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Allergology International (2001) **50**: 163–169

Original Article

Passive anaphylaxis and IgE antibody production are compromised in tumor necrosis factor- and in granulocyte–macrophage colony stimulating factor-deficient mice

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Abstract

Background: A number of recent studies has demonstrated a critical role for mast cells and mast cell-derived cytokines, especially tumour necrosis factor (TNF), in the control of host defense mechanisms during inflammation. In the presesnt study, we investigated whether TNF-deficient ($TNF^{-/-}$) and granulocyte–macrophage colony stimulating factor (GM-CSF)-deficient ($GM\text{-}CSF^{-/-}$) mice expressed defects in normal mast cell function.
Methods: Because the first step in the passive cutaneous anaphylactic (PCA) reaction is fixation of the antibody to mast cells, we tried to obtain a PCA in $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice.
Results: While an anti-dinitrophenyl IgE monoclonal antibody induced a strong PCA reaction in wild-type mice, it was not possible to obtain a PCA reaction in either $TNF^{-/-}$ or $GM\text{-}CSF^{-/-}$ mice. We next examined whether mast cells were present in these mice and if so, did they have functional FcεRI receptors on their surface. The number of mast cells in smears from the peritoneal fluid of the $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice was similar to that seen in wild-type mice. However, the expression of FcεRI on mast cells from the peritoneal fluid of $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice, measured by either rosetting assay or FACScan analysis, was compromised compared with wild-type mice. Previous studies have established that defects in FCεRI expression often have found that IgE production was compromised in both $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice.
Conclusions: The observed defects may partially explain the immunodeficiency of these cytokine-deficient animals during infection.

Key words: granulocyte–macrophage colony stimulating factor-deficient mice, IgE, mast cell, passive cutaneous anaphylactic reaction, tumor necrosis factor-deficient mice.

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Received 8 August 2000. Accepted for publication 27 December 2001.

Introduction

Mast cells are derived from pluripotent cells of the bone marrow. Mast cell development in the mouse has been shown to be dependent on the cytokines interleukin (IL)-3 and IL-4, wherease granulocyte–macrophage colony stimulating factor (GM-CSF) appears to inhibit mast cell proliferation.[1] Recent studies point towards an unexpected but critical role for mast cells in resistance to infections[2,3] and these studies implicate a mast cell-derived cytokine, namely tumor necrosis factor (TNF), as a central element in this mast cell function.[2,3] However, the role of GM-CSF and TNF in mast cell function is still not established.

The most important role for mast cells has long been recognized as the effector cells of the immediate-type hypersensitivity reactions.[4] To examine the role of GM-CSF and TNF in the development and expression of mast cell effector function, we tested mice deficient for these factors. We report herein that mast cell function in both TNF$^{-/-}$ and GM-CSF$^{-/-}$ mice is compromised in immediate-type hypersensitivity.

METHODS

Mice

The generation of TNF$^{-/-}$ and GM-CSF$^{-/-}$ mice on the C57BL/6 × 129 background has been described previously.[5,6] Wild-type littermates, from the mating of heterozygous (TNF$^{+/-}$) mice were used as controls. BALB/c and C3H-scid/scid[7] mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a specific pathogen-free environment and were tested monthly to confirm their pathogen-free status. Experiments were performed on male 8–10-week-old mice in accordance with protocols approved by the New York University Institutional Animal Care and Use Committee.

Materials

Histamine dihydrochloride was obtained from Sigma Chemical Co. (St Louis, MO, USA) and was diluted in saline to 1 μg/mL. Heparin ammonium salt (Sigma Chemical Co.) was diluted in saline to 25 μg/mL for i.p. injections. Evans blue dye (Eastman Kodak, Rochester, NY, USA) was diluted in saline to 1%. Bisdiazotized benzidine (BDB) was prepared using a benzidine base (Eastman Kodak).[8] Aliquots of 0.5 mL BDB were stored at –70°C until use. Peroxidase-labeled avidin was obtained from Dako (Carpinteria, CA, USA) and bovine serum albumin (BSA) was from Armour (Kankakee, IL, USA). Sheep erythrocytes (SE) were from Colorado Serum (Denver, CO, USA). The preparation of dinitrophenylated proteins has been described elsewhere.[9,10] We used DNP25 BSA and DNP13KLH (Keyhole limpet hemocyanin (KLH)). The numbers are per one molecule of protein for BSA and for 100 000 MW for KLH. The generation of monoclonal anti-dinitrophenyl (DNP) IgE, B53 (passive cutaneous anaphylactic (PCA) reaction titer 1/16 000)[11] and rat monoclonal antimurine IgE antibody (HMK-12)[12] have been described elsewhere. The hybridoma cell line SPE7, derived from murine C57BL/6 cells, secreting monoclonal anti-DNP IgEb, was a generous gift from Dr Z Eshhar (Weizmann Institute, Rehovot, Israel). It was maintained in the Department of Rheumatology, Juntendo University, Medical School, Tokyo, Japan. The fluorescein isothiocyanate (FITC)-labeled SPE-7 was prepared using standard methods according to the manufacturer's instructions (Phar Mingen, San Diego, CA, USA). Antibody determinations were by ELISA as described elsewhere.[12,13]

Passive cutaneous anaphylactic reactions

Mice were manipulated under light ketamine anesthesia. This precaution is necessary because even minimal bruising may provoke an increase in the permeability of small venules. The dorsal skin of the mouse was shaved and injected intradermally with 0.05 mL of dilutions of B53. Four hours later, in other spots, saline (negative control) or histamine (positive control) were injected intradermally. Immediately after the intradermal injections of saline and histamine, animals were injected intravenously with 0.2 mL antigen and dye, a mixture in equal volume of 1% Evans blue in saline and 10 mg/mL DNP25BSA in saline. We also examined the PCA reaction using anti-DNP-IgG1 and antibody instead of B53. The reactions were read 20 min later and scored.[14] To confirm that mast cell numbers in the skin of mutant mice were similar to those in wild-type mice, samples of dorsal skin were harvested and mast cell numbers were quantitated. Skin samples were rolled, fixed in 10% buffered formalin and paraffin embedded in a vertical orientation. Sections were stained by Giemsa and 15 40× microscopic fields were analyzed for the prevalence of mast cells.

Mast cell isolation

Mast cells were obtained from the peritoneal cavity of mice as described elsewhere.[9,10] Briefly, 2 mL heparin (5 mg/mL) in saline was injected i.p. and the abdomen was then gently massaged for 2 min. Mice were killed by CO_2 asphyxiation and the contents of the peritoneal lavage was layered onto a gradient of BSA in Ringer's solution composed of 1 mL of 28% BSA over 1 mL of 35% BSA. The tube was centrifuged in a swinging head rotor at 270 *g* for 6 min at 4°C. The top layer and the upper half of the middle layer were removed and discarded and 3 mL Ringer's solution was added to the remaining material. The tube was then centrifuged at 250 *g* for 6 min. The cells in the bottom layer (enriched

mast cells), were washed and resuspended in 3 mL Ringer's solution for further experiments. Mast cell purity in these preparations ranged from 30 to 60%. For any one experiment, cells from all mice were prepared in parallel and the number of mast cells between preparations varied by approximately 10%.

Rosette formation

The DNP25BSA was prepared as described previously.[9] Briefly, a 0.05 mL volume of packed washed SE was added to a tube containing 1.6 mL saline, 3.5 mL of 0.11 mol/L phosphate buffer (pH 7.4) and 2.0 mL of a 500 μg/mL solution of DNP25BSA in saline. To 0.5 mL frozen BDB, 3.5 mL chilled phosphate-buffered saline was added and 0.5 mL of this solution was immediately added to the SE suspension, mixed well and allowed to react at room temperature for 10 min with occasional mixing. The coated SE were centrifuged at 500 *g* for 5 min, washed once with phosphate buffer and once with saline before being resuspended in saline to a concentration of 1% (volume). These DNP25BSA-coated SE (DNP-SE) showed a minimal tendency either to agglutinate spontaneously or to form non-specific rosettes around normal mast cells.

A 1 mL sample of the 1% DNP-SE solution was mixed with 1 mL of a 1/10 dilution in saline of B53, kept at room temperature for 90 min with frequent swirling, then centrifuged at 250 *g* for 5 min, washed twice with saline and finally suspended in 1 mL saline. This preparation was used to examine rosette formation with mast cell-enriched peritoneal cells.

A sample of 0.05 mL enriched mast cell suspension and 0.05 mL B53-fixed DNP-SE suspension (see above) was incubated for 30 min with frequent gentle mixing at room temperature and examined following the addition of 0.01 mL of 0.1% Toluidine blue by microscope in a counting chamber.

FACScan examination

To measure expression of FcεR1, we used FITC-labeled monomeric IgE, which binds preferentially to this high-affinity receptor.[13] A small aliquot (0.05 mL) of the enriched mast cell preparation was incubated with 0.05 mL FITC-labeled SPE-7 (10 μg/mL final dilution) for 1 h at 4°C, followed by centrifugation for 5 min at 250 *g*, washed twice and analyzed on a FACScan (Becton Dickinson, San Diego, CA, USA).

IgE production

Eight-week-old mice, were injected i.p. with 10 μg DNP13KLH and 4 mg aluminum hydroxide. Mice were re-injected i.p. with the same amount of this mixture 2 weeks later. Serum was obtained on days 14, 21 and 28 after the primary immunization for anti-DNP IgE determination by ELISA.[12,13]

RESULTS

Passive cutaneous anaphylactic reactions

Passive cutaneous anaphylactic reactions obtained in BALB/c, wild-type, GM-CSF$^{-/-}$, TNF$^{-/-}$ and SCID mice are given in Table 1. Analysis of the prevalence of mast cells in dorsal skin indicated no reduction in mast cell numbers in any of the mutant mice compared with wild-type mice. (The number of mast cells harvested from the dorsal skin dermis of each strain was as follows: BALB/c, $12.6 \pm 1.6 \times 10^4$ /mm^2; wild type $9.8 \pm 1.1 \times 10^4$ /mm^2; GM-CSF$^{-/-}$, $9.5 \pm 1.2 \times 10^4$ /mm^2; TNF$^{-/-}$, $10.7 \pm 1.9 \times 10^4$ /mm^2; SCID, $11.3 \pm 1.4 \times 10^4$ /mm^2.) None of the mice reacted to injection of saline (negative control) and

Table 1 Passive cutaneous anaphylaxis in wild-type and granulocyte–macrophage colony stimulating factor$^{-/-}$ and tumor necrosis factor$^{-/-}$ mice

Injection	Mouse strain				
	BALB/c	Wild type	GM-CSF$^{-/-}$	TNF$^{-/-}$	SCID
Saline	–	–	–	–	–
Histamine (1 μg/mL)	+	+	+	+	+
B53 1/4000	++	+	–	–	–
B53 1/8000	+	+	–	–	–
IgG1 1/1000	+	+	–	–	ND

Dilutions of anti-DNP IgE monoclonal antibody (B53) or anti-DNP IgG1, IgG2a were injected intradermally 4 h before challenge, while histamine and saline were injected just before challenge with 1 mg DNP25BSA in 0.5% Evans blue dye in saline. Reactions were read 20 min after challenge. –, no reaction; +, reactions between 10 and 15 mm; ++, reactions greater than 15 mm.

all reacted to intradermal injection of histamine (positive control). The threshold reaction in BALB/c and wild-type mice was obtained with a dilution of 1/8000 anti-DNP B53 antibody. As shown in Table 1, no reaction was obtained with a 1/4000 dilution of B53 in GM-CSF$^{-/-}$, TNF$^{-/-}$ or SCID mice. Even with a 1/100 dilution of B53, we did not obtain any reactions in GM-CSF$^{-/-}$, TNF$^{-/-}$ or in SCID mice. The 1/1000 dilution of IgG1, which was able to produce a PCA reaction in wild-type mice was unable to induce a PCA reaction in GM-CSF$^{-/-}$ or TNF$^{-/-}$ mice.

Rosette formation with IgE-coated DNP-SE

Examination of mast cell-enriched peritoneal cell preparations after staining with Wright–Giemsa showed approximately equal numbers of mast cells in wild-type, TNF$^{-/-}$, GM-CSF$^{-/-}$ and SCID mice. (The number of mast cells isolated from the peritoneal cavity of each strain was as follows: BALB/c, $4.2 \pm 1.0 \times 10^4$ /mouse; wild type, $4.1 \pm 0.6 \times 10^4$ /mouse; TNF$^{-/-}$, $3.9 \pm 0.8 \times 10^4$ /mouse GM-CSF$^{-/-}$, $3.7 \pm 0.5 \times 10^4$ /mouse; SCID^{-}, $4.6 \pm 0.4 \times 10^4$ /mouse). Next, we examined fresh preparations of these peritoneal mast cell-enriched peritoneal fluid samples admixed with an equal volume of DNP25BSA and IgE (B53)-coated sheep erythrocytes in a rosetting assay. In the present study, a rosette was defined as only those nucleated cells around which at least four erythrocytes were found. We counted at least 100 rosettes on a slide covered with mast cell-enriched peritoneal fluid from BALB/c or wild-type mice, but we did not find any rosettes when peritoneal cells were taken from GM-CSF$^{-/-}$, TNF$^{-/-}$ or SCID mice (Fig. 1).

FACScan examination

The FACScan (Fig. 2) examination of the mast cell-enriched peritoneal cells incubated with the FITC-labeled

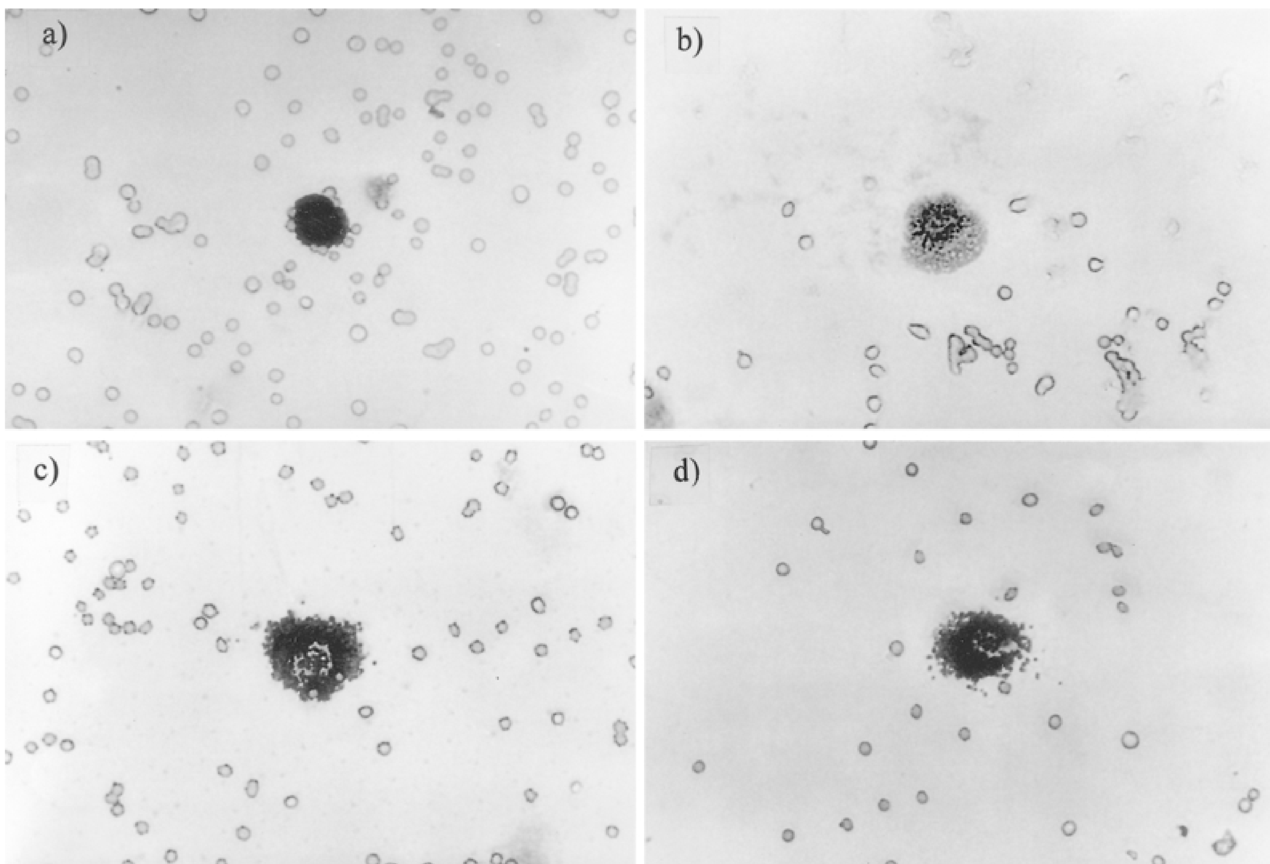

Fig. 1 Rosette formation of peritoneal mast cells with DMP25BSA-labeled sheep erythrocytes coated with anti-dinitrophenyl (DNP) IgE. A 0.050 mL sample of washed mast cell preparations was mixed with 0.05 mL of a 1/100 suspension of DNP25BSA and anti-DNP-coated sheep erythrocytes (see Methods). (a) Wild-type mice, (b) tumor necrosis factor (TNF)$^{-/-}$ mice, (c) granulocyte–macrophage colony stimulating factor (GM-CSF)$^{-/-}$ mice and (d) SCID mice.

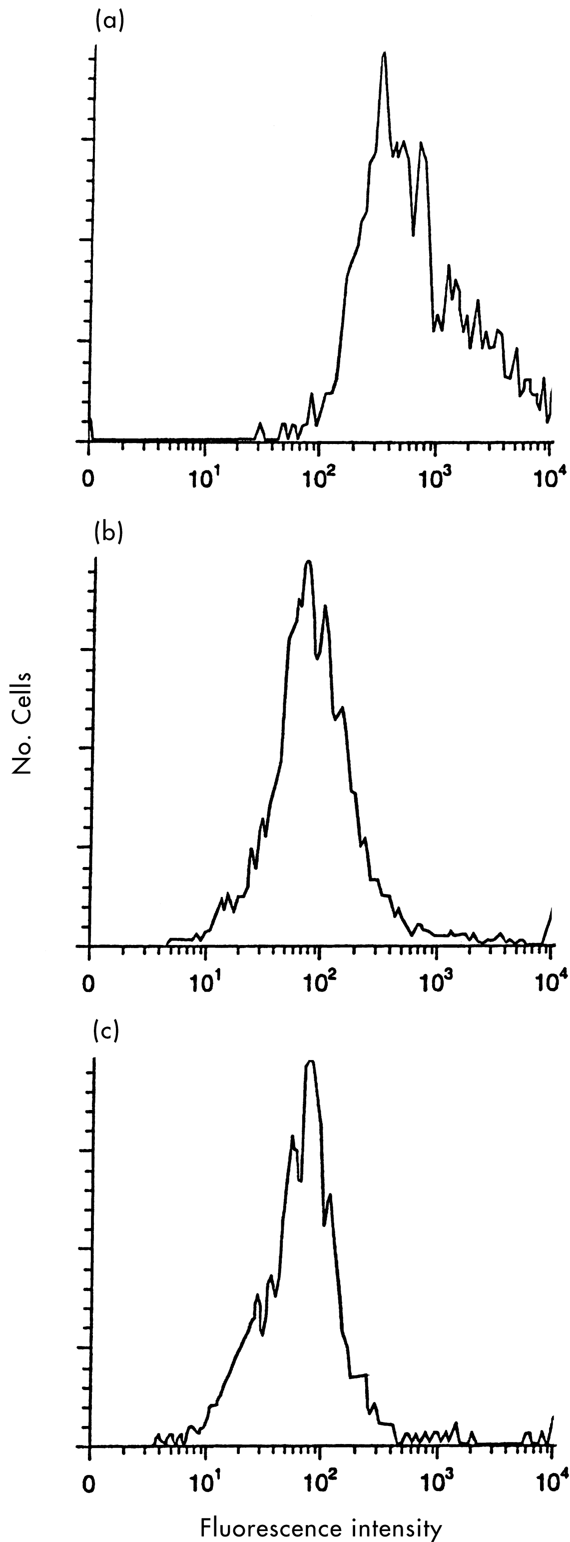

Fig. 2 FACScan analysis of FCεR1 expression of peritoneal mast cells. A sample of 1 × 10^5 mast cell-enriched peritoneal cells was suspended in 50 μL staining buffer, to which 5 μg fluorescein isothiocyanate-labeled SPE-7 in 50 μL staining buffer was added, and this mixture was incubated for 1 h at 4°C with occasional mixing, then washed three times with phosphate-buffered saline and examined by flow cytometry. (a) Wild-type mice, (b) granulocyte–macrophage colony stimulating factor (GM-CSF)$^{-/-}$ mice, (c) tumor necrosis factor (TNF)$^{-/-}$ mice.

murine monoclonal IgE (SPE-7) from both GM-CSF$^{-/-}$ and TNF$^{-/-}$ mice showed a very similar image and differed from that of wild-type mice. The image seen for mast cell-enriched peritoneal cells from wild-type mice treated with the same FITC-labeled monoclonal IgEb was shifted to the right and had a shoulder.

IgE production

Anti-DNP IgE antibody production in wild-type, GM-CSF$^{-/-}$ and TNF$^{-/-}$ mice is shown in Fig. 3. None of the mice produced much anti-DNP IgE antibody after primary immunization at day 14. However, at days 21 and 28 (7 and 14 days after boost injections) significantly less antibody was produced in both GM-CSF$^{-/-}$ and TNF$^{-/-}$ mice compared with wild-type controls.

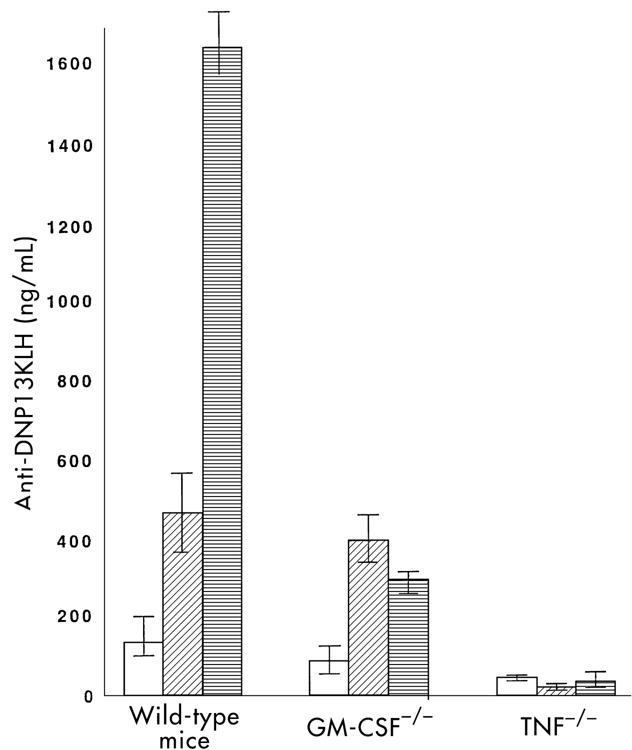

Fig. 3 IgE antibody production. Anti-dinitrophenyl (DNP) antibody production in wild-type, tumor necrosis factor (TNF)$^{-/-}$ and granulocyte–macrophage colony stimulating factor (GM-CSF)$^{-/-}$ mice. Data are mean (±SD) serum anti-DNP13KLH levels (ng/mL) in three mice measured at day 14 (□), 21 (▨) and 28 (▤) after immunization.

Discussion

In the present study, we demonstrated that: (i) we could not get a PCA reaction in $TNF^{-/-}$, $GM\text{-}CSF^{-/-}$ and in SCID mice; (ii) we could not see rosettes on peritoneal mast cells incubated with anti-DNP IgE-labeled SE; and (iii) FACScan analysis confirmed these rosetting assays. We also showed that $GM\text{-}CSF^{-/-}$ and $TNF^{-/-}$mice differ from wild-type mice in the production of IgE antibody when immunized with the DNP13KLH at either 21 or 28 days. The anti-DNP IgE production is much less in both the $GM\text{-}CSF^{-/-}$ and $TNF^{-/-}$ strains than in wild-type mice.

We examined whether $GM\text{-}CSF^{-/-}$, $TNF^{-/-}$ and SCID mice could be sensitized with murine IgE antibodies to produce anaphylactic reactions. The PCA reaction eliminates the problem of antibody production, a significant concern in TNF- and GM-CSF-deficient mice, both of which have been shown to exhibit defects in antibody production.[5,15–17] Although the number of mast cells harvested from the dorsal skin dermis of each strain was not significantly different, the PCA reaction of both $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice was compromised similar to SCID mice, which have been shown, using a different model,[18] to have a defective anaphylactic response.

It is well known that the first step of such anaphylactic reactions is mainly the bridging of two IgE molecules fixed on mast cells[19] mediated by binding through the Fc domain.[20] Mast cells normally possess on their surface the high-affinity receptors for IgE (FcεRI), a complex multimolecular structure.[21–23] Mast cells from wild-type mice are expected to express FcεRI molecule on their cell surface. Therefore, we examined whether the FCεRI is present or absent on the surface of mast cells of wild-type, $GM\text{-}CSF^{-/-}$, $TNF^{-/-}$ or SCID mice by direct examination of peritoneal mast cells with SE coupled to a DNP protein and covered with a monoclonal anti-DNP IgE antibody. The number of peritoneal mast cells in $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice was not significantly different compared with wild-type mice. However, in the case of rosette formations, many mast cell rosettes were found in peritoneal cells of wild-type mice, but we did not find any rosettes in peritoneal cells of the $TNF^{-/-}$, $GM\text{-}CSF^{-/-}$ or SCID mice. We also studied PCA reactions using anti-DNP IgG1 antibody because anti-DNP IgG1 antibody can induce a PCA reaction via FcγRIII in BALB/c mice.[24] The wild-type mice could be sensitized with IgG1 antibody to produce anaphylactic reactions, like BALB/c mice; however, the PCA reaction of both $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice was compromised. We could not induce rosette formation with IgG1-coated DNP-SE in deficient mouse and also in wild-type mice because the affinity of FcγRIII and FcγRIIB is very low. Further studies are needed to establish the expression of the low-affinity FcγRIII and FcγRIIB on mast cells of these deficient mouse strains, but there is a possibility that FcγRIIB expression may be depressed and/or FcγRIIB expression may be increased in $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice.

In other experiments, we examined peritoneal mast cells from wild type, $TNF^{-/-}$ or $GM\text{-}CSF^{-/-}$ mice with FITC-labeled SPE-7, a murine monoclonal IgE antibody, for the detection of FcεRI on peritoneal mast cells using FACScan. For the FACScan experiments, peritoneal mast cells were incubated with FITC-labeled SPE-7. As shown in Fig. 2, the image of the control cells from wild-type mice (Fig. 2a) was displaced to the right, whereas cells from both the $TNF^{-/-}$ (Fig. 2b) and $GM\text{-}CSF^{-/-}$ (Fig. 2c) were the same and both were shifted to the left relative to control.

The importance of mast cells in biological reactions, as versatile effector cells, was reviewed recently.[21] Two extensive reviews of Fc receptors, including those on mast cells, were recently published.[22,23] Moreover, it has been shown recently that mice genetically manipulated to have no genes for IgE production and, therefore, deprived of IgE antibody, can still produce general and also cutaneous anaphylactic reactions.[18] This fact is not unexpected, because it has been known that mice can be sensitized to produce anaphylactic reactions, not only by IgE, but also by IgG1[23] and, less effectively, by IgG2, but not by IgM or IgA antibodies.[10] In this respect, it is interesting to note that it was the study of guinea pig antibodies[25–28] that led to the understanding that different IgG molecules exist (i.e. IgG1, IgG2 etc.). Each IgG differs from other IgG by its Fc fragment and the Fc fragment carries the biological activities, as first shown in 1961.[20,29] Recently, it has also been shown that the expression of FCεRI on mast cells may be amplified by the IgE molecule. Decreased expression of FcεRI often coexists with compromised expression of IgE antibody, so that FcεRI on mast cells in $IgE^{-/-}$ mice is 81% less than in $IgE^{+/+}$ mice.[30,31] Our finding that $TNF^{-/-}$ and $GM\text{-}CSF^{-/-}$ mice have a parallel depression in IgE production and FcεRI expression is consistent with these previous observations. Taken together, our results indicate a central regulatory role for TNF and GM-CSF in the expression of critical mast cell functions.

ACKNOWLEDGMENTS

We thank Dr Yuji Noguchi (Department of Immunology, Okayama University, Okayama, Japan) for helpful suggestions and the generous gift of both TNF$^{-/-}$ and GM-CSF$^{-/-}$ mice.

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