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Role of STAT6 in IgE Receptor/FcεRI-Mediated Late Phase Allergic Responses of Mast Cells

Ravi Malaviya* and Fatih M. Uckun[1†]

In this study we show that IgE receptor engagement triggers activation of STAT6 in mast cells. We sought to determine the role of STAT6 activation in IgE receptor-mediated mast cell responses using STAT6 knockout mice. After IgE receptor engagement, bone marrow mast cells from STAT6$^{-/-}$ mice exhibited normal histamine and leukotriene C_4 release, but their cytokine release was markedly reduced. In accordance with these in vitro data, IgE/Ag-challenged STAT6$^{-/-}$ mice showed normal early phase, but severely impaired late phase, allergic reactions. These findings provide unprecedented evidence that STAT6 plays a pivotal role in mast cell responses to IgE/Ag stimulation. *The Journal of Immunology,* **2002, 168: 421–426.**

Mast cells are secretory cells that are distributed throughout the vascularized tissues, including skin, lung, gut, and urinary bladder. These cells are known to play a key role in allergic reactions by their ability to release proinflammatory vasoactive amines (e.g., histamine), arachidonic acid metabolites (e.g. leukotriene C_4 (LTC_4)[2]) and a number of cytokines (e.g., TNF-α and IL-6) (1). These proinflammatory mediators are released from sensitized mast cells upon activation through the Ag-mediated cross-linking of their high affinity cell surface IgE receptors/FcεRI (1). IgE receptor is a heterotetramer with an α- and a β-chain and a dimer of two γ-chains (2, 3). Both β and γ subunits of the IgE receptor/FcεRI contain immunoreceptor tyrosine-based activation motifs, which allow interaction with protein tyrosine kinases (PTK) and PTK substrates via their SH2 domains (3). The engagement of IgE receptors by Ag triggers a cascade of biochemical signal transduction events, including activation of multiple PTK (4, 5). Notably, the activation of the tyrosine kinases, LYN, SYK, and BTK has been shown to correlate with mast cell mediator release (3, 6–8).

Recent studies from our laboratory revealed that Janus kinase 3 (JAK3), a member of the Janus family protein tyrosine kinases (9–11), also plays a pivotal role in mast cell-mediated allergic and asthmatic responses (12–14). Specifically, JAK3$^{-/-}$ mast cells from JAK3-null mice release substantially reduced amounts of inflammatory mediators upon IgE/Ag stimulation (14). Furthermore, JAK3-specific tyrosine kinase inhibitors effectively prevented mast cell-mediated allergic as well as asthmatic responses in mice (12, 13).

STAT6, a member of the STAT family, has been shown to regulate the response of mast cells to cytokine stimulation (15). Conventionally STAT6 is a 94-kDa protein, but mast cells express a distinct 65-kDa isoform of STAT6 in addition (16). A recent study by Suzuki et al. (17) has shown that this isoform of STAT6 is central to the JAK3-dependent IL-4 signaling in mast cells. In addition, STAT6 has been shown to regulate IgE/FcεRI receptor expression in mast cells (18). However, the role of STAT6 in IgE receptor-mediated mast cell responses has not been studied. In the present study we examined the role of STAT6 in IgE/FcεRI-mediated mast cell responses using in vitro as well as in vivo models.

In this study we report that STAT6 is activated in mast cells upon IgE receptor ligation by an appropriate Ag. Although IgE/Ag-induced histamine and LTC_4 releases by STAT6$^{-/-}$ mast cells are normal, STAT6$^{-/-}$ mast cells are significantly impaired in their ability to release cytokines in response to IgE/Ag challenge. Consistent with these in vitro results, STAT6$^{-/-}$ mice showed severely impaired late phase allergic reactions; however, the early phase cutaneous anaphylactic reaction was not affected.

Materials and Methods

Mice

Male C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories (Wilmington, MA). Breeder pairs of STAT6-null mice (19) were obtained from Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). Animals were caged in groups of five in a pathogen-free environment in accordance with the rules and regulations of the U.S. Animal Welfare Act and the National Institutes of Health. Animal care and experimental procedures were conducted in agreement with institutional guidelines.

FBS was obtained from HyClone (Logan, UT). LTC_4 ELISA kits were purchased from Cayman (Ann Arbor, MI). Histamine ELISA kits were purchased from Immunotech (Westbrook, ME). Mouse TNF-α, and IL-6, ELISA kits were purchased from R&D Systems (Minneapolis, MN). The preparations of DNP-BSA (20) and monoclonal anti-DNP-IgE (21) have been described previously. Anti IgE-FITC Ab was purchased from BD PharMingen (San Diego, CA). Dinitrofluorobenzene (DNFB) was obtained from Sigma (St. Louis, MO). HRP-conjugated anti-phosphotyrosine Ab was obtained from Transduction Laboratories (Lexington, KY).

Bacterial strain

The *Escherichia coli* strain ORN103(pSH2) was cultured in Luria broth containing 50 μg/ml chloramphenicol (22, 23).

Mast cell cultures

Mast cells were cultured from the bone marrow specimens of STAT6-null (STAT6$^{-/-}$) and STAT6$^{+/+}$ control mice in a medium supplemented with 25% WEHI-3 cell supernatant for 3 wk as previously described (12, 24). Cell density was adjusted to 2×10^5 cells/ml on a weekly basis. After 3 wk mast cells were characterized by staining with toluidine blue and Alcian blue (25). RBL-2H3 cells were a gift from Dr. R. P. Siraganian (Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD). The cells were maintained as

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[2] Abbreviations used in this paper: LTC_4, leukotriene C_4; BMMC, bone marrow mast cells; DNFB, dinitrofluorobenzene; JAK, Janus kinase; PCA, passive cutaneous anaphylaxis; PMN, polymorphonuclear; PTK, protein tyrosine kinase.

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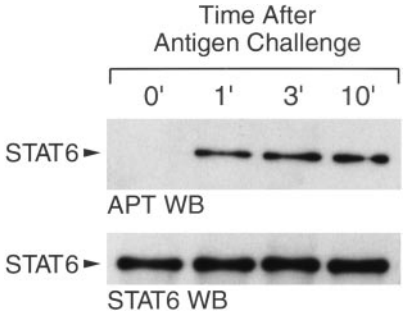

FIGURE 1. Activation of STAT6 in mast cells after IgE receptor cross-linking. To study IgE/Ag-induced activation of STAT6 in mast cells, RBL-2H3 cells were sensitized with monoclonal anti-DNP IgE and then challenged with DNP-BSA for the times indicated. Mast cells were lysed using a Nonidet P-40 lysis buffer, and STAT6 immune complexes from these cell lysates were subjected to anti-phosphotyrosine (APT) Western blot analysis to examine the phosphorylation of the STAT6 (*upper panel*). In parallel, STAT6 immune complexes were examined by anti-STAT6 immunoblotting (*lower panel*) to confirm that the increased tyrosine phosphorylation in APT blots was not due to differences in the amount of STAT6 immunoprecipitated.

monolayer cultures in 75- or 150-cm^2 flasks in Eagle's essential medium supplemented with 20% FCS (26).

Stimulation of mast cells

Bone marrow mast cells (BMMC) cultured from the bone marrow cells of STAT6$^{-/-}$ or STAT6$^{+/+}$ mice were sensitized with monoclonal anti-DNP IgE Ab (0.24 mg/ml) for 1 h at 37°C. Unbound IgE was removed by washing the cells with PBS. After washing the BMMC were resuspended in RPMI-HEPES buffer at a cell density of 1×10^6/ml. The cells were challenged with 20 ng/ml DNP-BSA at 37°C.

RBL-2H3 cells were sensitized with monoclonal anti-DNP IgE Ab (0.24 mg/ml) overnight at 37°C in a 48-well tissue culture plate. Unbound IgE was removed by washing the cells with PBS. After washing the PIPES-buffered saline containing 1 mM calcium chloride was added to the monolayers of the RBL-2H3 cells. The cells were challenged with 20 ng/ml DNP-BSA for 30 min at 37°C. The plate was centrifuged at $200 \times g$ for 10 min at 4°C. Supernatants were removed, and the cell pellets were lysed in lysis buffer.

To examine the role of STAT6 in *E. coli*-mediated mast cell responses, BMMC cultured from the bone marrow specimens of STAT6$^{-/-}$ or STAT6$^{+/+}$ mice were resuspended in RPMI-HEPES buffer at a cell density of 1×10^6/ml. The cells were challenged with *E. coli* 1×10^8 ORN103(pSH2) at 37°C for 1 h. After incubation the cells were centrifuged at $200 \times g$ for 10 min at 4°C. TNF-α levels were quantitated in cell-free supernatants.

Western blot analyses of mast cell lysates

Mast cells were sensitized with IgE and stimulated with Ag. Cells were harvested, lysed (10 mM Tris (pH 7.6), 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 100 μM Na_3VO_4, 50 μg/ml PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and the kinases were immunoprecipitated from the lysates, as reported using Abs directed against STAT6 (27, 28). Commercially available M-20 Abs reactive with STAT6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitations and immunoblotting using the ECL chemiluminescence detection system (Amersham Life Sciences, Arlington Heights, IL) were conducted as described previously (12).

Mediator release assays

Histamine content in cell-free supernatants and in the solubilized cell pellets was estimated using a commercially available enzyme immunoassay (29). LTC_4 levels were estimated in cell-free supernatants by immunoassay (24). TNF-α and IL-6 levels were estimated in cell-free supernatants using commercially available enzyme immunoassays.

IgE binding assay

Mast cells were incubated with 0.24 mg/ml monoclonal anti-DNP IgE Abs for 1 h at 37°C. The cells were washed thoroughly three times with saline containing 1% BSA and labeled with 10 μg/ml anti-IgE-FITC (BD PharMingen) for 30 min at 4°C. After washing the cells were analyzed by flow cytometry for FcεRI receptor expression.

Passive cutaneous anaphylaxis

To examine the role of STAT6 in FcεRI-mediated passive cutaneous anaphylaxis in mice, dorsal sides of the ears of STAT6$^{+/+}$ and STAT6$^{-/-}$ mice were injected intradermally with 20 ng anti-DNP IgE (left ears) or PBS (right ears) in a 20-μl volume using a 30-gauge needle, as previously described (30). After 24 h mice were challenged with 100 μg Ag (DNP-BSA) in 200 μl 2% Evans blue dye i.v. Mice were sacrificed 30 min after the Ag challenge. For quantitation of Evans blue dye extravasation as a measure of anaphylaxis-associated vascular hyperpermeability, 8-mm skin specimens were removed from the ears of mice, minced in 2 ml formamide, and incubated at 80°C for 2 h in water bath to extract the dye. The absorbance of the extracted dye was read at 590 nm. In some experiments, mice were injected i.v. with 0.5 μg anti-DNP IgE. After 24 h a skin reaction was elicited by painting 25 μl 0.15% DNFB on both the sides of the ear as has been described previously (31). Ear edema was measured at the times indicated by plythesmometer.

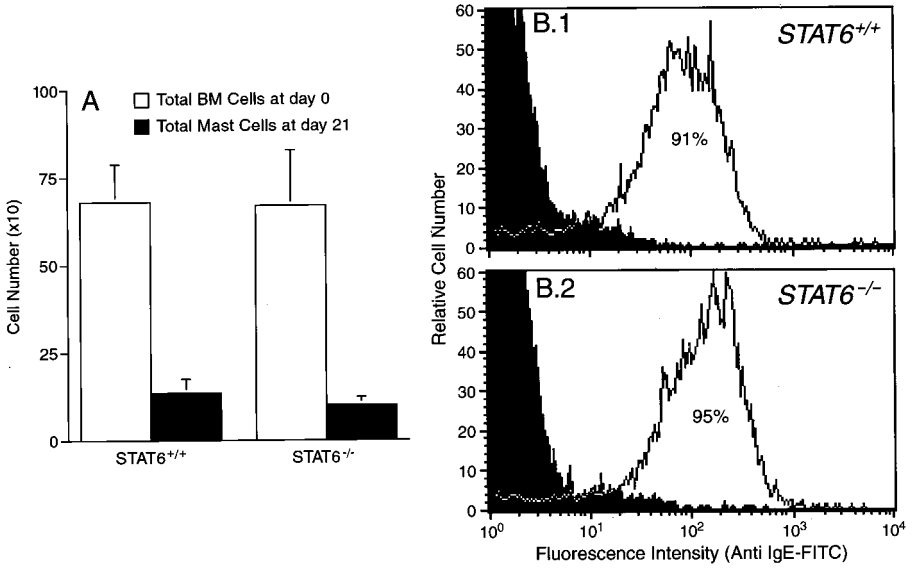

FIGURE 2. Characterization of STAT6$^{-/-}$ and STAT6$^{+/+}$ mast cells and their IgE receptor expression. Mast cells were cultured from the bone marrow (BM) of STAT6-null (STAT6$^{-/-}$), and wild-type (STAT6$^{+/+}$) control mice. *A*, Development of STAT6$^{-/-}$ and STAT6$^{+/+}$ mast cells cultured from the bone marrow specimens of STAT6$^{-/-}$ and STAT6$^{+/+}$ mice. *B*, Flow cytometric comparison of IgE receptor expression on STAT6$^{+/+}$ (B.1) vs STAT6$^{-/-}$ (B.2) mast cells was performed by staining mast cell-bound IgE with anti IgE-FITC Ab.

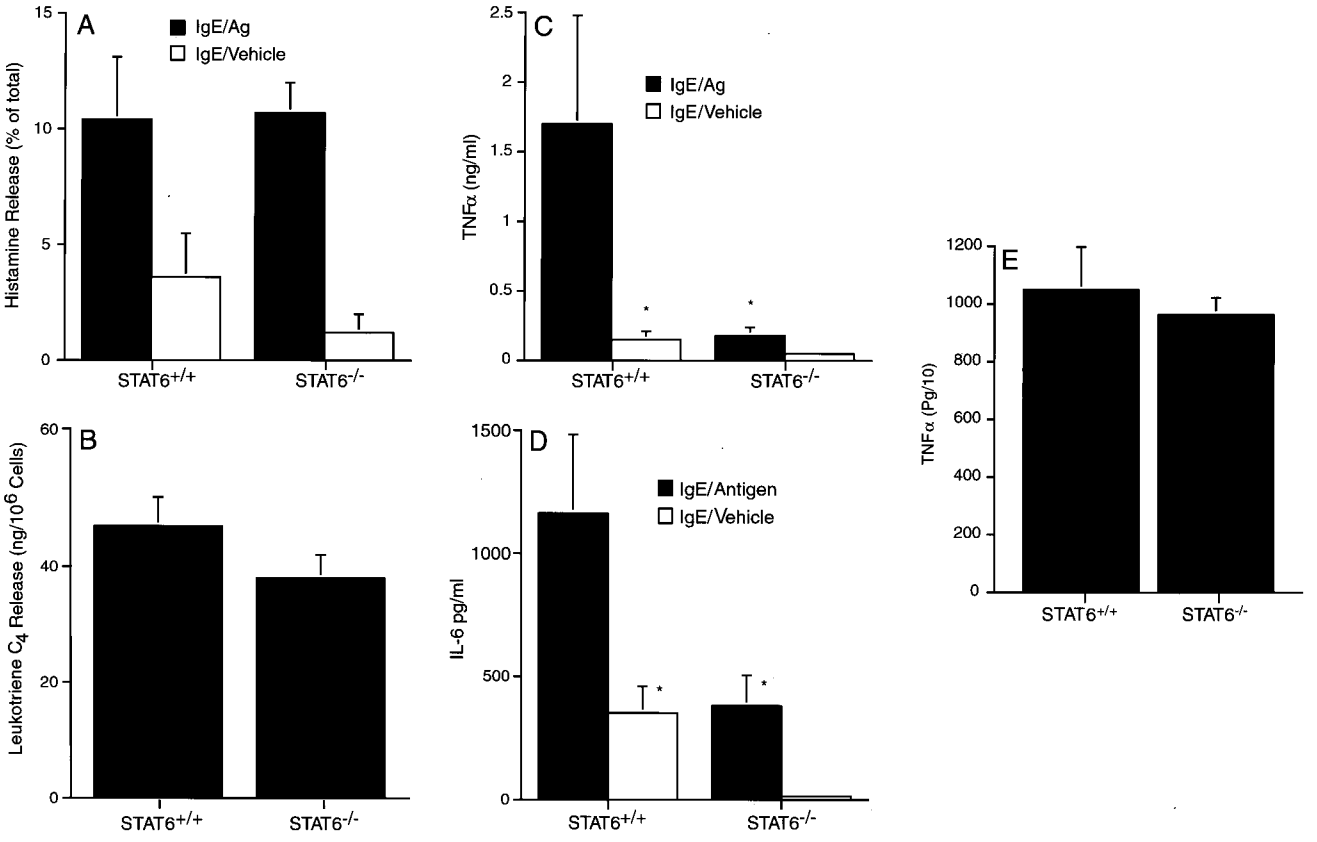

FIGURE 3. Inflammatory mediators released by STAT6$^{-/-}$ and STAT6$^{+/+}$ BMMC. STAT6 $^{-/-}$ and STAT6$^{+/+}$ BMMC were sensitized with a monoclonal anti-DNP IgE, and suspended in RPMI-HEPES buffer (*A* and *B*) or culture medium (*C–E*). Cells were then challenged with DNP-BSA for 30 min (*A* and *B*) or 24 h (*C* and *D*). Histamine (*A*) levels were quantitated in cell-free supernatants and Triton X-100-solubilized cell pellets. Results are expressed as the percent release of total cellular content. LTC_4 (*B*) and cytokine (*C* and *D*) levels were estimated in cell-free supernatants of BMMC by ELISA. To examine the effect of STAT6 deficiency on bacteria-mediated mast cell TNF-α release, STAT6 $^{-/-}$ and STAT6$^{+/+}$ BMMC were exposed to *E. coli* for 1 h, and extracellular TNF-α was quantitated by ELISA (*E*). No LTC_4 (*B*) or TNF-α (*E*) release was observed from vehicle-treated mast cells. The data points represent the mean $\pm$ SEM values obtained from three to eight independent experiments. $*$, $p < 0.05$ compared with control as determined by Student's *t* test.

Results

IgE receptor/FcεRI-mediated activation of STAT6 in mast cells

We first examined IgE/FcεRI receptor-mediated STAT6 activation in mast cells. To achieve this, anti-DNP IgE-sensitized RBL-2H3 mast cells were challenged with Ag (DNP-BSA) for indicated times and phosphorylation of STAT6 was examined by Western blot analysis. As shown in Fig. 1, cross-linking of the IgE receptors on mast cells resulted in enhanced tyrosine phosphorylation of a 94-kDa STAT6 protein.

Role of STAT6 in IgE/FcεRI receptor-mediated in vitro mast cells responses

We next sought to determine the physiologic significance of STAT6 activation in IgE/Ag-stimulated mast cells using STAT6 knockout (STAT6$^{-/-}$) mice. As shown in Fig. 2*A*, similar numbers of mast cells were obtained from bone marrow specimens of STAT6$^{+/+}$ and STAT6$^{-/-}$ mice after 3 wk of culture in medium supplemented with IL-3. STAT6$^{-/-}$ mast cells displayed typical staining pattern with toluidine blue (data not shown). The surface IgE/FcεRI receptor expression levels of STAT6$^{+/+}$ and STAT6$^{-/-}$ mast cells were virtually identical (Fig. 2*B*), reminiscent of the findings by Ryan et al. (18). Thus, STAT6 deficiency does not affect mast cell population size, morphology, or IgE/FcεRI receptor expression levels.

We next compared the IgE receptor/FcεRI-mediated release of inflammatory mediators from STAT6$^{+/+}$ and STAT6$^{-/-}$ mast cells. Mast cells cultured from the bone marrow of STAT6$^{+/+}$ and STAT6$^{-/-}$ mice were sensitized with anti-DNP IgE and challenged with DNP-BSA. The release of histamine, arachidonic acid

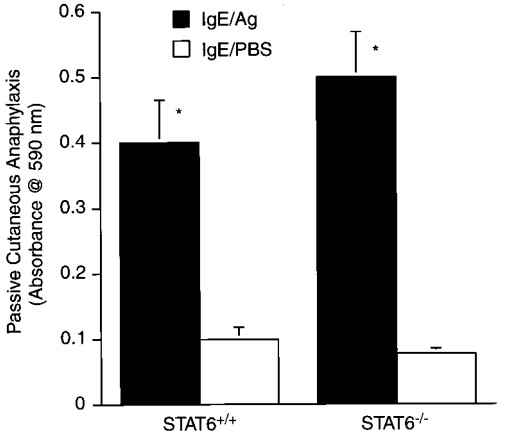

FIGURE 4. PCA in STAT6$^{+/+}$ and STAT6$^{-/-}$ mice. Anaphylaxis-associated vascular hyperpermeability was examined by evaluating the cutaneous extravasation of albumin-bound Evans blue dye in STAT6$^{+/+}$ and STAT6$^{-/-}$ mice. IgE-sensitized mice were challenged with 100 μg DNP-BSA in 2% Evans blue dye, and the plasma exudation indexes were determined. The data points represent the mean $\pm$ SEM values ($n = 10$–15). $*$, $p < 0.05$ compared with control (IgE/PBS) as determined by Student's *t* test.

metabolite LTC_4, and cytokine secretion was measured. No significant differences were detected in IgE/Ag induced histamine release (Fig. 3*A*) or LTC_4 release (Fig. 3*B*) from $STAT6^{+/+}$ vs $STAT6^{-/-}$ mast cells. However, the release of TNF-α (Fig. 3*C*) as well as the release of IL-6 (Fig. 3*D*) after IgE receptor/FcεRI cross-linking were substantially reduced in $STAT6^{-/-}$ mast cells compared with $STAT6^{+/+}$ mast cells. We next asked whether STAT6 deficiency also affects non-IgE receptor/FcεRI-mediated responses of mast cells. Mast cells release large amounts of TNF-α in response to *E. coli* challenge through CD48 receptor cross-linking (32). We therefore challenged $STAT6^{+/+}$ and $STAT6^{-/-}$ mast cells with type 1 fimbriated *E. coli* ORN103(pSH2) and quantitated their TNF-α release. As shown in Fig. 3*E*, STAT6 deficiency did not affect the TNF-α release from mast cells exposed to *E. coli*. Thus, STAT6 plays an important role in IgE receptor/FcεRI-mediated, but not *E. coli*-mediated, mast cell cytokine release.

Role of STAT6 in IgE/FcεRI receptor mediated in vivo mast cells responses

Mast cell-derived mediators produce a biphasic allergic reaction. A number of inflammatory mediators, including histamine, serotonin, and LTs, that are released immediately after mast cell activation have been shown to play a key role in induction of vascular permeability changes (30, 33, 34), whereas mast cell-derived cytokines such as IL-4, IL-5, GM-CSF, and TNF-α regulate the development of late phase inflammatory responses (35). We first examined the effect of STAT6 deficiency on vascular permeability in a well-characterized murine model of passive cutaneous anaphylaxis (PCA) (30). $STAT6^{+/+}$ and $STAT6^{-/-}$ mice were primed with intradermal injections of anti-DNP-IgE. Twenty-four hours later mice were challenged with Ag (DNP-BSA), and Evans blue dye extravasation was measured as a surrogate marker of plasma exudation. There was no significant difference in the PCA

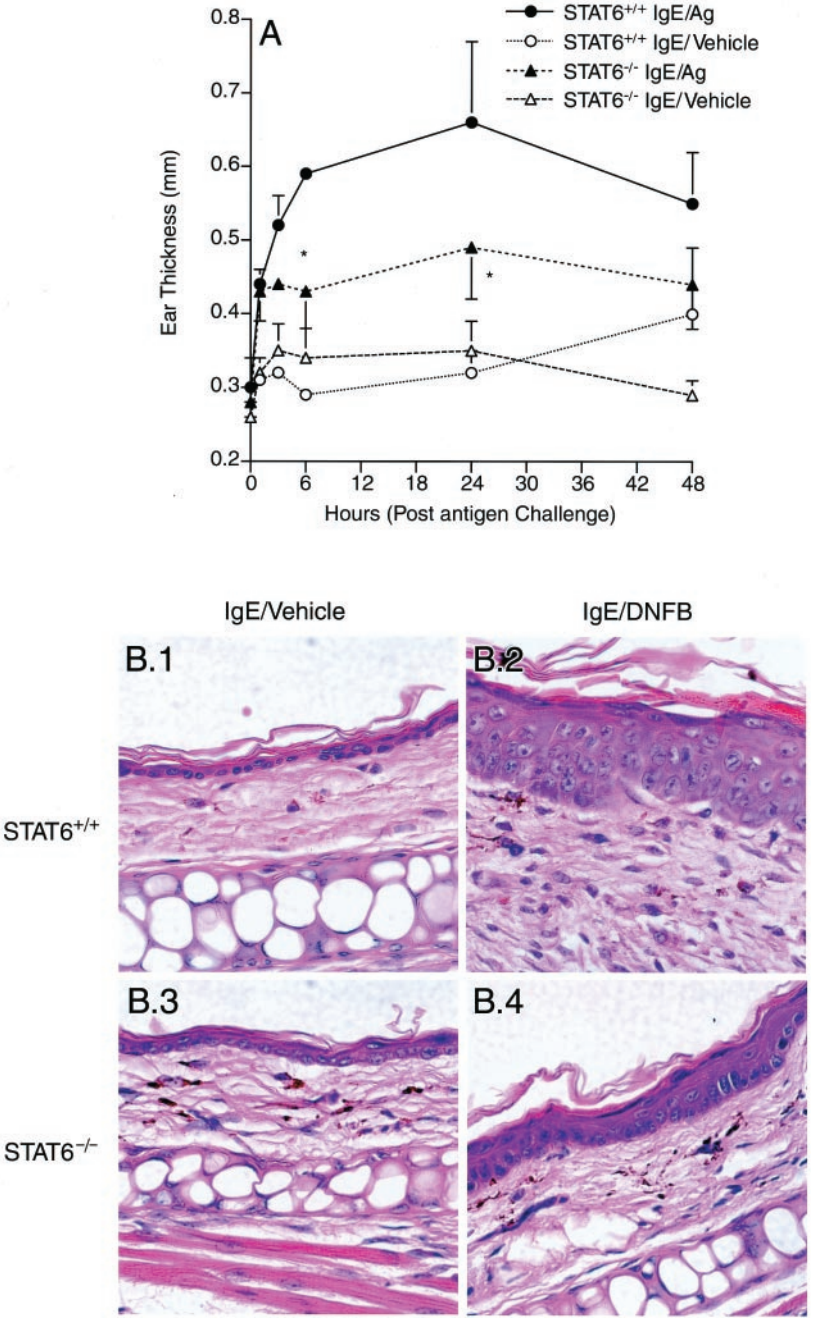

FIGURE 5. Effect of STAT6 deficiency on late phase PCA reaction in mice. $STAT6^{+/+}$ and $STAT6^{-/-}$ mice were sensitized i.v. with anti-DNP-IgE. Twenty-four hours later the left ears of mice were painted with 25 μl 0.15% DNFB, whereas the contralateral ears were painted with vehicle alone. *A*, Ear thickness of the mice was measured for the times indicated. The results are the mean ± SEM values (n = 5–7). *, $p < 0.05$ compared with $STAT6^{+/+}$ IgE/Ag mice as determined by Student's t test. *B*, For histopathologic evaluation ears were removed 24 h after Ag or vehicle challenge, and formalin-fixed thin sections (3–5 μm) of ears were stained with H&E. Histopathological evaluation of the coded ear tissues was performed by the pathologist in a blinded fashion. Sections shown are representative of sections of mouse ears from four mice in each group.

reaction between STAT6$^{+/+}$ and STAT6$^{-/-}$ mice (Fig. 4), which is in accord with our in vitro data showing that STAT6 deficiency does not affect histamine and LTC_4 release from mast cells. We next used a well-characterized mouse model of late phase allergic reactions (31, 35), which depends on mast cell-derived TNF-α (36). STAT6$^{+/+}$ and STAT6$^{-/-}$ mice were sensitized i.v. with 50 μg anti-DNP IgE. Twenty-four hours later a solution of 0.15% DNFB was painted on both sides of the left ear. Contralateral ears were painted with vehicle (acetone) alone. As shown in Fig. 5*A* STAT6$^{-/-}$ mice showed significantly less edema than STAT6$^{+/+}$ mice. We also examined the effects of STAT6 deficiency on IgE/hapten-induced histological changes in ear skin. The control ear skin (IgE/vehicle) typically has a two- or three-cell layer-thick epidermis and contains a few scattered inflammatory cells in the dermis (Fig. 5*B*). The ear skin of STAT6$^{+/+}$ mice showed thickened epidermis with four or five cell layers, and a large number of neutrophils were accumulated in the dermis (Fig. 5*B*). In contrast, the ear skin of STAT6$^{-/-}$ mice showed reduced epidermal cell thickening with fewer inflammatory cells than STAT6$^{+/+}$ mice. A histopathological analysis of ear skin of IgE/DNFB-challenged STAT6$^{+/+}$ and STAT6$^{-/-}$ mice was also performed to evaluate the magnitude of polymorphonuclear (PMN) cell infiltration. The tissue sections were scored on a scale of 1+ to 3+; 1+ was considered mild infiltration (1–10 PMN/section), 2+ was considered moderate infiltration (11–20 PMN/section), and 3+ was considered marked infiltration (21 or more PMN/section). Three of four IgE/DNFB-challenged STAT6$^{-/-}$ mice showed mild (1+) PMN infiltration (75%), whereas one STAT6$^{-/-}$ mouse showed no PMN infiltration (25%). Of five IgE/DNFB-challenged control STAT6$^{+/+}$ mice, three showed marked (3+) PMN infiltration (60%), one moderate (2+), and one mild (1+). These results demonstrate that STAT6 is a key regulator of IgE receptor-mediated late phase allergic responses of mast cells in vivo.

Discussion

STAT6 has been known as a regulator of IL-4-dependent immune responses (15, 19, 37, 38). It has also been shown to play an essential role in allergic airway inflammation by regulating Th2-type cytokine production of T cells (39–41). In the present study we examined the role of STAT6 in IgE receptor/FcεRI-mediated mast cell responses in experimental models of allergic inflammation. Our results demonstrate that STAT6 plays a pivotal role in IgE receptor/FcεRI-mediated cytokine release from mast cells.

STAT6-deficient bone marrow mast cells from STAT6$^{-/-}$ mice released significantly less TNF-α and IL-6 than wild-type mast cells from STAT6$^{+/+}$ mice upon cross-linking of their IgE receptor/FcεRI. The poor cytokine response of STAT6$^{-/-}$ mast cells was not due to reduced IgE receptor expression levels, because STAT6$^{+/+}$ and STAT6$^{-/-}$ mast cells expressed similar levels of IgE receptor/FcεRI on their surface, which is in accord with the findings of Ryan et al. (18), who previously reported that STAT6 deficiency does not affect mast cell IgE receptor expression. The impaired cytokine release from STAT6$^{-/-}$ mast cells was restricted to IgE receptor/FcεRI stimulation, because STAT6-deficient mast cells released normal amounts of TNF-α in response to bacterial stimulation.

In an effort to determine the significance of impaired mast cell cytokine response in STAT6$^{-/-}$ mast cells, we used a mouse model of cytokine-dependent late phase reaction. In this model mice are sensitized with IgE and subsequently challenged with DNFB (42). This challenge results in a biphasic inflammatory response, an early phase that peaks at 1 h, and a late phase that peaks at 24 h (42). Many studies indicate that the cutaneous late phase reaction is in part mediated by mast cell-derived cytokines (35, 36). Mast cell-derived TNF-α is known to play a critical role in late phase reactions of hypersensitivity (36). However, since STAT6 deficiency also affects the cytokine responses of other inflammatory cells, such as macrophages (43), a significant contribution of cells other than mast cells in the impaired late phase allergic responses of STAT6$^{-/-}$ mice cannot be excluded.

Histamine and LTs are critical for mast cell-mediated vascular permeability changes, a hallmark of early phase allergic reaction (30, 33, 34). Notably, STAT6 deficiency of mast cells did not affect the IgE receptor-mediated in vitro histamine or LTC_4 release from mast cells. When we compared the IgE/Ag-induced vascular permeability changes between STAT6$^{+/+}$ and STAT6$^{-/-}$ mice in an in vivo mouse model of passive cutaneous anaphylaxis, we noticed no significant differences. Thus, STAT6 deficiency impairs late (but not acute) phase allergic responses by selectively impairing the release of proinflammatory cytokines such as TNF-α.

While expanding our knowledge of the physiologic significance of STAT6, our study provides novel insights into the complex signal transduction network of mast cell activation. Our efforts to date to reconstitute STAT6$^{-/-}$ mast cells with wild-type or mutant STAT6 have failed. Therefore, the role of STAT6 protein in mast cell functions remains to be further elucidated at the cellular level using STAT6-reconstituted STAT6$^{-/-}$ mast cells. Further studies involving transfection of STAT6$^{-/-}$ mast cells with appropriately designed mutant STAT6 proteins would also help us determine which portions of STAT6 participate in signal transduction pathways stimulated by engagement of the high affinity IgE receptor of mast cells.

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