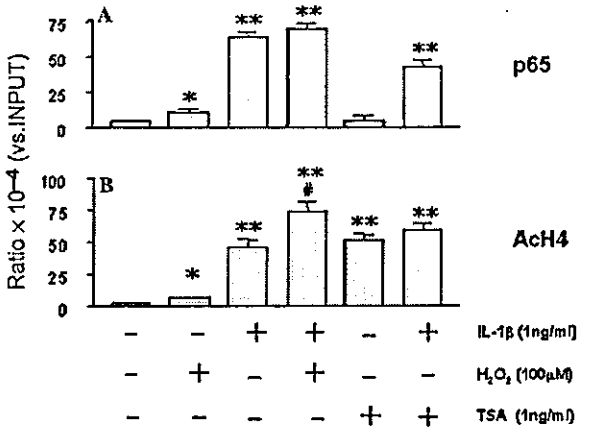

Fig. 3. Chromatin immunoprecipitation assay shows that H_2O_2 does not enhance p65 DNA binding but increases histone H4 acetylation at the IL-8 promoter. BEAS-2B cells were pretreated with H_2O_2 (100 μM) for 4 h before incubation with IL-1β (1 ng/ml). After 30 min, proteins and DNA were cross-linked by formaldehyde treatment and chromatin pellets were extracted. Following sonication, NF-κB p65 subunit (A) and acetylated histone H4 (B) were immunoprecipitated and the associated DNA was amplified by PCR. Results are representative of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with control; $^\#p < 0.05$ compared with IL-1-stimulated.

against acetylated histone 4 resulted in the enrichment for the DNA segments encompassing the GM-CSF promoter following IL-1β treatment (Fig. 3B). H_2O_2 did not effect IL-1β-induced enrichment of p65-associated IL-8 promoter fragments but caused an enhancement in the enrichment of acetylated H4-associated IL-8 promoter fragments (Fig. 3). This suggests that enhanced histone acetylation at the NF-κB site is related to either enhanced HAT activity or reduced HDAC activity.

H_2O_2 represses HDAC activity and expression

Pretreatment with H_2O_2 (100 μM, 4 h) reduced basal HDAC activity (330 ± 15 versus 499 ± 91 dpm/μg protein) and inhibited IL-1β-stimulated HDAC activity (487 ± 20 versus 898 ± 64 dpm/μg protein) (Fig. 4A). This effect occurred prior to changes in HDAC2 protein expression since H_2O_2 decreased HDAC2 expression only after 24 h (Fig. 4b). This effect of H_2O_2 was concentration-dependent and reached plateau at 100 μM (Fig. 4C).

Oxidative stress inhibits HDAC activity

IL-1β induced tyrosine phosphorylation of HDAC2, which was associated with a concomitant increase of HDAC activity (data not shown). This IL-1β-induced increase in HDAC2 activity was inhibited by alkaline phosphatase pre-treatment (basal, 381 ± 84; IL-1β, 667 ± 140; IL-1β + alkaline phosphatase, 410 ± 94 dpm/μg protein).

H_2O_2 induced nitration of HDAC2 from 0.5 h after stimulation, which peaked at 4 h, and was still elevated at 24 h (Fig. 5A). Peroxynitrite (500 nM) also enhanced tyrosine nitration of HDAC2 but this did not affect basal HDAC2 activity, but inhibited IL-1β-stimulated HDAC activity (Fig. 5B). Furthermore, SIN-1 (500 μM), a peroxynitrite generator, did not affect basal HDAC activity but significantly ameliorated the IL-1β-induced HDAC2 activity (basal, 107 ± 12; SIN-1, 76 ± 8; IL-1β, 165 ± 7; and IL-1β + SIN-1, 76 ± 6 dpm/μg protein) (Fig. 5C). This correlated with the ability of SIN-1 to enhance IL-1β-induced IL-8 production (2196 ± 22 versus 1091 ± 89 ng/ml) (Fig. 5C). SIN-1 also

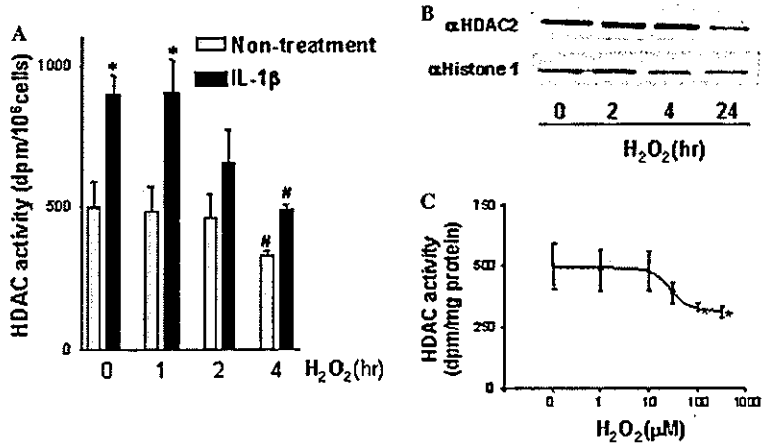

Fig. 4. Time- and concentration-dependent effect of H_2O_2 on HDAC activity and expression. (A) H_2O_2 (100 μM) directly inhibits HDAC activity in BEAS-2B cells at 4 h. Cells were collected at 1 h after IL-1β (1 ng/ml) stimulation, nuclear protein was extracted, and total HDAC activity was measured using [^{3}H]acetate incorporated histone. Results are expressed as means ± SEM for three experiments. $^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to $t = 0$. (B) Western blot analysis of HDAC2 in nuclear extracts of BEAS-2B cells shows decreased expression following H_2O_2 (100 μM) stimulation for 24 h. Histone 1 protein expression was detected as control. (C) Concentration-dependent inhibition of HDAC activity by H_2O_2 after 4 h incubation. $^*p < 0.05$ compared to control

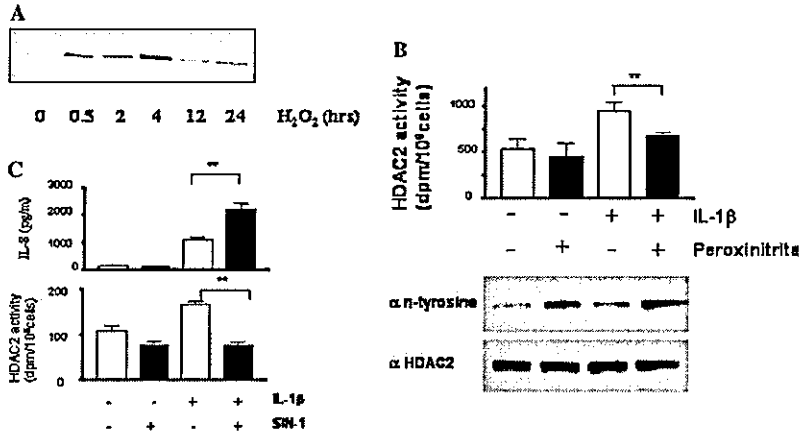

Fig. 5. H_2O_2 and peroxynitrite induce HDAC2 tyrosine nitration and suppression of HDAC activity. (A) Western blotting analysis of H_2O_2-induced tyrosine nitration of HDAC2 in BEAS-2B cells. Cells were collected after the indicated time, HDAC2 immunoprecipitated, and nitrotyrosine levels were determined. (B) Peroxynitrite directly regulates HDAC2 activity and nitration. Cells were stimulated with IL-1β (1 ng/ml) or control medium and after 1 h were collected, lysed with immunoprecipitation buffer, and immunoprecipitated with anti-HDAC2 antibody. Peroxynitrite (500 nM) was incubated with the immunoprecipitates from IL-1β-treated or non-treated cells for 10 min at 30 °C. HDAC activity assay and Western blotting for nitro-tyrosine and HDAC2 protein were performed. Results are expressed as means ± SEM ($n = 3$). $^{**}p < 0.01$ compared to IL-1β-stimulation. (C) SIN-1 (500 μM) attenuates HDAC2 immunoprecipitated HDAC2 activity and enhanced IL-8 production. Results are expressed as means ± SEM ($n = 3$). $^{**}p < 0.01$ compared to IL-1β-stimulation.

decreased HDAC1 and HDAC3 activity by 71% and 65% of IL-1β-induced HDAC activity, respectively. H_2O_2 (100 μM) and SIN-1 (500 μM) produced peroxynitrite at 3.6 ± -0.65 and 17.4 ± 0.61 μM, respectively.

Discussion

In this study we have demonstrated that H_2O_2 and peroxynitrite enhanced IL-1β-induced expression of inflammatory cytokines, such as GM-CSF and IL-8. This effect could be blocked by the anti-oxidant NAC. Using chromatin immunoprecipitation assays in cells treated with IL-1β plus H_2O_2, the IL-8 promoter region was associated with much greater levels of acetylated histone H4 than those after IL-1β stimulation alone. Under the same conditions, there was no change in p65-associated IL-8 promoter suggesting no increased NF-κB DNA binding at this time. A lack of enhancement of NF-κB activation was confirmed by Western blotting, immunocytochemistry, and DNA binding activity assay (TransAM).

H_2O_2 alone reduced HDAC2 activity after 4 h but its expression reduced only at 24 h or longer. H_2O_2 also reduced IL-1β-stimulated HDAC activity at 4 h-pretreatment and the reduction in total HDAC activity correlated with the enhancement of IL-1β-induced cytokine production. At this time point there was no reduction in HDAC expression, suggesting that a post-translational modification of HDAC is involved in attenuating HDAC activity. These data suggested that enhancement of IL-1β-induced cytokine production by H_2O_2 is not associated with NF-κB activation, but with enhancement of histone acetylation by inhibition of HDAC2 activity.

Peroxynitrite, the product of the reaction between nitric oxide (NO) and superoxide anions, is a much stronger oxidant than NO or H_2O_2. As well as having a strong cytotoxic effect [2], it is also reported to change protein function via the nitration of the *ortho*-position of tyrosine residues. For example, peroxynitrite-mediated nitration of a single tyrosine residue in the cell cycle kinase cdc2 prevents tyrosine phosphorylation in endothelial cells [16]. In addition, peroxynitrite-induced nitration of tyrosine residue of EGF receptor prevents their dimerization [17]. Peroxynitrite-mediated nitration of tyrosine residues has also been shown to inactivate the mitochondrial Mn superoxide dismutase [18], the lipid aggregatory activity of surfactant protein A [19], and glutamine synthetase activity [20]. In our system, nitration of HDAC inhibited the phosphorylation-associated increase in HDAC activity induced by IL-1β (data not shown) and confirm previous data showing that enhanced HDAC activity is associated with phosphorylation [21]. This initial increase in HDAC activity may be associated with a feedback resolution of gene transcription [22]. H_2O_2 nitrated HDAC2, which is peaked at 4 h. This correlated with the HDAC activity and enhancement of IL-1β-induced cytokine production. SIN-1, a peroxynitrite generator, gave the same effect as peroxynitrite. In these cells we obtained similar levels of peroxynitrite formation with SIN-1 and H_2O_2 possibly due to the low endogenous levels of cNOS present in the cells since iNOS is not induced until much later time points [23]. Further experiments using mass

spectrometry and peptide fingerprinting are required to determine whether nitration of distinct tyrosine residues antagonizes the enhanced HDAC activity associated with phosphorylated tyrosine residues.

Neutrophils, eosinophils, and alveolar macrophage produce ROS and nitric oxide, which interact to produce peroxynitrite. Nitro-tyrosine, which is a footprint of peroxynitrite formation, is increased in COPD and bronchial asthma [2]. Reduction in HDAC activity by oxidative stress might be one of the factors that worsen inflammatory disease.

In summary, we have shown in vitro ROS enhanced IL-1β-induced inflammatory cytokine release by reduction of HDAC activity acting via tyrosine nitration. This is a novel mechanism for the enhancement of inflammation. These studies suggest that there is a potential to develop novel therapeutic agents with improved anti-inflammatory properties that have improved HDAC activation properties and highlight the potential for anti-oxidant therapy in the treatment of chronic inflammatory diseases.

Acknowledgments

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TWEAK/Fn14 interaction stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF

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Abstract

TNF-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor (TNF) family, is a multifunctional cytokine that regulates cellular proliferation, angiogenesis, inflammation, and apoptosis. In this study, we investigated the effect of TWEAK on human bronchial epithelial cells. A human bronchial epithelial cell line, BEAS2B, expressed a TWEAK receptor, fibroblast growth factor-inducible 14 (Fn14), and produced IL-8 and GM-CSF upon TWEAK stimulation in a dose-dependent manner, which was abrogated by anti-Fn14 blocking antibody. TWEAK induced phosphorylation of IκBα and BAY11-7082, a selective inhibitor of IκBα phosphorylation, inhibited the TWEAK-induced IL-8 and GM-CSF production by BEAS2B cells. Moreover, primary cultured human bronchial epithelial cells also expressed Fn14 and produced IL-8 and GM-CSF upon TWEAK stimulation. Collectively, TWEAK stimulated human bronchial epithelial cells to produce IL-8 and GM-CSF through Fn14. Because IL-8 and GM-CSF are associated with inflammatory conditions, these results suggest that TWEAK/Fn14 interaction may play some roles in airway inflammatory responses.

Keywords: TWEAK; Fn14; IL-8; GM-CSF; Bronchial epithelial cell; IκBα

TNF-like weak inducer of apoptosis (TWEAK) was first identified as a new member of the tumor necrosis factor (TNF) superfamily which induced cell death in some tumor cell lines [1]. However, recent studies have revealed that TWEAK is a multifunctional cytokine that regulates cellular proliferation, angiogenesis, and inflammation [2]. TWEAK not only induced cell death in certain tumor cell lines [3,4], but also induced proliferation of endothelial cells and angiogenesis [5], upregulated cell surface expression of adhesion molecules and induced secretion of chemokines such as IL-8 and MCP-1 in human umbilical vein endothelial cells [6], and induced IL-8, IP-10, and RANTES in human dermal fibroblasts and synoviocytes [7].

The human TWEAK gene is expressed in many different cell types and encodes for a ~30 kDa type II transmembrane protein that can be cleaved to generate an ~18 kDa soluble factor with biological activity [2]. Recently, Wiley et al. [8] have identified fibroblast growth factor-inducible 14 (Fn14) as a TWEAK receptor with physiological affinity. Fn14 is a type I transmembrane protein composed of only one cysteine-rich domain in the extracellular region and a short cytoplasmic region containing a TRAF-binding motif [9]. Saitoh et al. [10] recently reported that TWEAK stimulated two NF-κB signaling pathways; IκBα phosphorylation and p100 processing via TRAF molecules. These findings suggest that NF-κB pathway mediates TWEAK/Fn14 signaling.

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In this study, we investigated Fn14 expression on human bronchial epithelial cells and examined the effect of TWEAK on chemokine/cytokine production by human bronchial epithelial cells. We found that TWEAK stimulated human bronchial epithelial cells to produce IL-8 and GM-CSF via Fn14. The results suggest that TWEAK/Fn14 interaction may contribute to the development of airway inflammatory responses through production of proinflammatory IL-8 and GM-CSF by bronchial epithelial cells.

Materials and methods

Reagents. Recombinant human TWEAK was purchased from Alexis Biochemicals (San Diego, CA). Anti-human Fn14 monoclonal antibodies (mAbs) (ITEM-1 and ITEM-2) were generated in our laboratory as previously described [11]. ITEM-2 is a blocking antibody preventing the interaction between TWEAK and Fn14 [11]. A selective inhibitor of IκBα phosphorylation, BAY11-7082, was purchased from Calbiochem (La Jolla, CA).

Cell culture. A human bronchial epithelial cell line, BEAS2B [12], was maintained in F-12 nutrient mixture medium (HAM) with L-glutamine (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml streptomycin. Normal human bronchial epithelial cells were purchased from BioWhittaker/Clonetics (Walkersvile, MD) and cultured in serum-free bronchial epithelial cell growth medium, SABM (BioWhittaker/Clonetics, Walkersvile, MD), containing epidermal growth factor (EGF) (0.5 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine (5 μg/ml), transferrin (10 μg/ml), retinoic acid (0.1 ng/ml), triiodothyronine (6.5 ng/ml), BSA-FAF (0.5 mg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and bovine brain pituitary extract (30 μg/ml) at 37 °C in humidified atmosphere in presence of 5% CO_2. The experiments were carried out using the cells at third or fourth passage.

Flow cytometric analysis for Fn14 expression. Cells (1×10^6) were incubated with biotinylated anti-human Fn14 mAb (ITEM-1) for 1 h at 4 °C, followed by PE-labeled avidin (BD Pharmingen, San Diego, CA). After washing with PBS, the cells were analyzed on a FACSCalibur (BD, San Jose, CA) and the data were analyzed by using the CellQuest program (BD).

ELISA. BEAS2B cells or normal human bronchial epithelial cells (1×10^6/well) were stimulated with recombinant human TWEAK or, in some experiments, with recombinant human TNF-α (R&D, Minneapolis, MN), for the indicated time period and the concentrations of human IL-8, TARC, Eotaxin, and GM-CSF in the culture supernatants of the cell cultures were determined by using the ELISA kits (R&D, Minneapolis, MN) according to the manufacturer's instruction. In some experiments, 10 μg/ml anti-human Fn14 mAb (ITEM-2) or isotype-matched control Ig, or 2 μM of BAY11-7082, was added in the cell cultures 1 h before the addition of TWEAK. This dose of BAY11-7082 did not affect cell viability and inhibited IκBα phosphorylation in our preliminary experiments and previous literatures [13,14].

Western blot. BEAS2B cells stimulated with recombinant human TWEAK (100 ng/ml) for the indicated time periods were suspended in RIPA buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, and 20 mM β-glycerophosphate. Extracts were cleared by centrifugation. Whole cell extracts (10 μg) were fractionated on 9–15% SDS–polyacrylamide gels and transferred onto immobilon-P membrane. Immunoblotting was performed using the phosphoPlus IκBα (Ser32) antibody kit (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's instruction or using antibodies against phosphorylated (Ser536) or non-phosphorylated NF-κB p65 (Cell Signaling Technology, Beverly, MA, USA).

Data analysis. Data are summarized as means ± SD of triplicate samples. Statistical analysis was performed by the unpaired Student's *t* test. A value of $P < 0.05$ was considered to be significant.

Results

A human bronchial epithelial cell line, BEAS2B, expresses Fn14

To determine whether TWEAK can act on human bronchial epithelial cells, we first examined the expression of Fn14 on a human bronchial epithelial cell line, BEAS2B. Flow cytometric analysis using anti-Fn14 mAb [11] showed a higher fluorescence from cells incubated with anti-Fn14 mAb compared to those incubated with control antibody (Fig. 1), indicating that Fn14 was significantly expressed on the cell surface.

TWEAK induces IL-8 and GM-CSF production by BEAS2B cells

Since BEAS2B cells expressed Fn14, we then investigated whether TWEAK could stimulate BEAS2B cells through Fn14. Human bronchial epithelial cells are known to produce several chemokines/cytokines associated with inflammation such as IL-8 and GM-CSF upon various stimuli [15]. We thus examined the effect of recombinant TWEAK on IL-8 and GM-CSF production by BEAS2B cells. As shown in Figs. 2A and B, TWEAK induced IL-8 production in a dose- or time-dependent manner, which was almost completely inhibited by blocking the TWEAK/Fn14 interaction with anti-Fn14 mAb (Fig. 2C). We also found that

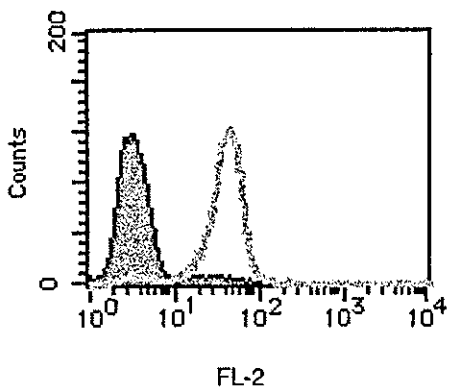

Fig. 1. Cell surface expression of Fn14 on BEAS2B cells. BEAS2B cells were stained with biotinylated anti-human Fn14 mAb (open histogram) or control Ig (filled histogram), followed by PE-labeled avidin, and then analyzed by flow cytometry. A representative of three repeated experiments with similar results is shown.

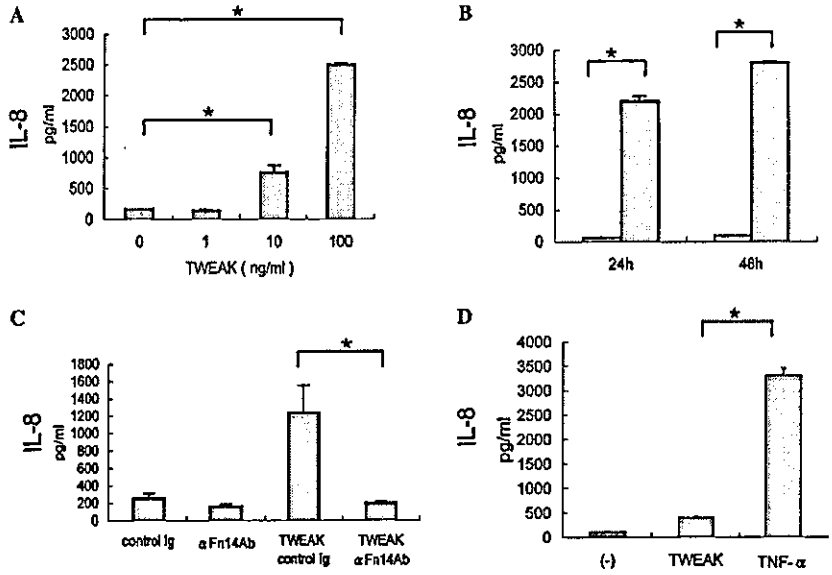

Fig. 2. Tweak induces IL-8 production by BEAS2B cells via Fn14. BEAS2B cells were stimulated with the indicated doses of TWEAK for 48 h (A) or stimulated with 100 ng/ml TWEAK for 24 or 48 h (B), or stimulated with 10 ng/ml TWEAK for 48 h in the presence of 10 μg/ml anti-Fn14 blocking mAb or control Ig (C) or stimulated with 10 ng/ml TWEAK or 10 ng/ml TNF-α for 48 h (D). Human IL-8 concentration in the culture supernatant was measured by ELISA. Data are indicated as means ± SD of triplicate samples. $*p < 0.05$. Similar results were obtained in three repeated experiments.

TWEAK induced GM-CSF production by BEAS2B cells, which was again inhibited by anti-Fn14 mAb (Figs. 3A and B). No significant production of MCP-1, TARC, or Eotaxin was observed upon TWEAK stimulation of BEAS2B cells (data not shown). The IL-8 and GM-CSF production induced by TWEAK was significantly less than that induced by TNF-α (Figs. 2D and 3C). These findings indicated that TWEAK stimulated BEAS2B cells to produce IL-8 and GM-CSF via Fn14.

Phosphorylation of IκBα is critical for TWEAK-induced IL-8 and GM-CSF production by BEAS2B cells

To explore the mechanisms by which TWEAK stimulated BEAS2B cells, we investigated IκBα phosphorylation in TWEAK-stimulated BEAS2B cells. Saitoh et al. [10] showed that TWEAK induced phosphorylation of IκBα, resulting in NF-κB activation in fibroblasts. As shown in Fig. 4A, we found that TWEAK induced phosphorylation of IκBα and also NF-κB p65 at 20–60 min after the stimulation in BEAS2B cells. Moreover, a selective inhibitor of IκBα phosphorylation, BAY11-7082, substantially inhibited TWEAK-induced IL-8 and GM-CSF production by BEAS2B cells (Fig. 4B). These results suggested that phosphorylation of IκBα was critical for TWEAK-induced IL-8 and GM-CSF production by BEAS2B cells.

Primary cultured human bronchial epithelial cells express Fn14 and produce IL-8 and GM-CSF upon TWEAK stimulation

In order to address the physiological relevance, we examined the effect of recombinant human TWEAK on primary cultured human bronchial epithelial cells. We found that normal human bronchial epithelial cells expressed Fn14 and produced IL-8 and GM-CSF upon TWEAK stimulation (Fig. 5). These results indicated that TWEAK induced IL-8 and GM-CSF production not only in an immortalized bronchial epithelial cell line (BEAS2B) but also in primary cultured bronchial epithelial cells.

Discussion

In this study, we demonstrated that TWEAK induced IL-8 and GM-CSF production by BEAS2B cells through Fn14 expressed on their surface (Figs. 1–3). Phosphorylation of IκBα was critical for the TWEAK-induced IL-8 and GM-CSF production by BEAS2B cells (Fig. 4). In addition, not only BEAS2B cells but also primary cultured human bronchial epithelial cells expressed Fn14 and produced IL-8 and GM-CSF upon TWEAK stimulation (Fig. 5). These findings suggest that TWEAK/Fn14 interaction stimulates human bronchial epithelial cells to produce proinflammatory

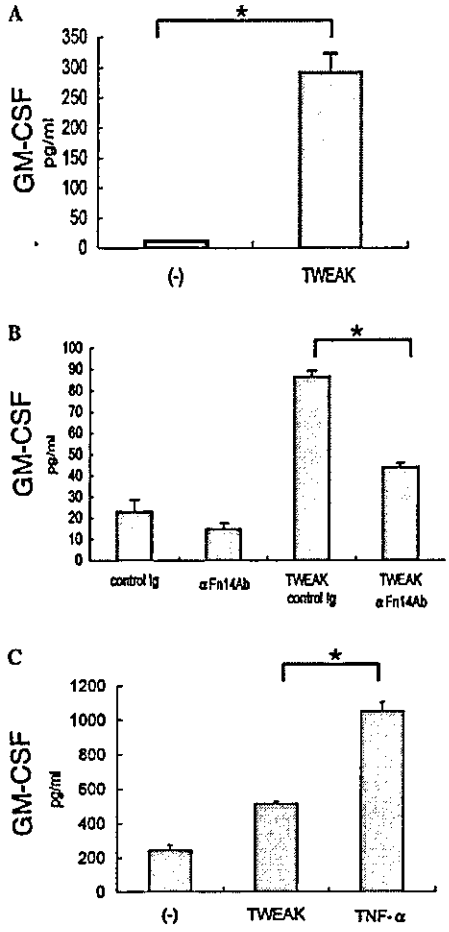

Fig. 3. TWEAK induces GM-CSF production by BEAS2B cells via Fn14. BEAS2B cells were stimulated with 100 ng/ml TWEAK for 48 h (A) or stimulated with 20 ng/ml TWEAK for 24 h in the presence of 10 μg/ml anti-Fn14 blocking mAb or control Ig (B) or stimulated with 10 ng/ml TWEAK or 10 ng/ml TNF-α for 48 h (C). Human GM-CSF concentration in the culture supernatant was measured by ELISA. Data are indicated as means ± SD of triplicate samples. $*p < 0.05$. Similar results were obtained in three repeated experiments.

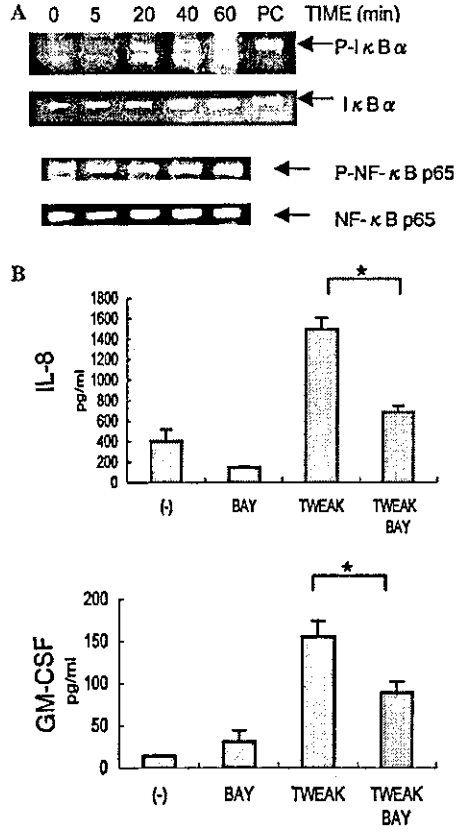

Fig. 4. Critical role of IκBα phosphorylation in TWEAK-induced IL-8 and GM-CSF production by BEAS2B cells. (A) BEAS2B cells were stimulated with 100 ng/ml TWEAK for the indicated time periods. Then, the cell lysates were immunoblotted with anti-phosphorylated IκBα (Ser32) antibody or anti-IκBα antibody or anti-phosphorylated NF-κB p65 antibody (Ser536) or NF-κB p65 antibody. PC: positive control (HeLa cell extract stimulated with TNF-α). Representative of three repeated experiments with similar results. (B) BEAS2B cells were stimulated with 100 ng/ml TWEAK for 24 h in the presence or absence of 2 μM BAY 11-7082. Human IL-8 or GM-CSF concentration in the culture supernatant was measured by ELISA. Data are indicated as means ± SD of triplicate samples. $*p < 0.05$. Similar results were obtained in three repeated experiments.

IL-8 and GM-CSF and thus may contribute to the development of airway inflammatory responses.

TWEAK induced IL-8 and GM-CSF production by BEAS2B cells and primary cultured bronchial epithelial cells (Figs. 2, 3, and 5). However, MCP-1, TARC, or Eotaxin was not produced by BEAS2B cells upon TWEAK stimulation (data not shown). We and others have previously shown that TWEAK induces several cytokines/chemokines; for example, IL-8 and MCP-1 from human umbilical vein endothelial cells (HUVEC) [6], TNF-α from certain tumor cell lines [16], IL-8, IP-10, and RANTES from human dermal fibroblasts and synoviocytes [7]. Thus, the pattern of cytokines/chemokines induced by TWEAK varies depending on the cell types.

Anti-Fn14 mAb did not completely inhibit GM-CSF production as in Fig. 3B, which was quite different from the inhibition observed in IL-8 production (Fig. 2C). This may suggest the existence of other receptors than Fn14 mediating TWEAK effect leading to GM-CSF production. Polek et al. [17] recently proposed the second receptor for TWEAK that was responsible for osteoclast differentiation. The second receptor for TWEAK may thus exist in human bronchial epithelial cells.

We found that TWEAK induced phosphorylation of IκBα (Fig. 4A). This is consistent with the recent findings by Saitoh et al. [10] that TWEAK stimulated IκBα phosphorylation, resulting in activation of NF-κB in

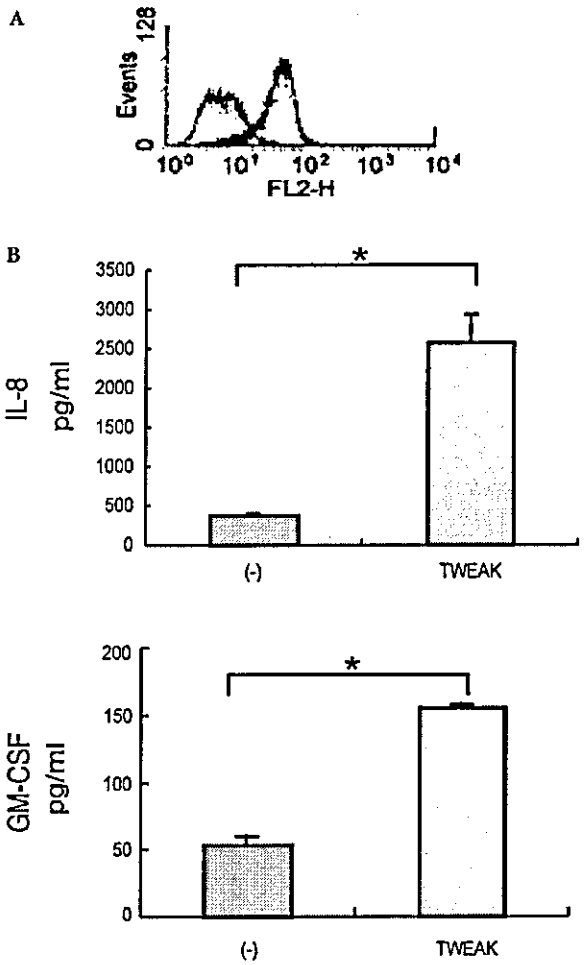

Fig. 5. TWEAK induces IL-8 and GM-CSF production by primary cultured human bronchial epithelial cells. (A) Primary cultured normal human bronchial epithelial cells were stained with biotinylated anti-human Fn14 mAb (right histogram) or control Ig (left histogram), followed by PE-labeled avidin, and then analyzed by flow cytometry. A representative of three repeated experiments with similar results is shown. (B) Primary cultured normal human bronchial epithelial cells were stimulated with 100 ng/ml TWEAK for 48 h. Human IL-8 or GM-CSF concentration in the culture supernatant was measured by ELISA. Data are indicated as means ± SD of triplicate samples. $^*p < 0.05$. Similar results were obtained in three repeated experiments.

fibroblasts. The finding that BAY11-7082, a specific inhibitor of IκBα kinase, abrogated the TWEAK-induced IL-8 and GM-CSF production by BEAS2B cells (Fig. 4B) is also consistent with previous observations that the expressions of IL-8 and GM-CSF genes were NF-κB-dependent [18,19]. However, it should be noted that BAY11-7082 incompletely inhibited IL-8 and GM-CSF production, suggesting the possibility that signaling pathways other than NF-κB might also contribute to TWEAK-induced IL-8 and GM-CSF production by human bronchial epithelial cells.

It remains unclear regarding the role of TWEAK in respiratory disease. Nakayama et al. [4] showed that IFN-γ-stimulated monocytes expressed TWEAK and induced cell death in some tumor cell lines. Thus, we speculate that alveolar or infiltrating monocytes/macrophages might express TWEAK upon inflammation and interaction between the activated monocytes/macrophages and bronchial epithelial cells might play some role in the development of certain inflammatory lung diseases through IL-8 and GM-CSF production. We are currently doing immunohistochemical studies with anti-TWEAK antibodies to identify TWEAK producers in inflammatory lung disorders. We are also examining TWEAK expression in bronchoalveolar lavage fluids obtained from patients with various pulmonary inflammatory disorders.

In summary, we demonstrated a novel biological activity of TWEAK on human bronchial epithelial cells. Our results suggest that TWEAK/Fn14 interaction in bronchial epithelial cells may be involved in the pathogenesis of inflammatory lung diseases.

Acknowledgments

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MD-2 is required for the full responsiveness of mast cells to LPS but not to PGN

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Abstract

To address the role played by MD-2 in mast cell recognition of LPS, we examined bone marrow-derived mast cells (BMMCs) from MD-2 gene-targeted mice. BMMCs from MD-2−/− mice showed impaired cytokine production (TNF-α, IL-6, IL-13, and IL-1β) in response to LPS from *Escherichia coli*, but not to peptidoglycan (PGN) from *Staphylococcus aureus*. In a mast cell-dependent acute septic model, MD-2 deficiency of mast cell resulted in significantly higher mortality due to defective neutrophil recruitment and the production of cytokines in the peritoneal cavity, which was similar to mice with TLR4-deficient mast cells. The TLR2-dependent activation of skin mast cells by PGN was not altered by the absence of MD-2 in vivo. Collectively, MD-2 is essential for the recognition of LPS by TLR4 but not for that of PGN by TLR2 of mast cells.

Keywords: Mast cells/basophils; Toll-like receptor; MD-2; Bacterial; Rodent; Lipopolysaccharide

Mast cells are primarily known as effector cells of allergic diseases and chronic inflammatory responses. However, recent accumulating evidence has shown that mast cells play an important role in host defense against bacterial infection [1–4]. Innate immune responses elicited by mast cells also rely on the ability of the cells to recognize the molecular motifs of microorganisms through pattern recognition receptors called toll-like receptors (TLR). A wide variety of bacterial components including LPS, peptidoglycan (PGN), lipoteichoic acid (LTA), lipoarabinomannan, lipopeptides, and bacterial DNA are capable of stimulating the innate immune system through specific TLR [5–7]. However, the precise mechanisms for the recognition of each ligand by respective TLR are complex and poorly understood.

LPS from gram-negative bacteria is among the best-characterized ligands for TLR4. The lipid A portion of LPS is conserved among gram-negative bacteria and appears to be responsible for the biological toxicity of LPS [8,9]. At least four cellular proteins are known to participate in LPS recognition and cellular activation, LPS-binding protein (LBP), CD14, TLR4, and MD-2 [6,7,10,11]. The plasma protein, LBP, dramatically accelerates the binding of monomer LPS to CD14, which usually forms aggregates in aqueous environments, thereby enhancing the sensitivity of cells to

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LPS [12,13]. CD14 is a glycosylphosphatidylinositol (GPI)-linked cell surface protein that serves as the major LPS receptor but is itself devoid of signaling [14]. Experiments using TLR4 gene-targeted mice or TLR4-mutated C3H/HeJ mice have shown that TLR4 is a signal transducer of cell activation via LPS [15,16]. MD-2 was cloned through its homology with MD-1 that associates with and is required for the signaling and surface expression of RP105, a leucine-rich-repeat-containing protein similar to TLRs [17–19]. Since MD-2 lacks a transmembrane domain, it associates with the cell through its interaction with the extracellular domain of TLR4. Evidence that both TLR4 and MD-2 participate directly in the discrimination of LPS structures has been demonstrated using mutated MD-2 lacking one or two N-glycosylation sites [20–22]. Also, da Silva et al. [23] observed that direct physical contact occurs between LPS and CD14–TLR4–MD2. Thus, the physical association of MD-2 with TLR4 is critical for LPS recognition by TLR4. Furthermore, it has been shown that MD-2 is important for the correct distribution of TLR4 on the surface of cells such as MD-1, which also plays an important role in the surface expression of RP105 on B cells [18,24]. Thus, it appears that MD-2 is an indispensable molecule in the LPS-sensing complex.

We have previously demonstrated that mouse mast cells express TLR4 and are activated by LPS through that [3,4]. However, the roles played by MD-2 in mast cells for both LPS recognition and TLR4 distribution remain unclear. In this study, we demonstrate that MD-2 is required for the full responsiveness of mast cells against LPS but not PGN. We also demonstrate that MD-2, as well as the TLR4 molecule, plays a crucial role in the recognition and activation of peritoneal mast cells in acute septic bacteria infection.

Methods

Mice. MD-2-deficient (MD-2−/−) and heterozygous (MD-2+/−) mice were kindly provided by Dr. K. Miyake of Tokyo University, Japan [24]. We used MD-2+/− mice as control mice since no phenotypic difference was observed between MD-2+/− and MD-2+/+ mice. TLR4-deficient (TLR4−/−) or TLR2-deficient (TLR2−/−) mice were kindly provided by Dr. S. Akira of Osaka University, Japan [15,25]. WBB6F$_1$-W/W^v mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed under the approval of the Institutional Review Board of Juntendo University.

Generation of bone marrow-derived mast cells. Bone marrow-derived mast cells (BMMCs) were generated from the femoral bone marrow cells of mice and maintained in the presence of 10% pokeweed mitogen-stimulated spleen-conditioned medium (PWM-SCM) as a source of mast cell growth factors as previously described [3,4]. After 4 weeks of culture, more than 98% of cells were identifiable as mast cells as determined by toluidine blue staining and FACS analysis of cell surface expressions of both *c-kit* and FcεRI.

RT-PCR analysis of the mRNA expression of TLR and MD-2. Total RNA was extracted from BMMCs using STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. First-strand cDNA was constructed from 3 μg of total RNA with oligo(dT)$_{12-18}$ as the primer using Superscript II RNase H$^-$ reverse transcriptase (Life Technologies, Rockville, MD). PCR was performed using specific primers for mouse MD-2 (5′-ATG TTG CCA TTT ATT CTC TTT TCG ACG-3′ and 5′-ATT GAC ATC ACG GCG GTG AAT GAT G-3′) and for mouse TLR4 (AF110133) [26] and mouse GAPDH (5′-AGT ATG ACT CCA CTC ACG GCA A-3′ and 5′-TGT CGC TCC TGG AAG ATG GT-3′).

FACS analysis. Single cell suspensions were incubated at 2×10^5 cells/100 μl in staining buffer (PBS containing 0.5% BSA and 0.01% NaN_3) with anti-TLR4–MD-2 (MTS510: Hycult Biotechnology, PB Uden, The Netherlands), followed by FITC-rabbit F (ab′)$_2$ anti-rat IgG (ICN Pharmaceuticals, Aurora, OH) or mouse IgE Ab (BD Biosciences, San Jose, CA), followed by FITC-anti-mouse IgE (BD Biosciences, San Jose, CA), or FITC-anti-*c*-kit Ab (BD Biosciences, San Jose, CA). Flow cytometric analysis was performed using FACS caliber (BD Biosciences, San Jose, CA).

Measurement of cytokine concentrations. BMMCs (2×10^6 cells/ml) in complete culture medium were stimulated with the indicated concentration of LPS from *Escherichia coli* (serotype 0111:B4; Sigma–Aldrich) or PGN from *Staphylococcus aureus* (Sigma–Aldrich) in the presence of 10% FCS and PWM-SCM. For IgE-mediated stimulation, BMMCs (5×10^6/ml) were incubated with 1 μg of anti-TNP mouse IgE (BD Biosciences, San Jose, CA) on ice for 2 h. After washing of the cells twice, BMMCs (2×10^6 cells/ml) in complete culture medium were stimulated with 250 ng/ml of anti-mouse IgE (BD Biosciences, San Jose, CA). Cells were incubated at 37 °C, 3 h for TNF-α or 6 h for IL-1β, IL-6, and IL-13. The amount of each cytokine in the supernatant was measured by ELISA kit (Genzyme Techne, Minneapolis, MN, USA).

Reconstitution of W/W^v mice with BMMCs. Mast cell-deficiency of W/W^v mice in the peritoneal cavity was selectively reconstituted by injecting 2×10^6 BMMCs from MD-2+/−, MD-2−/− or TLR4−/−, mice into the peritoneal cavity as previously described [3,4]. The reconstitution of the skin mast cells of W/W^v mice was performed using an intradermal injection of 1×10^6 BMMCs from MD-2+/−, MD-2−/−, TLR4−/−, or TLR2−/− mice [27,28]. Five weeks after injection of BMMCs, the mice were used for experiments. Reconstitution of the mast cells was confirmed by toluidine blue or alcian blue/safranin staining of the cytospun preparation of peritoneal cells or formalin-fixed tissue section of the skin.

Cecal ligation and puncture. Cecal ligation and puncture (CLP) was performed as previously described [3]. Briefly, mice were anesthetized and the cecum was ligated with 4–0 silk and punctured using a 21-gauge needle. After CLP, mice were observed for mortality over a period of 10 days.

Differential cell counts and estimation of cytokine concentrations in peritoneal exudates. Peritoneal exudates were collected 6 h after CLP induction. The total cell number was counted, and the differential cell count of infiltrating leukocytes was performed by counting 500 leukocytes under an oil immersion field after staining cytospun preparations with Diff-Quik (International Reagents, Kobe, Japan). The amount of each cytokine in the peritoneal fluid was determined by ELISA as described above.

Induction of skin inflammation. PGN-induced skin inflammation was performed as previously described [4]. Mice were intradermally injected with 20 μl PGN (100 μg/ml in saline) or saline into each ear. For the PCA reaction, anti-TNP IgE (25 μg in 20 μl) was injected intradermally into the ears 2 h before Ag (TNP-BSA) application. Vascular leakage was visualized by an intravenous injection of 0.5% Evans blue (0.25 ml) 5 min before PGN or antigen application. Thirty minutes after the application of PGN or the antigen, the ears were removed and the amount of dye in the ear was measured as previously described [4]. The amount of dye was calculated according to the standard curve of known concentration of Evans blue.

Statistical analysis. Statistical analysis was performed using Student's *t* test. Statistical analysis of survival data in the CLP experiment was performed using the Log-rank test.

Results

Expression of the MD-2 transcript in BMMCs

To address the role played by MD-2 in the activation of mast cells by gram-negative bacteria-derived LPS, we generated BMMCs from MD-2 gene-targeted mice. The mRNA and protein expression of MD-2 in BMMCs was investigated by RT-PCR and FACS analysis using anti-TLR4 mAb (MTS510), which specifically detects the TLR4–MD-2 complex [29]. Although TLR4 mRNA was present in BMMCs from MD-2+/– or MD-2–/– mice (Fig. 1A), the TLR4–MD-2 complex was not expressed on BMMCs from MD-2–/– mice (Fig. 1B), demonstrating the absence of MD-2 protein on the cell surface. TLR4–/– mice also did not express TLR4–MD-2 detected by MTS510, due to the absence of TLR4, although the amount of mRNA expression of MD-2 in TLR4–/– mice was not altered in the absence of TLR4 (Figs. 1A and B). The expressions of other surface molecules, such as the *c-kit* or IgE receptor, were not different between the BMMCs of these mice (Fig. 1B).

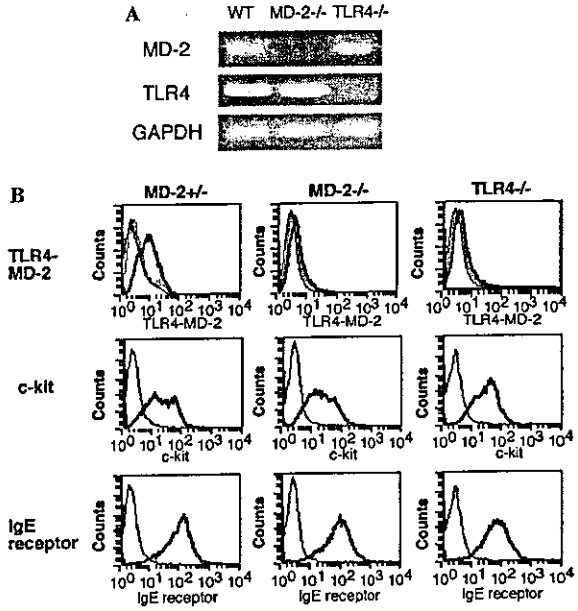

Fig. 1. Expression of the MD-2 molecule of mast cells. (A) BMMCs were prepared from MD-2+/–, MD-2–/–, or TLR4–/– mice and used for RT-PCR analysis to detect the mRNA of MD-2, TLR4 and GAPDH. (B) BMMCs from MD-2+/–, MD-2–/–, or TLR4–/– mice were stained with mAbs to TLR4–MD-2 (MTS510), followed by FITC-anti-rat IgG, or mouse IgE, followed by FITC-anti-mouse IgE or FITC labeled anti-*c*-kit Abs. The solid line shows staining with isotype-matched Ab; the dotted line shows isotype-matched Ab and the second Ab alone. The bold line shows staining with respective specific Abs and the second Ab.

Requirement of MD-2 for the full responsiveness of BMMCs to LPS but not to PGN in vitro

As we have previously reported that LPS induced the secretion of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-13) from the BMMCs in a TLR4-dependent manner [3,4], we examined the LPS responsiveness of BMMCs of MD-2–/– mice by stimulation with increasing concentrations of LPS (from *E. coli*) or PGN (from *S. aureus*). LPS induced the secretion of these cytokines from the BMMCs of MD-2+/– mice, but not from those of MD-2–/– or TLR4–/– mice (Fig. 2A). In contrast, there were no significant differences in TNF-α, IL-6, and IL-13 secretion by the BMMCs of MD-2+/–, MD-2–/–, and TLR4–/– mice when stimulated with PGN (a TLR2 ligand) or with IgE receptor cross-linking (Figs. 2A and B). PGN or IgE receptor cross-linking did not lead to detectable levels of IL-1β secretion in any BMMCs as has been reported [4]. Consistent with previous data, LPS did not induce the degranulation of mast cells of any mice (MD-2+/–, MD-2–/–, and TLR4–/–), although there were no differences in the ability of these BMMCs to degranulate upon IgE receptor cross-linking when evaluated by β-hexosaminidase release (data not shown).

Requirement of MD-2 of mast cells for host-innate immune response against gram-negative bacteria infection

To address the roles played by mast cell-MD-2 in bacterial infection in vivo, we used an acute septic peritonitis model in which host protection against bacteria was known to depend on mast cells in the peritoneal cavity. Peritoneal mast cells of mast cell-deficient W/W^v mice were reconstituted with BMMCs from MD-2+/–, MD-2–/– or TLR4–/– mice and subjected to CLP. Five weeks after reconstitution, there were no significant differences in the number of peritoneal mast cells between W/W^v mice reconstituted with different BMMCs (MD2+/–, MD-2–/–, and TLR4–/–: $4.45 \pm 0.64 \times 10^4$, $5.43 \pm 1.12 \times 10^4$, and $5.41 \pm 1.16 \times 10^4$ cell per mouse, respectively). More than 95% of these mast cells showed positive staining with alcian blue/safranin that was the property of connective tissue-type mast cells (data not shown). Consistent with previous reports [3,4], mast cell-deficient W/W^v mice all died within 3 days after CLP induction (Fig. 3A). In contrast, the reconstitution of W/W^v mice with BMMCs from MD-2+/– mice protected the host from acute peritonitis-induced death ($p < 0.001$ vs W/W^v) (Fig. 3A). Similar reconstitution of W/W^v mice with

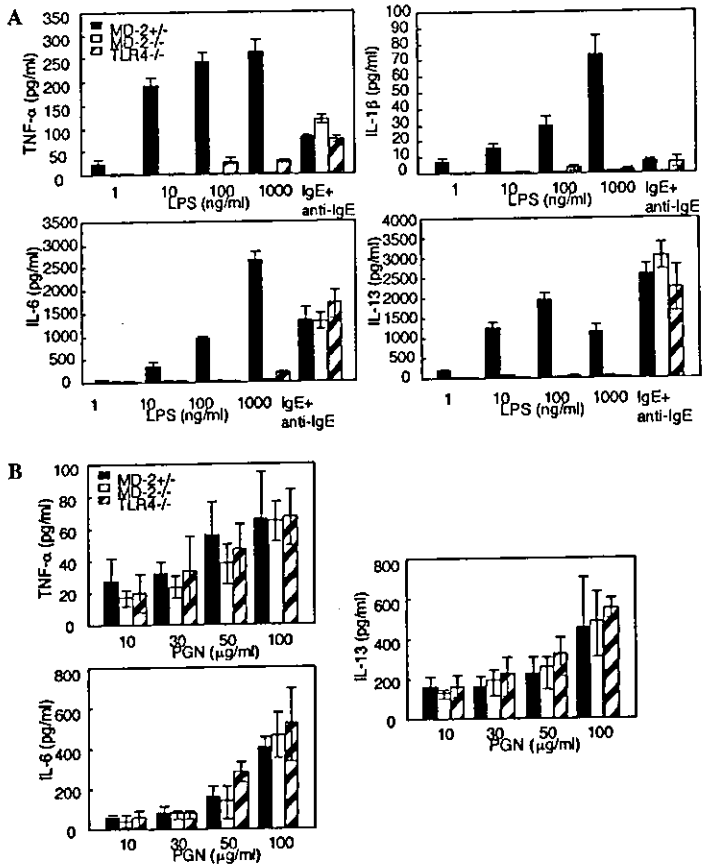

Fig. 2. MD-2 is required for the activation of BMMCs by LPS. BMMCs from MD-2+/– (filled bars), MD-2–/– (open bars), or TLR4–/– (striped bars) were stimulated with the indicated concentrations of LPS (A) or PGN (B), 3 h for TNF-α and 6 h for other cytokines. For IgE receptor-mediated stimulation, BMMCs were sensitized with IgE and challenged with anti-IgE as described in Methods. The cytokine concentrations in the supernatant were determined as described in Methods. Data shown are means ± SD of three to four experiments conducted with different BMMC preparations.

BMMCs from MD-2–/– or TLR4–/– slightly improved the survival rate, but the rate was significantly lower than that of W/W^v mice reconstituted with MD-2+/– BMMCs ($p < 0.05$) (Fig. 3A). The survival rate of W//W^v mice with MD-2+/– BMMCs was not significantly different from that of W//W^v mice with WBB6F$_1$-+/+ BMMCs (data not shown). These results indicate again that mast cells play an important role in host protection from early enterobacterial infection via MD-2 in addition to the TLR4 molecule.

As a previous study suggested that defective production of TNF-α and early recruitment of neutrophils in the absence of mast cell are responsible for host protection from acute septic peritonitis-induced death [2], we investigated whether defective leukocyte recruitment in the peritoneal cavity after CLP was associated with MD-2 deficiency of mast cell. When we examined the number of infiltrating cells and the amount of inflammatory cytokines in the peritoneal cavity, W/W^v mice without mast cell or with MD-2/TLR4-deficient mast cells showed significantly lower levels of cytokine and neutrophil infiltration 6h after CLP than those with intact mast cells ($p < 0.01$) (Figs. 3B and C). Thus, early leukocyte, especially neutrophil recruitment into the peritoneal cavity by the production of inflammatory cytokines was highly dependent on mast cell activation by enterobacteria via MD-2/TLR4 and was one of the crucial factors to protect the host from acute septic peritonitis-induced death. These results indicate that MD-2, as well as the TLR4 molecule, plays an important role in mast cell activation by enterobacteria infection in vivo.

MD-2 deficiency did not affect TLR2 of mast cell-dependent acute skin response

To verify the roles played by MD-2 in the TLR2-dependent activation of skin mast cells by PGN in vivo, we compared the responses elicited by an intradermal injection of PGN into the ear of W/W^v mice and W/W^v mice reconstituted with BMMCs from MD-2+/–, MD-2–/–,

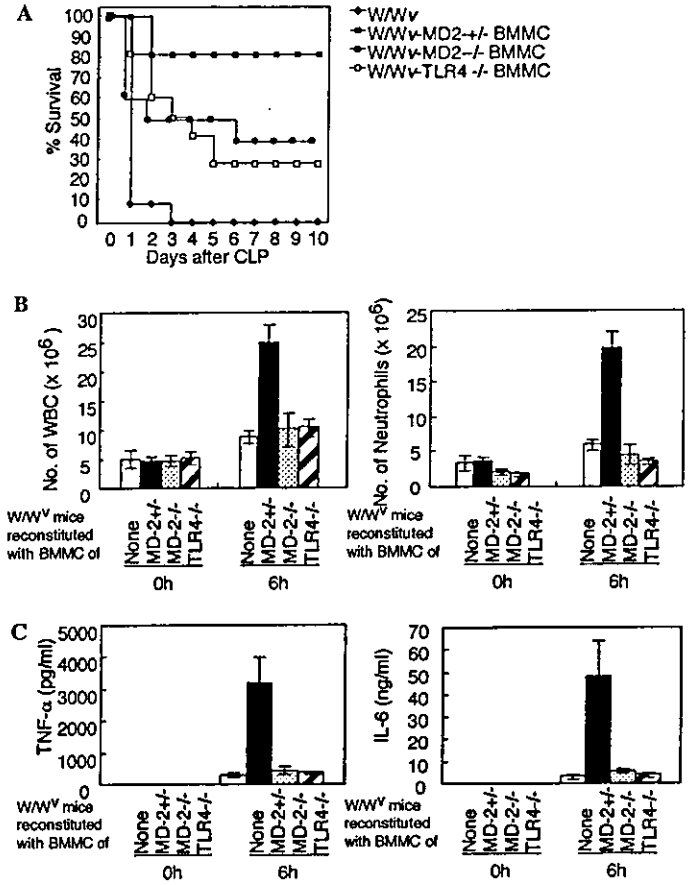

Fig. 3. Both MD-2 and TLR4 of mast cells are required for the full expression of innate immunity in a mast cell-dependent sepsis model. (A) W/W^v mice without reconstitution (♦) or reconstitution with BMMCs from MD-2+/− (■), MD-2−/− (●) or TLR4−/− (□) mice (10 mice/group) underwent CLP. The mortality was observed and expressed as percent survival. (B) Six hours after CLP, total numbers of leukocytes and neutrophils in the peritoneal cavity were estimated by differential cell count and expressed as total cells/mouse. (C) The levels of individual cytokines were measured by ELISA. Data shown are means ± SD of four mice.

TLR4−/−, or TLR2−/− mice. Vascular permeability, evaluated by the extravasation of Evans blue dye, was significantly increased by PGN ($p < 0.001$) in wild-type mice but not in mast cell-deficient W/W^v, suggesting that this reaction was dependent on mast cells in the skin (Fig. 4). The reconstitution of skin mast cells of W/W^v mice with BMMCs from MD-2+/−, MD-2−/−, and TLR4−/− but not from TLR2−/− mice resulted in increased extravasation of the dye, indicating that the deficiency of MD-2 as well as TLR4 in skin mast cells did not confer the TLR2-mediated skin inflammation by PGN even in vivo. Consistent with the previous data [4], LPS did not cause severe vasodilatation as much as PGN within 30 min (data not shown). The number of skin mast cells 5 weeks after the reconstitution of each BMMC was not different between these mice (data not shown). Also, there were no differences in the functional ability of reconstituted and differentiated mast cells to degranulate in response to IgE receptor-mediated activation (Fig. 4). Thus, unlike CD14, which seems to bind soluble PGN from *S. aureus* and lipoarabinomannan from *Mycobacterium* [30], MD-2 associated with TLR4 directly interprets LPS structures and does not confer the responsiveness of the TLR2 ligand.

Discussions

The roles played by MD-2 in the activation of mast cells by gram-negative bacteria-derived LPS were examined both in vitro and in vivo. We generated BMMCs from MD-2−/− mice as well as MD-2+/− and TLR4−/− mice. There were no differences in the growth rate between these mast cells during the course of experiment. Also the properties of mast cells evaluated by the expression of the *c-kit* and IgE receptor were not different between these mast cells (Fig. 1B). The importance of MD-2 in LPS responsiveness has been implicated in

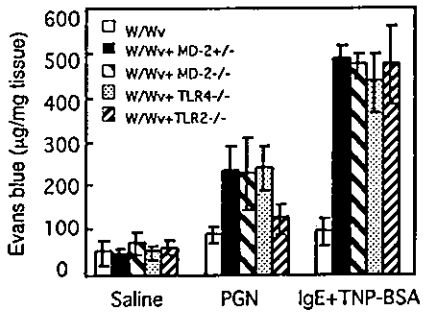

Fig. 4. PGN-TLR2 activation of skin mast cell is not affected by the absence of MD-2. Skin mast cells of W/W^v mice without reconstitution (□) or reconstituted with BMMCs from MD-2+/− (■), MD-2−/− (▨), TLR4−/− (▤) or TLR2−/− (▨) mice were intravenously injected with 0.5% of Evans blue 5 min before intradermal administration (20 μl) of PGN (100 μg/ml) to the ears. The dye content in the tissues was measured 30 min after PGN administration as described in Methods. The concentration of dye content in the tissues by IgE-mediated stimulation was measured as described in Methods. Data shown are means ± SD of three mice.

both LPS recognition and the proper surface distribution of TLR4 [24]. It has been reported that an association between MD-2 and TLR4 occurs in the endoplasmic reticulum (ER) in which the endoplasmic reticulum chaperone gp96 plays an important role [31]. Thus, it is generally thought that the distribution of TLR4 alone in the Golgi apparatus does not necessarily lead to LPS recognition and signaling. Although, there is an exception of intestinal epithelial cells, in which LPS recognition occurs in the Golgi apparatus after LPS internalization [32]. The result that MD-2-deficient mast cell could not respond against LPS in vitro and in vivo, suggesting that TLR4 alone in the Golgi apparatus may not be enough to recognize LPS and signal in the mast cells. An approach using a mutated form of MD-2 by replacing Cys95 with Tyr95, which abolishes the LPS responses but not the cell surface expression of TLR4 [20–22], will help to clarify this point.

Also, our in vivo results indicate that soluble forms of MD-2 which might circulate in the serum [33,34] did not confer or restore the responsiveness of peritoneal mast cells against gram-negative bacteria. Although an in vitro experiment has shown that soluble MD-2 as low as 50 pM can rescue the hypo-responsiveness of cells to LPS [33,34], little is known about the physiological role of soluble MD-2 in vivo. As serum concentrations of soluble MD-2 have never been determined, but are probably below 50 pM, the concentrations of circulating MD-2 in vivo may not be sufficient to induce the responsiveness of peritoneal mast cells against bacteria-derived LPS in our experimental system.

Unlike CD14, which is implicated in the recognition of ligands for both TLR4 and TLR2 [30], it seems that MD-2 is required only for the recognition and binding of TLR4 ligand. In contrast to the observation that TLR2-associated MD-2 enhances the cell responsiveness against several TLR2 ligands including PGN [35], we could not observe any defect in the TLR2-mediated mast cell activation in MD-2-deficient mice. These discrepancies may arise from differences in the species (human vs mouse), or the cells used (artificially transfected-HEK293 vs naturally developed-mast cell).

It has been demonstrated that MD-2 is required for the recognition of various TLR4 ligands other than LPS, such as Taxol [36], HSP60 [37], and extra domain A of fibronectin [38]. We confirmed that BMMCs could be activated by extra domain A of fibronectin in a TLR4-dependent manner (manuscript in preparation), it would be interesting to examine how these structurally different molecules are recognized by MD-2/TLR4 in mast cells.

In the CLP model, a slight improvement of the survival rate in W/W^v mice reconstituted with BMMCs from MD-2−/− or TLR4−/− was observed but was not significantly different from W/W^v mice without receiving mast cell reconstitution. The exact reason for this improvement was not clear from this experiment. It is possible that other molecules than TLR4/MD-2 on mast cells, such as CD48 and complement receptor, may confer the activation of mast cells by enterobacteria infection as has been reported [39,40].

Taken together, this study first shows in vivo that MD-2 is essential for the full responsiveness of mast cells against LPS but not PGN, a TLR2 ligand. Also, this study shows that in vivo circulating forms of MD-2 may not be enough to rescue the responsiveness of reconstituted-mast cells against enterobacteria. To generalize the requirement of both TLR4 and MD-2 molecules for the proper distribution of TLR4 on the surface of cells, a careful examination of TLR4 distribution in mast cells in the absence of MD-2 is required (under investigation). The clarification of the mechanism of mast cell activation by microorganisms through these receptors would be helpful to understand the physiological roles played by mast cells in innate immune responses.

Acknowledgments

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Dual Role of Fcγ Receptor in Transient Focal Cerebral Ischemia in Mice

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Background and Purpose—Cerebral ischemia/reperfusion injury is associated with the development of inflammatory response, including pathological contributions by vascular leukocytes and endogenous microglia. Expression of Fc receptors (FcRs) on macrophages and microglia is thought to be involved in the inflammatory cascade. The present study assessed the role of FcγR in ischemia/reperfusion injury, using FcγR knockout ($Fc\gamma R^{-/-}$) mice and bone marrow chimera $Fc\gamma R^{-/-}$ mice, which express enhanced green fluorescent protein (EGFP).

Methods—Mice underwent occlusion of the middle cerebral artery for 60 minutes, followed by reperfusion. Infarct volume and mortality were calculated at several time points after ischemia. To clarify the function and distribution of microglia/macrophages, immunohistochemical staining and immunoblotting of ionized calcium-binding adapter molecule 1 (Iba-1), inducible nitric oxide synthase, and nitrotyrosine were performed.

Results—$Fc\gamma R^{-/-}$ mice showed significantly reduced mortality (20%) and smaller infarcts (19.7 ± 3.63 versus 33.28 ± 7.98 mm^3; $P<0.001$) than wild-type (WT) mice at 72 hours after reperfusion. Western blotting revealed that microglial activation ($P<0.002$) and induction of inducible nitric oxide synthase ($P<0.005$) were reduced in $Fc\gamma R^{-/-}$ mice compared with WT mice. At 7 days after reperfusion, sections double-immunostained for EGFP and Iba-1 showed less activation and migration of EGFP-positive bone marrow–derived macrophages in $Fc\gamma R^{-/-}$ chimera mice than in WT mice.

Conclusions—Our results demonstrated that the neuroprotective effect of FcγR deficiency in our model may be primarily attributed to the suppression of activation and infiltration of inflammatory cells. (***Stroke*. 2004;35:958-963.)**

Key Words: microglia ■ macrophages ■ Fc-gamma receptor ■ inflammation ■ ischemia/reperfusion injury

The inflammatory response in the central nervous system is considered important in the pathological process after the onset of cerebral ischemia and is a risk factor for the initial development of cerebral ischemia.[1] Brain ischemia induces a marked response of resident microglia and hematopoietic cells, including monocytes and macrophages, and elicits a strong intrinsic inflammatory response involving activation of microglia, recruitment of granulocytes, and infiltration of macrophages in the ischemic area.[2] However, whether the molecular mechanisms underlying these inflammatory responses are beneficial or detrimental in cerebral infarction is still unclear. Understanding the intracellular signaling mechanism and cell-to-cell interaction in the inflammatory cascade may help in the design of therapeutic strategies for cerebral infarction.

Recent studies have emphasized the critical roles of Fc receptors (FcRs) expressed on macrophages and microglia in the inflammatory cascade.[3,4] Although several studies stressed the importance of the FcγR in the inflammatory response in immunological[5] and degenerative diseases[6] of the central nervous system, to our knowledge no report has described a link between FcγR and cerebral ischemia.

In a previous study we provided direct evidence for the migration and distribution of bone marrow–derived monocytes/macrophages and the relationship between resident microglia and infiltrated hematogenous elements in the ischemic brain of bone marrow chimera mice that expressed enhanced green fluorescent protein (EGFP).[7] It is conceivable that microglia/macrophages may serve a dual and paradoxical role after ischemic injury. To assess the role of Fcγ receptor in ischemia/reperfusion injury, middle cerebral artery (MCA) occlusion/reperfusion was performed in the FcγR knockout ($Fc\gamma R^{-/-}$) mice and the bone marrow chimera $Fc\gamma R^{-/-}$ mice with the use of a model system established previously.

Materials and Methods

Transient Focal Cerebral Ischemia

The protocol described here received prior approval by the Committee on Animal Experimental Guidelines of Juntendo University School of Medicine. $Fc\gamma R^{-/-}$ mice (Jackson Laboratory) were generated by the homogeneous recombination method, as described previously.[8] Studies were conducted in 8-week-old $Fc\gamma R^{-/-}$ and C57BL/6 (wild-type [WT]) (n=50 per group) mice of the same

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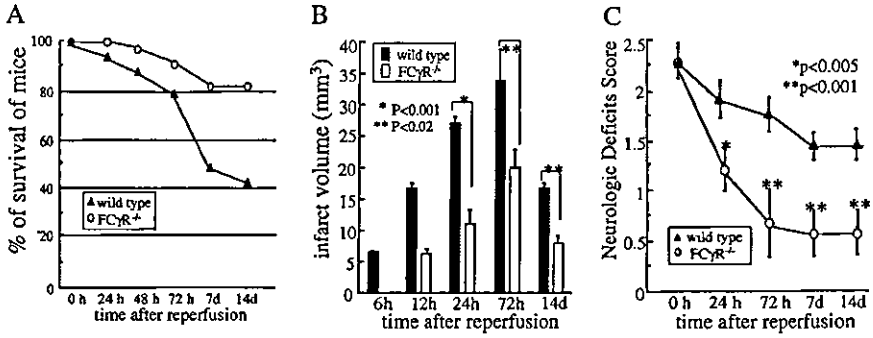

Figure 1. A, Survival analysis during 14 days after reperfusion in WT and $Fc\gamma R^{-/-}$ mice. B, Effects of FcγR depletion on stroke outcome. Infarct volume was compared among $Fc\gamma R^{-/-}$ mice and WT littermates at different time points after reperfusion. C, Neurological deficit scores in WT and $Fc\gamma R^{-/-}$ mice at different time points after reperfusion.

genetic background. Animals were housed under diurnal lighting and provided with food and water ad libitum.

Mutant and WT mice, weighing 20 to 25 g, were initially anesthetized with 4.0% isoflurane and maintained on 1.0% to 1.5% isoflurane in 70% N_2O and 30% O_2 with the use of a small-animal anesthesia system. The tip of the laser-Doppler probe was placed on the area selected for regional cerebral blood flow monitoring, which corresponded to the territory of the occluded MCA. The left MCA was occluded for 60 minutes and then reperfused as described previously.[9] In another group of mice (n=10), reperfusion was not performed (permanent MCA occlusion group). Body temperature was kept at 37°C during the experiment with a heating pad. We generated the $Fc\gamma R^{-/-}$/EGFP transgenic model by bone marrow transplantation of EGFP into $Fc\gamma R^{-/-}$ mice, using the method reported previously,[7] and induced transient cerebral ischemia in these animals 6 weeks later.

Estimation of Infarct Volume

At 6, 12, 24, or 72 hours or 14 days after reperfusion, the mice were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (n=5 per group) and decapitated. The brains were coronally sectioned into six 1-mm-thick slices. The slices were incubated for 20 minutes in 2% solution of 2,3,5-triphenyltetrazolium chloride at 37°C and immersion-fixed in 4% buffered formalin solution. To compensate for brain edema, the correct infarct volume was calculated as described in detail previously.[10]

Neurological Evaluation

Neurological examination was performed daily after reperfusion until the animals were killed. The observer was blinded to the study protocol and scored the postural reflexes using a modified neurological scoring system described previously.[9] In this system, score 0 represents no observable neurological deficits; 1, failure to extend the left forepaw on lifting the whole body by the tail; 2, circling to the contralateral side; and 3, loss of walking or righting reflex.

Immunohistochemistry

Five animals of each group were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) at 12, 24, and 72 hours and 7 and 14 days after reperfusion. The brain was removed immediately and postfixed for 24 hours in 4% paraformaldehyde in PBS at 4°C before cryoprotection by bathing in 30% sucrose. It was then frozen, and 20-μm-thick consecutive coronal sections were prepared on a cryostat (CM-1900, Leica). Immunohistochemical staining was performed for CD64, ionized calcium-binding adapter molecule 1 (Iba-1; a kind gift from Dr Kohsaka[11]), and inducible nitric oxide synthase (iNOS, BD Bioscience). Sections were washed in PBS, incubated in 0.3% H_2O_2 in PBS for 30 minutes, and incubated overnight at 4°C with 10% normal goat serum (Dako Corporation) in PBS and anti-Iba-1 (1:1500) antibody, anti-iNOS (1:300) antibody, or anti-CD64 (1:300) antibody. Immunoreactivity was visualized by the avidin-biotin complex method (Vectastatin, Vector Laboratories) as described previously.[7]

Double-Immunofluorescence Staining

Free-floating sections of EGFP bone marrow chimera mouse were washed with PBS and incubated in a blocking solution, 3% Block-Ace (Yukijirushi) in T-PBS (0.5% Triton X-100), for 30 minutes at room temperature. Double-immunofluorescence staining was performed by simultaneous incubation of sections with anti-Iba-1 antibody, anti-iNOS antibody, or anti-nitrotyrosine antibody (1:50; Upstate Biotechnology) overnight 4°C. For double labeling, the primary antibodies were detected with Texas red–conjugated secondary antibody (1:500; Vector Laboratories) afterward for 2 hours at room temperature. The sections were washed with PBS and mounted on microslide glass with Vectorshield Mounting Medium (Vector Laboratories).

Western Blots

Four animals of each group were decapitated after 24 or 72 hours of reperfusion. Samples were taken from 2 regions: the ischemic region and the contralateral cortex. Protein extraction and Western blotting were performed.[11] Aliquots containing 50 μg of protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein bands were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) with the use of an electrophoretic transfer system (Trans-blot Semi-dry Transfer Cell, Bio-Rad). After they were blocked with BlockAce for 1 hour, the membranes were incubated overnight at 4°C with anti–Iba-1 antibody (1:5000), anti-iNOS antibody (1:1000), or anti–α-tubulin antibody (1:1000; Santa Cruz Biotechnology Inc). After incubation with the appropriate horseradish peroxidase–conjugated secondary antibody (1:25 000; Amersham) for 1 hour at room temperature, immunoreactive bands were visualized in the linear range with enhanced chemiluminescence (ECL Western blotting system, (Amersham). For quantitative evaluation, the immunoreactive bands were subjected to densitometric analysis.

Cell Count and Statistical Analysis

In each coronal section of Iba-1 staining, the numbers of Iba-1–positive cells at the transition area were counted independently by 2 investigators. Values presented in this study are expressed as mean±SD. After acquisition of all data, the randomization code was broken, and the data were assigned to the respective group. One-way ANOVA and subsequent post hoc Fisher protected least significant difference test were used to determine the statistical significance of differences in physiological variables, neurological score, and volume of infarction between the 2 groups.

Results

Effects of FcγR Deficiency on Transient Focal Cerebral Ischemia

Figure 1A shows that the survival rate of $Fc\gamma R^{-/-}$ mice (80%) was significantly increased 14 days after reperfusion compared with WT mice. In the permanent MCA occlusion group, 10% of WT mice and 50% of $Fc\gamma R^{-/-}$ mice died with signs of brain swelling and herniation within 24 hours (data not shown). At 24

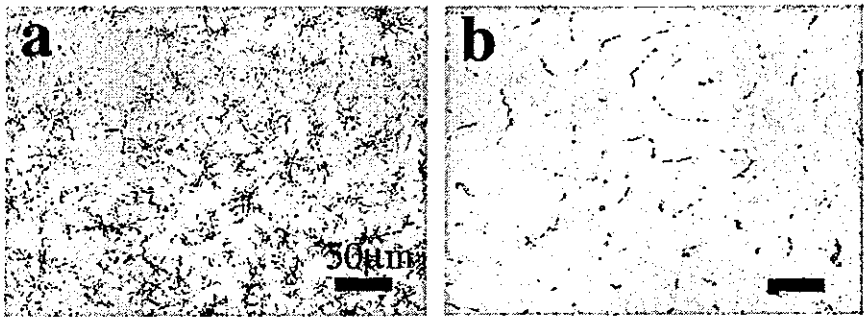

Figure 2. Photographs show FcγRI (CD64) immunostaining in untreated WT and FcγR$^{-/-}$ mice. a, FcγR immunostaining was observed in many glial cells with microglial morphology in the cortex of WT mice. b, However, there was no specific FcγR immunostaining in FcγR$^{-/-}$ mice. Magnification ×200.

hours after reperfusion, the infarct size (10.89±1.26 mm^3) in FcγR$^{-/-}$ mice was significantly smaller (P<0.001) than that in WT littermates (26.59±1.26 mm^3) (Figure 1B). After 72 hours of reperfusion, the infarct size was 19.7±3.63 mm^3 in FcγR$^{-/-}$ mice and 33.28±7.98 mm^3 in WT littermates. The neurological deficit scores are shown in Figure 1C. The scores of FcγR$^{-/-}$ mice recorded at several time points after reperfusion were significantly lower (P<0.005) than those of WT mice.

Microglial Activation in FcγR$^{-/-}$ Mice After Ischemia/Reperfusion

FcγRI immunostaining was detected in glial cells, which morphologically resembled microglia (Figure 2a). However, no specific FcγRI immunostaining was noted in the brain of FcγR$^{-/-}$ mice (Figure 2b).

The distribution of infarct area was analyzed with the use of cresyl violet–stained sections. We defined each ischemic lesion by location in 3 areas (ischemic core, transition area, and peri-infarct area), as shown schematically in Figure 3Aa as areas C, B, and A, respectively. In WT mice, activation of microglia was identified by Iba-1 antibody in the peri-infarct and transition areas. Ramified Iba-1–positive microglia were detected in the ischemic core at 12 hours after reperfusion (Figure 3Aa). Such microglial activation was widely distributed and gradually increased in the peri-infarct and transition areas until 7 days after MCA reperfusion and then tended to decrease (Figure 3Aa to 3Af). On the other hand, the microglial response was less evident in the ischemic core in a time-dependent manner. In FcγR$^{-/-}$ mice, microglial activity in the transition area was weak (Figure 3Ag to 3Al)

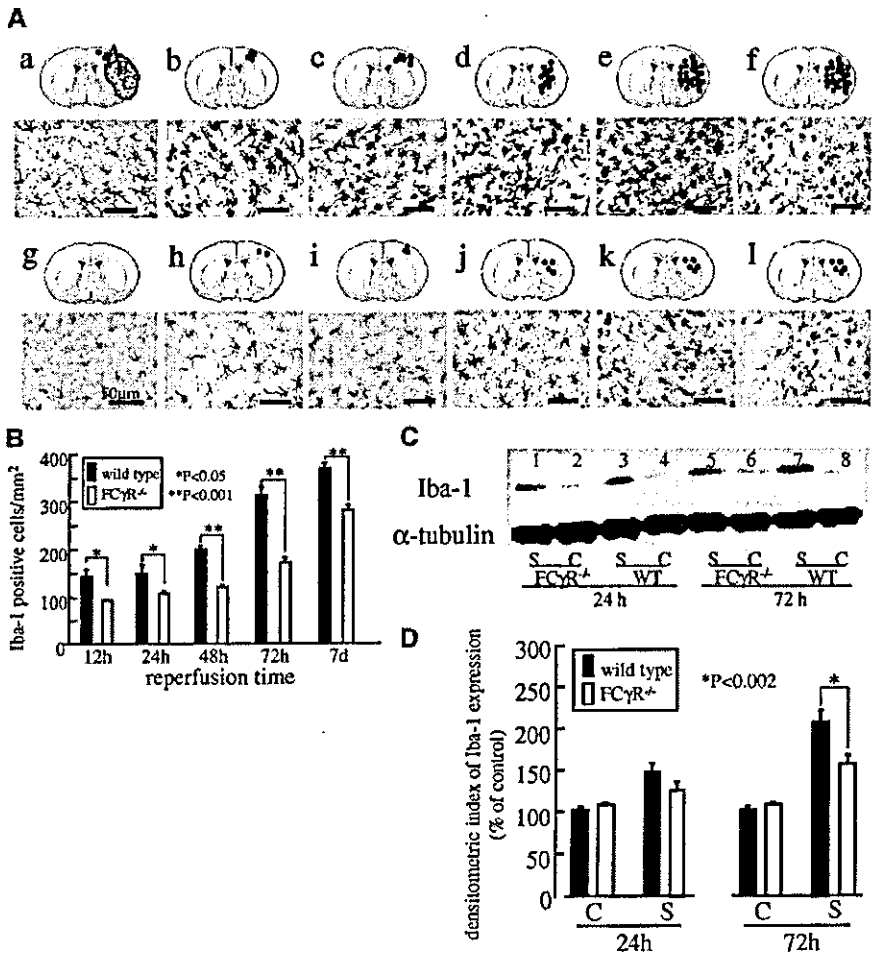

Figure 3. A, Schematic representation of distribution of neuronal damage in mouse brain after reperfusion. Shaded area represents the infarct zone (a). Three areas subjected to immunohistochemical analysis are illustrated: A, peri-infarct area; B, transition area; C, ischemic core area. Photographs show Iba-1 immunostaining in the transition area of representative WT (a to f) and FcγR$^{-/-}$ (g to l) mice. Shown are 12 hours (a, g), 24 hours (b, h), 48 hours (c, i), 72 hours (d, j), 7 days (e, k), and 14 days (f, l) after reperfusion. Dots on the brain schema represent the distribution of Iba-1–immunoreactive cells. Magnification ×200. B, Numbers of Iba-1–positive microglia at different time points. C, Western blot analysis. Samples were prepared from the brain at 24 hours (FcγR$^{-/-}$ mice: lanes 1 and 2; WT mice: lanes 3 and 4) and 72 hours (FcγR$^{-/-}$ mice: lanes 5 and 6; WT mice: 7 and 8) after reperfusion. A 17-kDa band corresponding to Iba-1 protein was clearly detected in the ischemic lesion, and the intensity of the band increased in the stroke side in a time-dependent manner. A weaker band was noted in FcγR$^{-/-}$ mice than in WT littermates. D, Densitometric analysis. Values are expressed as percentage of control. C indicates contralateral lesion; S, stroke side.