absence of 40 µg/ml FITC at 37°C for 72 h. Proliferation was assessed by pulsing with 0.25 µCi [3H]thymidine (Amersham Bioscience) for 6 h, harvesting the cells using a Harvester 96 Mach BIM (Tomtec), and measuring incorporated [3H]thymidine using the Micro β System (Amersham Bioscience).

Quantification of numbers of DCs resident in the lung by FACS

Single-cell suspensions of lungs were prepared as described elsewhere (23). Lung DCs were stained with FITC-anti-mouse CD11c (N418; eBioscience), PE-anti-mouse I-A/E (M5/114.15.2; eBioscience), and biotin-anti-mouse 3D11 (3D1; eBioscience) after FeR blocking. The cells were then incubated with allophycocyanin-streptavidin (BD Pharmingen) and 7-aminoactinomycin D-negative, I-A/E+CD11c+3D1+ cells were counted on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

Quantification of numbers of lung mast cells

Single-cell suspensions of lungs were prepared as described above and mast cells were quantified after 2 × 10⁶ of these cells were cytoospun onto glass slides. The glass slides were air-dried overnight, fixed in methanol for 5 min, and then incubated with blocking buffer (Protein Block Serum-Free, Ready-to-Use; DakoCytomation) at room temperature for 1 h. After removal of blocking buffer, the specimens were incubated with rhodamine-conjugated avidin (Vector Laboratories) in DakoCytomation Ab diluent with background-reducing components (DakoCytomation) at room temperature for 4 h. The specimens were then washed five times in PBS at room temperature for 30 min each time. Rhodamine-positive cells (i.e., mast cells) were counted by fluorescence microscopy.
Statistics
The Student t test (two-tailed), paired or unpaired, as appropriate, was used for statistical evaluation of the results; unless otherwise specified, all results are presented as mean ± or or ± SEM.

Results
Optimal expression of CHS responses to FITC is both mast cell and TNF dependent

Wild-type (WT) C57BL/6 mice developed robust CHS responses to FITC, as assessed by tissue swelling at the site of hapten challenge (Fig. 1A), and also exhibited infiltration of PMNs at these sites (Fig. 1D). By contrast, the responses elicited by hapten challenge in the congenic mast cell-deficient Kitw-sh/sh mice developed only slightly, albeit significantly, greater swelling than the control reactions elicited by vehicle (Fig. 1A) and exhibited significantly reduced numbers of PMNs compared with the corresponding values in the WT mice (Fig. 1D). TNF−/− mice on the C57BL/6 background also exhibited weak CHS responses to FITC, with minimal levels of tissue swelling (Fig. 1A) and significantly lower than WT levels of PMN infiltration (Fig. 1D).

As shown in Fig. 1, B and C, the ear pinnae of Kitw-sh/sh mice, which were the sites of sensitization and challenge for CHS, were profoundly mast cell-deficient (containing no detectable dermal mast cells), whereas levels of mast cells at this site in the TNF−/− mice were statistically indistinguishable from those in the WT animals. In the central part of the ear pinnae, mast cell counts per mm² of dermis were nearly identical in WT and TNF−/− mice (Fig. 1B), whereas, over the entire length of the ear pinnae, numbers of mast cells per mm² were slightly lower in the TNF−/− mice than in the WT mice, a difference that did not achieve statistical significance (Fig. 1C).

To determine whether the abnormalities in CHS in Kitw-sh/sh mice reflected the lack of mast cells in these animals, as opposed to other consequences of their c-kit mutations, we tested Kitw-sh/sh mice that had been selectively engrafted with mast cells in the ear pinnae. We found that engraftment with WT BMCMCs fully restored CHS reactivity in Kitw-sh/sh mice, as judged by tissue swelling over the course of the reaction (Fig. 1A) or by PMN infiltration at 24 h after hapten challenge (Fig. 1D). However, Kitw-sh/sh mice that had been engrafted with TNF−/− BMCMCs exhibited only partial reconstitution of both the tissue swelling response associated with CHS to FITC (to ~50% of the WT level) (Fig. 1A) and only minimal enhancement of the PMN infiltration associated with the response compared with the levels observed in mast cell-deficient Kitw-sh/sh mice (Fig. 1D).

Engraftment of Kitw-sh/sh mice with TNF−/− BMCMCs resulted in numbers of mast cells in the central part of the ear dermis (the site injected with BMCMCs) that were statistically indistinguishable from those in WT mice or in Kitw-sh/sh mice that had been engrafted with WT BMCMCs (Fig. 1B). As expected, when measured over the entire length of the ear pinna, numbers of mast cells per mm² of dermis were substantially lower in the WT BMCMC- or TNF−/− BMCMC-engrafted Kitw-sh/sh mice than in the WT or TNF−/− mice; mast cells per mm² also were slightly lower in the Kitw-sh/sh mice that had been engrafted with WT as opposed to TNF−/− BMCMCs, but this difference was not statistically significant (Fig. 1C).

Taken together, these findings show that, under the conditions tested in our experiments, mast cells, and mast cell-associated TNF, are required for optimal expression of CHS to FITC. These results are in accord with those of Biedermann et al. (13), who showed that mast cell-derived TNF contributes to optimal expression of CHS reactions to TNCB.

Mast cells, and mast cell-associated TNF, are required for optimal DC migration to LNs during the first 24 h of the sensitization phase of CHS to FITC

Skin LCs can firmly adhere to keratinocytes through interactions of adhesion molecules such as E-cadherin (24). After epicutaneous exposure to hapten, such adhesion molecule expression is downregulated and LCs can then migrate from the epidermis. We found that the numbers of LCs in epidermal sheets were significantly reduced (by >30%) 24 h after epicutaneous application of FITC to the ear pinnae of WT C57BL/6 mice, compared with the corresponding values after vehicle treatment (Fig. 2A). In accord with
these findings, application of the sensitizing dose of FITC to WT C57BL/6 mice also resulted in the appearance of many FITC+ DCs in the local LNs, whereas LNs draining the contralateral sites that had been challenged with vehicle contained very few FITC+ DCs (Fig. 2, B and C).

In mice exposed epicutaneously to high levels of FITC, some FITC can enter the lymphatic or systemic circulation and thereby reach DCs already present in the LNs (25). To assess whether the levels of FITC used in this study might influence the numbers of FITC+ DCs we detected in the LNs draining sites of FITC application, we used an approach described by Grabbe et al. (22) to deplete epidermal LCs at sites of FITC application. We found that epicutaneous treatment of the skin with corticosteroids (~0.2 g of corticosteroid cream (0.05% clobetasol-17-propionate) per mouse), topically to both sides of the ear skin once per day for 1–5 consecutive days (22) resulted in nearly complete depletion of skin LNs by 3 days after initiation of corticosteroid treatment (Fig. 3A). At 24 h after application of FITC to the ear skin of corticosteroid-or vehicle-treated mice on day 3 after initiation of treatment, there were significantly fewer FITC+ DCs in draining LNs of the LC/DC-corticosteroid-depleted vs vehicle-treated mice (Fig. 3B).

![Figure 2](image-url)

**FIGURE 2.** Mast cells and TNF are required for optimal migration of FITC-bearing LCs/DCs to draining LNs at sites treated epicutaneously with FITC. A, At 24 h after FITC or vehicle treatment, ear skin epidermal sheets and submaxillary and brachial LNs were collected as described in **Materials and Methods**. The number of I-A<sup>b+ </sup>LCs in epidermal sheets were counted in C57BL/6 WT mice (n = 6), TNF<sup>−/−</sup> mice (n = 6), mast cell-deficient Kit<sup>W-sh/W-sh</sup> mice (n = 6), and Kit<sup>W-sh/W-sh</sup> mice that had been selectively repaired of their mast cell deficiency by the i.d. injection of WT BMCMCs. B and C, The proportion (percentage) of all I-A<sup>b+ </sup>CD11c<sup>high</sup> cells that were FITC<sup>+</sup> (i.e., FITC+ DCs) were determined by FACS analysis in C57BL/6 WT mice (n = 23), TNF<sup>−/−</sup> mice (n = 11), mast cell-deficient Kit<sup>W-sh/W-sh</sup> mice (n = 16), and Kit<sup>W-sh/W-sh</sup> mice that had been selectively repaired of their mast cell deficiency by the i.d. injection of WT BMCMCs. B, Representative flow cytometry data for individual mice (numbers = percent of FITC+ cells among I-A<sup>b+ </sup>CD11c<sup>high</sup> DCs). Shaded areas represent LNs draining vehicle-treated ears; solid lines represent LNs draining FITC-treated ears. A and C, data are mean ± SEM of values for individual mice; ++ ++ + + + ++ + = p < 0.01, 0.001 or <0.0001 vs corresponding values for vehicle-treated ears (A) or vs LNs draining vehicle-treated ears (C); + + + + + = p < 0.05 or <0.0001 vs FITC-treated ear skin (A) or vs LNs draining FITC-treated ears (C) of Kit<sup>W-sh/W-sh</sup> mice; + + + + + + = p < 0.005, <0.01, or <0.0001 vs FITC-treated ear skin (A) or vs LNs draining FITC-treated ears (C) of TNF<sup>−/−</sup> mice; and + + + + + + + = p < 0.05, <0.01, or <0.0001 vs FITC-treated ear skin (A) or vs LNs draining FITC-treated ears (C) of Kit<sup>W-sh/W-sh</sup> mice + TNF<sup>−/−</sup> BMCMCs.

![Figure 3](image-url)

**FIGURE 3.** Depletion of epidermal LCs by topical treatment with a corticosteroid is associated with reduced numbers of FITC+ DCs in the draining LNs after epicutaneous application of FITC. A, Corticosteroid cream was applied to both sides of the ear skin as described in **Materials and Methods**. Before beginning treatment with corticosteroids (day 0) and at the time intervals shown after initiation of corticosteroid treatment, the numbers of I-A<sup>b+ </sup>LCs in epidermal sheets were counted after their staining with an anti-I-A<sup>b+</sup> mAb. Data show the average percentage ± SEM of LCs vs the corresponding baseline value on day 0 (100%); n = 3 mice/group. B, At 4 days after initiation of daily corticosteroid (or vehicle) treatment, 2.0% FITC (on skin of left ear) or vehicle alone (on skin of right ear) were applied epicutaneously. At 24 h after FITC or vehicle treatment, the draining LNs were collected and the proportion (percentage) of I-A<sup>b+ </sup>CD11c<sup>high</sup> cells that were FITC+ (i.e., FITC+ DCs) was determined by FACS analysis as shown in Fig. 2, B and C. Results shown are representative of data obtained from individual mice in two independent experiments, each of which gave similar results; data are from one mouse which had not been treated with corticosteroids (A) and two mice which had been treated with corticosteroids (B and C). Based on comparison to the mean level of fluorescence intensity of FITC on MHCII+CD11c+ DCs in the LNs draining the FITC-treated ears in mice which had not received corticosteroid treatment, the level of fluorescence intensity of FITC on MHCII+CD11c+ DCs in the LNs draining FITC-treated ears in mice which had received corticosteroid treatment was significantly reduced (percent of FITC+ DCs in LNs (mean ± SEM) draining ear skin not treated with corticosteroids = 43.4 ± 2.5% (n = 6) vs corticosteroid-treated ear skin = 2.3 ± 1.3 (n = 6), p < 0.0001). In the mice in the experiments shown in B, the LC number (mean ± SEM) was, for ear skin not treated with corticosteroids: 590 ± 34 cells/mm<sup>2</sup> (n = 6), and, for corticosteroid-treated ear skin: 19.2 ± 7.2 (n = 6), p < 0.0001.
The simplest interpretation of all of our findings is that most or all of the FITC+ DCs, which we detected in draining LNs after epicutaneous application of FITC to the ear pinnae, were those which originally had been resident in the skin as LCs or dermal DCs and then migrated to the LNs, rather than DCs originally resident in the LNs, which acquired FITC via the systemic distribution or local lymphatic drainage of the hapten.

The migration of LCs 24 h after application of FITC, as reflected in the reduced numbers of LCs in epidermal sheets, was profoundly impaired in mast cell-deficient KitW-sh/W-sh mice and, to a somewhat lesser extent, in TNF−/− mice (Fig. 2A). Consistent with these data, numbers of FITC+ DCs in draining LNs 24 h after hapten application also were markedly reduced in mast cell-deficient KitW-sh/W-sh or TNF−/− mice (by mean values, in comparison to the corresponding levels in WT mice, of 49 or 54%, respectively, Fig. 2, B and C).

Local transfer of BMCMCs did not significantly influence the number of LCs in the epidermis at the site of mast cell engraftment (LC numbers per mm2 (mean ± SEM) were, for KitW-sh/W-sh mice: 533 ± 14 (n = 5), for KitW-sh/W-sh mice + WT BMCMCs: 474 ± 31 (n = 3) and, for KitW-sh/W-sh mice + TNF−/− BMCMCs: 486 ± 25.3 (n = 3)). However, we found that local engraftment of KitW-sh/W-sh mice with WT BMCMCs fully restored WT levels of hapten-induced release of LCs from the epidermis and appearance of FITC+ DCs in draining LNs, as assessed 24 h after hapten application (Fig. 2). By contrast, KitW-sh/W-sh mice that had been engrafted with TNF−/− BMCMCs exhibited significantly lower levels of both hapten-induced release of LCs from epidermis and numbers of FITC+ DCs in local LNs (Fig. 2). Indeed, numbers of FITC+ DC in draining LNs from KitW-sh/W-sh mice that had been engrafted with TNF−/− BMCMCs were only slightly higher than those in KitW-sh/W-sh or TNF−/− mice, differences that did not achieve statistical significance (Fig. 2, B and C).

Two types of mast cell-engrafted genetically mast cell-deficient mice can now be used for studies of mast cell function in vivo: C57BL/6/J-KitW-sh/W-sh mice (26, 27) and WBB6F1-KitW-sh/W-sh mice (28–30). We found that both the migration of FITC+ DCs to draining LNs within 24 h of initial sensitization with FITC, and the expression of CHS reactions to FITC in sensitized animals, were also markedly reduced in WBB6F1-KitW-sh/W-sh mast cell-deficient mice in comparison to values in the congenic WT (i.e., WBB6F1-KitW+/+) mice; moreover, both of these abnormalities in WBB6F1-KitW-sh/W-sh mice were repaired in animals that had undergone local selective repair of their cutaneous mast cell deficiency (Fig. 4).

TNF can express biological functions in either its membrane- or its secreted form (17) and via interactions with cells bearing TNFR1 and/or TNFR2 (31). The findings shown in Fig. 5 indicate that C57BL/6J-memTNFΔΔΔ mice, which are defective in their ability to produce the secreted form of TNF, are able to sustain apparently normal levels of DC migration to LNs within the first 24 h after FITC application and that, in this setting, TNF acts via TNFR1 to a significantly greater extent than via TNFR2.

Taken together, our findings in the FITC-induced model of CHS show that, under the conditions tested in our experiments, mast cells, and mast cell membrane-associated TNF, were required for optimal migration of hapten-bearing cutaneous DCs to local draining LNs within the first 24 h of initial application of the hapten, as well as for optimal expression of the elicitation phase of the CHS response to this hapten. In contrast, detectable, albeit quite weak,
CHS reactions to this hapten occurred in the virtual absence of mast cells or in mice that completely lacked TNF (Fig. 1). Similarly, some migration of FITC$^+$ DCs from the skin to local draining LNs was elicited within 24 h of FITC application in mast-cell- or TNF-deficient mice, or in mice that contained only mast cells that were unable to produce TNF (Fig. 2).

Thus, while our results clearly show that mast cells and mast cell-associated TNF can significantly enhance both the FITC-induced migration of hapten-bearing DCs upon initial hapten exposure and the robust expression of CHS upon subsequent hapten challenge of the sensitized mice, neither mast cells nor TNF are absolutely required for either the FITC-induced DC migration (Figs. 2 and 4) or expression of CHS at sites of hapten challenge in sensitized mice (Figs. 1 and 4). Nakae et al. (9) previously reported that DC migration from the skin to draining LNs was impaired in TNF$^{-/-}$ mice at 24 h after epicutaneous application of FITC, but that the levels of DC migration in TNF$^{-/-}$ mice later caught up with those observed in wild-type mice. We found that TNF$^{-/-}$ or Kit$^{W-sh/W-sh}$ mice, which showed significantly impaired DC migration at 24 h after FITC treatment, exhibited significantly increased levels of DCs in the draining LNs, compared with corresponding levels in wild-type mice, at 48 h after FITC treatment (Fig. 6, A and B). At 72 h after FITC treatment, mice of all three genotypes (WT, TNF$^{-/-}$, or Kit$^{W-sh/W-sh}$) exhibited very similar levels of FITC-bearing DC in the draining LNs (Fig. 6, A and B).

These results indicate that the initial impairment of DC migration to local LNs observed in the absence of mast cells or TNF eventually resolves, presumably by the engagement of mast cell- or TNF-independent mechanisms. To assess whether the delay in DC migration observed in mast cell-deficient or TNF$^{-/-}$ mice resulted in significantly impaired expansion of populations of hapten-specific memory T cells, we harvested draining LNs 6 days after FITC sensitization and examined the proliferation induced in such LN cells in the presence of FITC. We found essentially identical levels of FITC-specific LN T cell proliferation in cells derived from wild-type, Kit$^{W-sh/W-sh}$, or TNF$^{-/-}$ mice (Fig. 6C). Similarly, no differences among the responses of wild-type, Kit$^{W-sh/W-sh}$, or TNF$^{-/-}$ mice were detected when FITC-induced proliferation was measured in draining LN T cells obtained from these mice at 5 days after the onset of sensitization with FITC, in experiments in which various numbers of T cells were challenged with any of multiple different concentrations of FITC (data not shown).

Thus, under the conditions of FITC sensitization examined, a deficiency in mast cells or TNF delayed the migration of hapten-bearing DCs to the local LNs but this did not significantly influence the expansion of hapten-specific memory T cells during the sensitization phase of FITC-induced CHS, at least as assessed 5 or 6 days after the initial application of hapten.

The mechanism(s) by which mast cell-associated TNF can influence the early stages of DC migration remain to be elucidated.

**FIGURE 6.** Delayed DC migration and normal FITC-specific LN responses in Kit$^{W-sh/W-sh}$ and TNF$^{-/-}$ mice. The migration of FITC$^+$ DCs at 48 and 72 h after FITC treatment was assessed, and the data expressed, as in Fig. 2, B and C. A and B. The proportion of I-A$^b$ b70 CD11c$^{high}$ FITC$^+$ cells (i.e., % FITC$^+$ DCs) was determined by FACS analysis in C57BL/6 wild-type (n = 5), Kit$^{W-sh/W-sh}$ (n = 5) and TNF$^{-/-}$ (n = 5) mice. A. Representative FACS data from one mouse of each group are shown. The upper (in bold) and lower numbers are the percentage of FITC$^+$ cells among the I-A$^b$ b70 CD11c$^{high}$ cells derived from LNs draining FITC- vs vehicle-treated ears, respectively. Shaded areas represent LNs draining vehicle-treated ears; solid lines represent LNs draining FITC-treated ears. B. Columns (□) cells from LNs draining vehicle-treated ears; ■ cells from LNs draining FITC-treated ears show the mean + SEM of values for individual mice. †, p < 0.05, ††, p < 0.005 vs corresponding values for LNs draining the FITC-treated ears of wild-type mice. For all individual groups of mice, the values for LNs draining the FITC-challenged vs corresponding vehicle-treated sites were significantly different at the p < 0.001 level. C. FITC-specific LN T cell proliferation responses in C57BL/6 wild-type (n = 5), Kit$^{W-sh/W-sh}$ (n = 5), and TNF$^{-/-}$ (n = 5) mice; data are shown as mean ± SEM.
We considered the possibility that, in CHS to FITC, as in CHS to Ox administered in ethanol (14), activation of mast cells via the FcR chain can contribute to the ability of mast cells to enhance the early stages of DC migration. We found that FcR chain−/− mice did exhibit a defect in their ability to express tissue swelling during the elicitation phase of CHS to FITC (Fig. 7A), a result that is in accord with our findings with FcR chain−/− mice in CHS to Ox (14). However, we detected no impairment of the ability of FcR chain−/− mice to exhibit FITC-induced migration of FITC DCs to draining LNs in the first 24 h of the sensitization phase of the response (Fig. 7B). Taken together, these results indicate that the requirements for FcR chain (and, by implication, Ab) function in various components of the sensitization and elicitation phases of CHS may vary, depending on the specific hapten tested and/or on other conditions of the experiment.

The same also appears to be true with respect to the roles of mast cells in the expression of CHS responses. Studies using genetically mast-cell-deficient KitW/W− mice have shown that, with some hapten/vehicle combinations, mast cells significantly contribute to the expression of various aspects of the responses (12–14). By contrast, with other hapten/vehicle combinations, no significant contributions of mast cells have been detected (32, 33). These findings suggest that the roles of mast cells in influencing different aspects of CHS responses may vary significantly depending on the hapten and/or conditions of sensitization and challenge tested.

**Mast cells, and mast cell-associated TNF, are required for optimal DC migration to LNs during the first 24 h after i.n. administration of FITC-OVA**

To examine possible effects of mast cells and mast cell-associated TNF on DC migration in a different context, we administered FITC-OVA to mice i.n. We used an Ab to 33D1 as a DC marker, rather than an Ab to MHC class II, to help to identify FITC+ DCs in a different context. We also found that migration of FITC+ DCs was assessed, and the data expressed, as in Fig. 2. A, ++++, ++++ = p < 0.001 or <0.0001 vs corresponding 24 h values for vehicle-treated ears; **, p < 0.01 vs corresponding 24 h values for wild-type (WT) mice; NS, (p > 0.05). B, ++++ = p < 0.0001 vs corresponding values for vehicle-treated ears.

**FIGURE 7.** CHS responses to FITC, and FITC-induced DC migration, in C57BL/6 WT and C57BL/6-FcRγ−/− mice. A, Ear swelling responses for vehicle-treated right ears and FITC-challenged left ears. The first and second numbers in parentheses show total number of mice in each group used for all measurements up to 24 h and at all intervals after 24 h, respectively; the numeral after # is the total number of independent experiments performed using mice in that group. Data for WT C57BL/6 mice are from Figs. 1A and 2C. B, Migration of FITC+ DCs was assessed, and the data expressed, as in Fig. 2. A, ++++, ++++ = p < 0.001 or <0.0001 vs corresponding 24 h values for vehicle-treated ears; **, p < 0.01 vs corresponding 24 h values for wild-type (WT) mice; NS, (p > 0.05). B, ++++ = p < 0.0001 vs corresponding values for vehicle-treated ears.
mast cell-derived mediators, as well as TNF, may also have sig- 

significant defects in both DC migration and the expression of CHS in mice after i.v. transfer, 8 wk before the experiment, of 1.0 × 10⁶ congenic WT BMCMCs. Then, the proportion (percent) of FITC⁺ cells among the 33D1⁺ CD11c⁺ DCs was determined by FACS. A. Representative FACS data from one mouse of each group. The upper (in bold) and lower numbers are the percentage of FITC⁺ cells among the thoracic and submaxillary LNs, respectively. Shaded areas, submaxillary LNs; bold lines, thoracic LNs. B. Columns (□), submaxillary LNs; ▽, thoracic LNs) show the average ± SEM proportion (percent) of FITC⁺ cells among the 33D1⁺ CD11c⁺ DCs in mice of each group. The numbers in parentheses represent the total number of mice in each group; the numeral after # is the total number of independent exper-

iments performed using mice in that group. ††††, p < 0.0001 versus corresponding values for thoracic LNs from Kit⁺/Kit⁺ (WT) mice or from Kit⁺/Kit⁺ mice which had been engrafted with congenic +/+ (WT) BMCMCs. For all individual groups of mice, the values for thoracic vs the corresponding submaxillary LNs were significantly different at the p < 0.0001 level.

FIGURE 8. Mast cells are required for optimal DC migration from the airways to thoracic LNs in response to intranasal administration of FITC-OVA. A and B. At 24 h after intranasal administration of FITC-OVA, thoracic and submaxillary LNs were separately collected from WBB6F₁/Kit⁺/Kit⁺ (WT) mice, Kit⁺/Kit⁺ mice and Kit⁺/Kit⁺ mice after i.v. transfer, 8 wk before the experiment, of 1.0 × 10⁶ congenic WT BMCMCs. Then, the proportion (percent) of FITC⁺ cells among the 33D1⁺ CD11c⁺ DCs was determined by FACS. A. Representative FACS data from one mouse of each group. The upper (in bold) and lower numbers are the percentage of FITC⁺ cells among the thoracic and submaxillary LNs, respectively. Shaded areas, submaxillary LNs; bold lines, thoracic LNs. B. Columns (□), submaxillary LNs; ▽, thoracic LNs) show the average ± SEM proportion (percent) of FITC⁺ cells among the 33D1⁺ CD11c⁺ DCs in mice of each group. The numbers in parentheses represent the total number of mice in each group; the numeral after # is the total number of independent exper-

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mice may have solely reflected defects in mast cell- and/or TNF-associated effector function at these sites.

The findings that mast cells, and mast cell-associated TNF, can make important contributions to the migration of hapten-bearing DCs during the initial stages of the sensitization phase of acquired, T cell-dependent immune responses are in accord with prior ob-

servations indicating that mast cells, and mast cell-derived TNF, can promote the migration of other hemopoietic cell types, includ-
ing neutrophils, monocytes, and T cells, in the context of both innate and acquired immune responses (13, 37–39). Our observa-
tions are also consistent with work with other haptons demonstrat-
ing defects in both DC migration and the expression of CHS in TNF⁻/⁻ mice (4, 9).

Although our experiments show that mast cell-associated TNF is necessary for optimal DC migration in response to FITC in the two model systems tested, we have not excluded the possibilities that other mast cell-derived mediators also can contribute to these responses or have important roles in other settings associated with DC migration. Mast cells represent a rich source of a diverse array of biologically active mediators, including histamine and many cytokines and chemokines (40, 41), many of which have potential effects on DC migration, maturation, or function (7, 42–45). And Jawdat et al. (15) showed that activation of skin mast cells with IgE and specific Ag can enhance epidermal LC migration by mechanisms that are, at least in part, both histamine and H2R dependent. Accordingly, it is possible that histamine and/or other mast cell-derived mediators, as well as TNF, may also have sig-

nificant effects on DC migration during responses to FITC.

Just as multiple mast cell-derived mediators have the potential to influence DC migration, maturation, or function during the sen-
sitzation phase of acquired immune responses, multiple mecha-
nisms probably can contribute to mast cell activation in such set-
tings. Bryce et al. (14) showed that Ag nonspecific IgE-FcɛRI signals, as well as mast cells, significantly promoted the egress of epidermal LCs when mice were sensitized with 2% Ox in ethanol. Demeure et al. (46) reported that mosquito saliva can directly induce mast cell degranulation in vitro and in vivo, in an apparently 

IgE- and specific Ag-independent manner, and that such mast cell activation can promote the migration of leukocytes, including skin DCs, to draining LNs. Although Demeure et al. (46) did not formally rule out a role for Ag-independent effects of IgE on mast cells in this model, their study showed that mosquito saliva can directly or indirectly promote the mast cell-dependent enhance-
ment of DC migration.

We found that the migration FITC⁺ DCs to local LNs during the first 24 h after application of FITC or FITC-OVA was not signifi-
cantly diminished in mice which lacked expression of FcRγ. Thus, in these skin or airway models, mast cells apparently exert their effects on DC migration independently of activation by either IgE/FcɛRI or IgG1/FcγRIII signaling. Although we have no data proving that FITC or FITC-OVA induced FcRγ-independent mast cell activation in our experiments, and, if so, by what mecha-
nism(s) this occurred, there are a number of possibilities which can be investigated.

For example, the vehicle used for administration of FITC con-
 sists of acetone and dibutylphthalate. A 1:1 mixture of acetone and dibutylphthalate can induce the migration of F4/80 Ag⁻ and macrophage C-type lectin macrophages from the mouse dermis by a mechanism which is at least in part IL-1β dependent (47). Perhaps the vehicle-induced local production of cytokines (e.g., by keratinocytes, which can produce TNF and other proinflammatory mediators; Ref. 1), can contribute to mast cell activation independently of FcRγ-dependent signals. Alterna-

tively, in our airway model, FITC-OVA might first activate alve-

olar macrophages to produce mediators which can then induce FcRγ-independent mast cell activation. Finally, dialkyl phosphates not only can enhance the Ag- and IgE-dependent activation of rat basophilic leukemia cells, but they also can directly increase concentra-
tions of cytosolic calcium ions in the cells and, at high con-
centrations, weakly promote degranulation independently of IgE and specific Ag (48). However, these represent only a few of the many possible direct or indirect mechanisms of mast cell activa-
tion which could contribute to our observation that mice lacking FcRγ exhibited no apparent defects in their ability to express mast cell-dependent enhancement of DC migration in response to FITC.

Moreover, in attempting to understand how mast cell function is elicited in this setting, it is important to recognize that the form of mast cell TNF which contributes to FITC-induced, mast cell-de-
pendent enhancement of DC migration appears to be the mem-
brane-associated form of the cytokine. Perhaps relatively subtle changes in the expression of the membrane-associated form of 

TNF by mast cells at sites of hapten challenge are sufficient to permit the elicitation of mast cell-dependent enhancement of DC migration. Possibly, even constitutive levels of expression of membrane-associated TNF are sufficient to allow mast cells to pro-

mote DC migration. In that case, initiation of DC migration by hapten may require a local signal other than an alteration of levels of mast cell-associated TNF.

Even though FcRγ-dependent signals were not essential for the enhancement of skin (or airway) DC migration, expression of 

FcRγ was required for the optimal local expression of CHS re-

ponses upon subsequent FITC challenge of sensitized mice. This may have reflected an important role for FcRγ in either the sen-
sitization or elicitation phases of CHS to FITC. For example, FcRγ
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0.001 vs corresponding values for wild-type (WT) mice. was assessed by FACS (mice that had not been challenged i.n. with FITC-OVA) prescribed in DCs in single-cell suspensions of lungs (prepared as de-

imated with TNCB, and mice that received TNCB-sensitized T cells were observed in LN T cells obtained from

mice (67). In contrast, normal levels of Ag-specific proliferation
tiation phase (66). Neutralization of TNF in the elicitation phase and this was associated with attenuated inflammation in the elic-

itters. One might even speculate that some of the clinical benefit of anti-TNF therapy in humans reflects suppression of either “effector/proinflammatory” or “immunoregulatory” functions of mast cell-derived TNF. Anti-TNF therapy has been reported to be helpful in rheumatoid arthritis (54), Crohn’s disease (55), and asthma (56), each of which has been associated with increased numbers and/or activation of mast cells at sites of disease (57–65). In such disorders, anti-TNF treatment may confer benefit in part by antagonizing effector functions of TNF derived from mast cells, as well as from the many other potential cellular sources of this cytokine.

Nevertheless, our data indicate that mast cell-associated TNF has the potential to contribute to the development of acquired immune responses involved in host defense or immunological disorders. One might even speculate that some of the clinical benefit of anti-TNF therapy in humans reflects suppression of either “effector/proinflammatory” or “immunoregulatory” functions of mast cell-derived TNF. Anti-TNF therapy has been reported to be helpful in rheumatoid arthritis (54), Crohn’s disease (55), and asthma (56), each of which has been associated with increased numbers and/or activation of mast cells at sites of disease (57–65). In such disorders, anti-TNF treatment may confer benefit in part by antagonizing effector functions of TNF derived from mast cells, as well as from the many other potential cellular sources of this cytokine.

Although the work reported herein indicates that anti-TNF treatment also has the potential to interfere with an “immunoregulatory” role of mast cell-associated TNF, the literature does not reveal a clear picture regarding the importance of TNF in the sensitization phase of CHS. On the one hand, anti-TNF Ab treat-

ment in the sensitization phase of a model of OX-induced CHS in mice almost completely suppressed DC migration to local LNs, and this was associated with attenuated inflammation in the elic-
tation phase (66). Neutralization of TNF in the elicitation phase also can suppress the development of CHS reactions to TNCB in mice (67). In contrast, normal levels of Ag-specific proliferation were observed in LN T cells obtained from TNF−/− mice sensi-
tized with TNCB, and mice that received TNCB-sensitized T cells (on DCs and/or other cell types) may be required for key steps in DC maturation or function, and/or for the optimal orchestration of the elicitation phase of the response.

The findings that mast cell-associated TNF can significantly enhance DC migration from the skin or airways in mice, as well as contribute to the effector phase of CHS responses, offer a new perspective on a large body of work implicating TNF, and specif-

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The findings that mast cell-associated TNF can significantly enhance DC migration from the skin or airways in mice, as well as contribute to the effector phase of CHS responses, offer a new perspective on a large body of work implicating TNF, and specifically mast cell-derived TNF, in a wide variety of mouse models of disease, and, by extension, in the corresponding human disorders. Thus, in mice, mast cell-derived TNF can contribute to the development of the pathology associated with certain models of sepsis (37, 49–51), cutaneous granuloma formation (52), skin vasculitis and glomerulonephritis (53), and CHS (13). In such settings, it is generally thought that mast cell-derived TNF mediates “proinflam-

matory” effects at sites of disease.

However, our data indicate that mast cell-associated TNF also can promote DC migration during the early stages of the sensitization phase of acquired immune responses, and thereby may contribute to the development of such responses. The actual extent to which mast cell-associated TNF can contribute to immune sensitization in various settings will have to be established on a case-by-case basis. For example, in the FITC-induced CHS model investigated herein, we so far have not detected a defect in immune sensitization in the absence of either mast cells or TNF, at least as can be assessed by quantifying Ag-induced proliferation of draining LN T cells obtained 5 or 6 days after application of FITC.
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Defective elicitation of delayed-type hypersensitivity in W/Wv and SI/SId

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