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Stat5 Expression Is Required for IgE-Mediated Mast Cell Function

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The mast cell (MC) inflammatory response is now linked not only to atopy, but also to arthritis, multiple sclerosis, heart disease, and resistance to bacterial infection. In the current study, we demonstrate that the signal transducer and activator of transcription 5 (Stat5) is rapidly activated by IgE cross-linkage, and that its expression is critical to the MC response. Stat5-deficient (Stat5KO) MC demonstrated a significant decrease in IgE-mediated degranulation, leukotriene B4 production, cytokine secretion, and survival signals. The defect in cytokine production may be caused by decreased cytokine mRNA stability. Stat5KO MC-induced cytokine mRNAs normally following IgE cross-linkage, but these mRNAs were not sustained over time and were degraded at twice the rate observed in WT cells. Interestingly, the RNA destabilizing protein tristetraprolin was induced following IgE cross-linkage in Stat5KO but not wild-type cells. Moreover, reducing tristetraprolin expression via short hairpin RNA transfection significantly increased IL-13 production in Stat5KO MC. Our work demonstrates that Stat5 is a critical factor in IgE-induced MC activation, acting in part via posttranscriptional control of cytokine mRNA stability. These data have a direct impact on MC-associated inflammatory and autoimmune diseases.

Mast cells (MC) are present in nearly all tissues. When activated, they direct inflammatory reactions through a biphasic response that controls vascular, respiratory, and immune cell function. The immediate phase of MC activation is characterized by the secretion of vasoactive and inflammatory substances such as histamine, heparin, proteases, prostaglandins, and leukotrienes (LT). This is followed by the late phase of MC activation, marked by synthesis and secretion of cytokines that elicit sustained inflammation (1, 2). This response has been best studied in atopic diseases such as asthma (1). However, MC are now implicated in the inflammatory responses associated with rheumatoid arthritis (3), multiple sclerosis (4), heart disease (5), and resistance to bacterial infection (6–9).

We recently showed that the transcription factor Stat5 is essential for MC survival and proliferation in vitro and in vivo (10). Stat5-deficient (Stat5KO) mice, although born with normal MC numbers, are completely MC deficient by 12 wk of age, due to lack of proper survival signals (Ref. 10 and C. Shelburne and J. Ryan, unpublished observations). Stat5KO MC can be derived in vitro, where they develop and expand normally if maintained in IL-3 plus stem cell factor (SCF), conditions that do not appear to exist in vivo. Thus, Stat5 expression is not required for normal MC development but is necessary to support subsequent survival and proliferation.

Given the critical role for Stat5 in survival, we questioned whether this transcription factor also might play a role in MC function. IgE-mediated signaling is a clinically relevant, well-studied model of MC activation. We determined the importance of Stat5 to the immediate and late phases of the IgE-induced MC response. These experiments revealed an essential role for Stat5 in IgE-mediated activation that impacts our understanding of MC-mediated inflammatory responses, possibly revealing a new clinical target for MC-associated diseases.

Materials and Methods

Cell culture

Bone marrow (BM) cells were cultured in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies) (10% FBS, 2 mM t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 1 mM HEPES; Biofluids), supplemented with IL-3 (5 ng/ml) and SCF (50 ng/ml). BM cells were harvested from the femurs and tibias of C57BL/6, BALB/c, Stat5A/B-deficient, and IL-3KO (provided by C. Lantz, Harri-sonburg, VA) mice as described (10). Bcl-2 transgenic (Bcl-2Tg) × Stat5KO mice were created by breeding H2K-Bcl-2Tg mice (a gift from J. Domen, Duke University, Durham, NC) to Stat5KO mice.

Reagents

Murine SCF and IL-3 were purchased from PeproTech and R&D Systems. IgE (clone C38-2), anti-CD16/CD32 (clone 2.4G2), PE-labeled anti-kit, anti-ERK, and phosphotyrosine-specific anti-ERK were purchased from BD Biosciences. PE-labeled rat IgG isotype control was purchased from eBioscience. FITC-labeled rat IgG isotype control and FITC-labeled rat anti-mouse IgE were purchased from Southern Biotechnology Associates. DNP-human serum albumin was purchased from Sigma-Aldrich. Phospho-

Western blotting

Western blotting was performed essentially as described previously (9) using 50 μg of total cell lysate per sample.
Stat5 IS REQUIRED FOR IgE-MEDIATED MC FUNCTION

Histamine release assay
Histamine release was measured using an ELISA kit (Neogen). BMMC were sensitized to IgE overnight in medium with IL-3 plus SCF, washed, and resuspended in Tyrode’s buffer with 1 mM MgCl₂ and 2.5 mM CaCl₂, without cytokines. Cells were then activated for 30 min with Ag at 37°C. Percent histamine release was calculated by dividing histamine reactivity in supernatant by reactivity in cell pellet.

LTB₄ assay
Cells were incubated with IgE (10 μg/ml) for 45 min at 4°C in cRPMI, washed, resuspended in cRPMI, then activated with DNP-human serum albumin (50 ng/ml) for 1 h in cRPMI without cytokines. LTB₄ was measured in culture supernatants using an ELISA kit (R&D Systems) as described by the manufacturer.

ELISA
Cytokine production was measured as described previously, (11). Briefly, BMMC were incubated with IgE (10 μg/ml) for 45 min at 4°C in cRPMI, washed, and resuspended in cRPMI supplemented with IL-3 plus SCF, then activated with DNP-human serum albumin (5–50 ng/ml) for 16–24 h. Cytokines were measured using OptEIA ELISA kits as described by the manufacturer (BD Biosciences).

Retroviral transduction
Stat5A expression was achieved using a murine stem cell virus-based bicistronic retroviral vector expressing Stat5A and coexpressing the GFP. This vector, termed pMSCV/Stat5A-IRES-GFP, has been described previously (10). Because Stat5A expression conveys a growth advantage, nearly 100% of the transfected cells were GFP⁺ at the time of use and expressed Stat5A at levels comparable with that of wild-type (WT) cells.

RNase protection assay
RNase protection assay was performed using the Riboquant system (BD Biosciences) as directed by the manufacturer. The ratio of gene of interest expression to housekeeping gene (L32 plus GAPDH) expression in the same sample was determined using a Typhoon phosphorimager (Molecular Dynamics). These normalized values were used to calculate the percentage change in gene expression.

Short hairpin RNA (shRNA) expression
To inhibit tristetrapolin (TTP) expression, Stat5KO BMMC were transfected with the TTP-A shRNA expression vector, encoding the sequence 5’act ggc att ctc tcc gct ct corresponding to nucleotides 160–179 of the murine TTP mRNA. Control cells were transfected with the pSuppressorNeo vector (Imgenex) alone. Plasmid was introduced into BMMC through use of the Nucleoporator, using Solution V, program T20 (Amaxa). Cells were selected in medium supplemented with 500 μg/ml G418 for 10 days before use.

Flow cytometric analysis of IgER expression
IgER expression was determined by flow cytometry as described previously (11).

Flow cytometric analysis of TNF production
Cells were stimulated with IgE plus anti-IgE for 5 h in the presence of monensin (2 μM), fixed in 4% paraformaldehyde for 20 min, then permeabilized and stained with PE-anti-TNF-α or PE-IgG in a solution of PBS containing 0.1% BSA and 0.5% saponin.

Analysis of monomeric IgE effects
Monomeric IgE was prepared by centrifuging IgE at 60,000 × g for 60 min. Monomeric IgE was added to BMMC cultures for 3 days at 0.1 or 1 μg/ml, which yielded similar results. FcεRI up-regulation was measured by flow cytometry. Cell survival was measured by propidium iodide staining.

Statistics
Results are the mean and SD for experiments with three to four samples. SE measurements are shown for data sets where >4 measurements were made. Values of p < 0.05, as determined by Student’s t test, were considered to be significant.

Results
FcεRI signaling activates Stat5
To investigate the possibility that Stat5 may function in IgE-mediated MC activation, we first examined Stat5 activation following cross-linkage with IgE plus Ag. As shown in Fig. 1, Stat5 is rapidly tyrosine phosphorylated by the addition of Ag to IgE-sensitized MC, with maximal activation after 5 min of stimulation. This activity was quite transient, as tyrosine phosphorylated Stat5 was nearly 100% of the transfected cells were GFP⁺ at the time of use and expressed Stat5A at levels comparable with that of wild-type (WT) cells.

FIGURE 1. FcεRI cross-linkage activates Stat5. BMMC were incubated in medium without cytokines for 3 h, then activated with IgE plus Ag (50 ng/ml) for the indicated times in the presence or absence of BFA (10 μg/ml). Total cell lysates were subjected to Western blotting with phosphotyrosine-specific anti-Stat5. The membrane was stripped and reprobed with anti-Stat5 and anti-actin to demonstrate protein loading. Data shown are representative of five experiments.

FIGURE 2. Stat5 deficiency does not alter ERK activation, but inhibits IgE-mediated degranulation and LT secretion. A, BMMC were in medium without cytokines for 3 h, then activated by IgE plus Ag (50 ng/ml) for 15 min. Total cell lysates were subjected to Western blotting with Abs specific for phosphorylated ERK proteins. The membrane was stripped and reprobed with anti-ERK to show protein loading. Data shown are representative of three independent experiments. B, BMMC were cross-linked with IgE plus Ag (50 ng/ml) for 30 min, and histamine release was measured by ELISA as described in Materials and Methods. Data are means and SD of three samples from one of three representative experiments. *, p = 0.035, when comparing activated WT with Stat5KO cells using Student’s t test. C, BMMC were cross-linked with IgE plus Ag (50 ng/ml) for 60 min as described in Materials and Methods. LTB₄ secretion was measured in culture supernatants by ELISA. Data are means and SD of four samples from one of three representative experiments. *, p < 0.001, when comparing activated WT with Stat5KO cells using Student’s t test.
not detectable after 30 min of IgE signaling. Addition of the vesicular transport inhibitor brefeldin A (BFA), which blocks MC cytokine secretion (12), did not affect Stat5 phosphorylation (Fig. 1). We also found that Stat5 was phosphorylated after IgE cross-linkage in IL-3KO BMMC, indicating that IgE-mediated IL-3 secretion is not required for Stat5 activation (data not shown). Hence, Stat5 activation may occur directly through FcεRI signaling rather than secondary to cytokine production.

**Stat5 deficiency does not affect IgE-mediated ERK activation, but reduces degranulation and LT production**

To determine the importance of Stat5 in IgE-mediated signaling, we compared responses in WT and Stat5KO BMMC. Importantly, we have shown previously that BMMC derived from Stat5KO mice develop normally and express FcεRI at levels comparable to that of WT BMMC (10). In keeping with the normal expression of IgER, some early events in FcεRI signaling appeared to be intact in Stat5KO MC. For example, the absence of Stat5 did not affect IgE-mediated MC ERK phosphorylation (Fig. 2A). In contrast, degranulation and secretion of the arachidonic acid metabolite LTB₄ during the first hour after IgE cross-linkage was reduced ~50% in Stat5KO BMMC (Fig. 2, B and C). Thus Stat5 has distinct and important roles in the early phase of the MC response.

**Stat5 deficiency prevents IgE-mediated cytokine production**

The late phase of MC activation is denoted by the production of cytokines that promote inflammation (1, 2). We determined the importance of Stat5 expression in IgE-mediated cytokine secretion by activating WT and Stat5KO BMMC with IgE plus Ag. Culture supernatants were tested for the presence of IL-6, IL-13, and TNF-α by ELISA. As expected, WT BMMC secreted significant amounts of TNF-α, IL-6, and IL-13 (Fig. 3A). Stat5KO BMMC were completely defective in secreting these cytokines, producing at most 5–10% of WT levels. To rule out a contribution of SCF or IL-3 in this process, BMMC were activated with IgE plus Ag for 18 h in the absence of growth factors. Because Stat5KO BMMC die rapidly after growth factor withdrawal, we used BMMC prepared from Stat5KO mice crossed to H2K-Bcl-2Tg mice in this experiment. These cells survived normally for 24 h in medium without growth factors (>95% viable by trypan blue exclusion; data not shown). As shown in Fig. 3B, Bcl-2Tg BMMC activated in medium without IL-3 and SCF produced TNF-α, whereas Bcl-2/Stat5KO Tg did not. Hence, the cytokine production defect in Stat5KO BMMC is consistent in the absence of IL-3 or SCF signaling. The reduction in cytokine production also was apparent when activated cells were stained for TNF-α and analyzed by flow cytometry (Fig. 3C).
expression observed by intracellular staining may indicate that Stat5 exerts its influence by controlling the degree of protein synthesis and secretion rather than completely inhibiting cytokine gene induction.

We have shown previously that Stat5A transduction rescues the survival defects of Stat5KO MC, indicating that the loss of Stat5, and not an altered development in vitro, explains the phenotype of these cells (10). To confirm the requirement for Stat5 in cytokine production, Stat5KO BMMC were transduced with retrovirus expressing Stat5A. Nearly 100% of these cells expressed the vector, as judged by expression of GFP from the IRES sequence. Transduction restored Stat5 production to levels comparable to that of WT cells (Fig. 3D). Importantly, Stat5A expression restored IgE-induced IL-13 secretion in Stat5KO MC (Fig. 3E). These results argue strongly that Stat5 expression is a critical aspect of IgE-induced cytokine production.

Decreased cytokine mRNA stability in Stat5KO BMMC

Cytokines are controlled by both mRNA induction and stability (13–15). To determine the effect of Stat5 deletion on IgE-mediated cytokine mRNAs, we measured the induction and maintenance of IL-13, a cytokine with critical inflammatory functions (16). IL-13 showed significant up-regulation that was matched by protein secretion. As shown in Fig. 4A, IgE cross-linkage greatly up-regulated IL-13 mRNA in both WT and Stat5KO BMMC. These mRNAs were nearly absent within 90 min of signaling in Stat5KO BMMC but were still overtly expressed at this time point in WT cells. A similar pattern of regulation was observed with IL-6 and TNF-α (data not shown). To determine whether these differences were related to mRNA stability, BMMC were activated for 30 min with IgE plus Ag, after which the transcriptional inhibitor actinomycin D (ActD) was added. RNA was then harvested during the 60 min following ActD addition to determine the rate of mRNA degradation. As shown in Fig. 4, B and C, the rate of IL-13 mRNA degradation in Stat5KO BMMC was twice that observed with WT MC. This was in contrast to c-kit mRNA, which showed a similarly slow rate of degradation in both WT and Stat5KO BMMC.

Stat5 deficiency alters TTP expression

The observed instability of cytokine mRNAs in Stat5KO BMMC suggests that Stat5 may control the expression of proteins that alter mRNA half-life, rather than directly eliciting cytokine transcription. One candidate is the mRNA destabilizing protein TTP, which is induced by Stat6 (17). We measured TTP protein levels during IgE cross-linkage in WT and Stat5KO BMMC and noted a striking increase in TTP expression that occurred only in Stat5KO cells (Fig. 5A). These results suggest that Stat5 may normally function to inhibit TTP expression in WT cells. To determine whether TTP expression had a causal relationship to cytokine production, Stat5KO BMMC were transfected with pSuppressorNeo plasmid expressing TTP shRNA, which has previously been shown to repress TTP expression (17). As shown in Fig. 5B, TTP shRNA expression significantly repressed TTP expression following IgE cross-linkage, when compared with Stat5KO cells transfected with vector alone. Moreover, TTP shRNA expression led to a significant decrease in IL-13 production (Fig. 5C), arguing that TTP has a role in the cytokine defect noted in Stat5KO MC.

Stat5 deficiency alters the effects of monomeric IgE

Separate from Ag-mediated effects, IgE has been reported to up-regulate FcεRI surface expression and to induce MC survival (18). Although the increase in IgE receptor expression is thought to occur via protein stabilization, IgE-mediated survival may be driven by IL-3 secretion (19). We found that monomeric IgE profoundly up-regulated FcεRI expression on both WT and Stat5KO BMMC, but only enhanced the survival of WT MC (Fig. 6). Thus, Stat5 is critical to the cytokine-related effects of IgE, both those elicited by Ag, and those that occur independent of IgE–Ag complexes.

Discussion

MC-mediated immune responses, once mainly the interest of allergists, are now broadly implicated in many inflammatory diseases. Data from the human system are still largely circumstantial; however, animal models show a clear and critical role for these granulated tissue cells in models of multiple sclerosis, rheumatoid arthritis, coronary artery disease, and host resistance to bacterial infection (3–9). Thus, understanding MC activation has taken on a
new degree of importance, especially given the range of MC-targeted therapies already in use for atopic diseases that could be expanded to other settings. It is in this arena that we find Stat5 to be a central factor and potential target for clinical intervention.

The MC response can be simplistically separated into early and late events, largely demarcated by degranulation and lipid mediator release in the first minutes after activation and cytokine secretion over the following 3–6 h. These phases collectively work to alter vascular flow and recruit leukocytes (1, 2). We find that Stat5 deletion inhibits histamine release and LT production, markers of the early phase, and essentially ablates late-phase cytokine secretion. Given this regulation of the inflammatory response and its obligatory role in MC survival (10), the utility of targeting Stat5 in MC diseases warrants exploration.

Because our data showed a complete loss of cytokine production in Stat5KO BMMC, we chose to pursue this issue in more detail. However, the 50% decline in histamine release and LTB4 secretion are clinically important, because these are key mediators of bronchoconstriction and vasodilation. More work is needed to understand how Stat5 contributes to the immediate phase of MC activation. For example, it is striking that MAPK, a pathway that appears to be intact in Stat5KO BMMC, has been implicated in activating the arachidonic acid cascade (20–24), yet LTB4 production remains low in Stat5KO MC. Our current study is aimed at understanding how Stat5 controls this response, focusing specifically on expression and function of phospholipase A2.

Loss of IgE-induced cytokine production was the most overt defect in Stat5KO MC. This is a clinically important finding, because MC-derived cytokines may be particularly important to disease pathology, perhaps because of the sentinel role these cells play in immunity. For example, IL-13 alone elicits many of the symptoms of asthma (16). Further, MC-derived TNF-α appears to be the critical determining factor in bacterial immunity and lymphadenopathy (6–9). The ability of cytokines to modulate the immune response makes Stat5 control of these factors particularly important.

Our data suggest that Stat5 may mediate repression of the mRNA-destabilizing protein TTP because of the mechanism controlling cytokine expression in MC. TTP expression was up-regulated following IgE cross-linkage only in Stat5KO BMMC, arguing that Stat5 may actively suppress TTP in WT MC. In support of this theory, TTP suppression by shRNA partially restored IL-13 secretion to 30% of WT levels. It is possible that TTP induction is only part of the cytokine defect in Stat5KO MC. Stat5-mediated repression of TTP is an interesting contrast to Stat6-mediated TTP induction (17). This is particularly striking because Stat6, as well as Stat3, is activated by FcεRI (25, 26). A unifying hypothesis to these opposing activities is that Stat transcription factors regulate MC cytokine production by recruiting transcriptional repressors or
activators to the TTP promoter. By this theory, Stats would effectively integrate extrinsic signals, providing homeostasis to a potentially pathological inflammatory response.

At present, we do not know the mechanism by which IgE cross-linkage activates Stat5. Given its rapid phosphorylation and the inability of BFA to hamper this process, it does not appear that autocrine signaling by cytokine receptors is the explanation. Further, the induction of IL-3 during IgE sensitization also does not conform to our findings. BMMC were sensitized in the cold, the induction of IL-3 during IgE sensitization also does not potentially pathological inflammatory response.

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