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Stat5B is Required for IgE-Mediated Mast Cell Function *in vitro* and *in vivo*

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Abstract

Mast cells are found primarily at interfaces with the external environment, where they provide protection from pathogens but also elicit allergic inflammation. Mast cell activation by antigen-induced aggregation of IgE bound to the high affinity receptor, FcεRI, is a critical factor leading to inflammation and bronchoconstriction. We previously found that Stat5 is activated by FcεRI and that Stat5B suppression decreased IgE-induced cytokine production *in vitro*, but *in vivo* responses have not been assessed. We now show that Stat5B-deficient (KO) mice have reduced responses to IgE-mediated anaphylaxis, despite normal mast cell tissue distribution. Similarly, Stat5B KO mast cells have diminished IgE-induced degranulation and cytokine secretion *in vitro*. These mice have elevated IgE production that is not correlated with an intrinsic B cell defect. The current work demonstrates that the Stat5B isoform is required for normal mast cell function and suggests it limits IgE production *in vivo*.

1. Introduction

Mast cells are tissue resident cells found at the boundaries between the external and internal environment such as mucosal surfaces, blood vessels, and nerve endings, where they act as sentinels quickly initiating the innate immune response[1]. They are characterized by their

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6. Author Contributions

All authors contributed to the writing and editing of this manuscript as well as data analysis. Experimental design was largely performed by: KNK, ERMK, RKM, and JJR. Data acquisition was largely performed by KNK, EMK, JJAM, BB, PAP, MPZ, TTH, AP, SAK, JD, KJ.

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large central nucleus and highly granulated cytoplasm. These granules contain preformed mediators that are released during the early phase of mast cell activation[2]. The preformed mediators include histamine, proteases, peptidoglycans such as heparin, and some cytokines. The late phase of mast cell activation involves the *de novo* synthesis and secretion of cytokines, leukotrienes, and prostaglandins. Collectively, the early and late phase reactions elicit many allergy symptoms and are particularly important in vasodilation and bronchoconstriction[3].

Mast cells have several receptors that are vital to their role as sentinels. This includes FcεRI, FcγR, toll-like receptors, complement receptors, and many chemokine and cytokine receptors. In the context of asthma and allergy, FcεRI aggregation by antigen-IgE complexes is one of the earliest and most potent signals inducing mast cell function. FcεRI signaling has been intensely studied and is revealing numerous drug targets for allergic disease. We previously identified a Fyn kinase-Stat5B pathway activated by IgE crosslinking that has a major effect on mast cell function[4].

Signal Transducers and Activators of Transcription (Stats) are ubiquitously expressed proteins found in the cytoplasm. Their primary role is to relay signals from the cell membrane to the nucleus. The binding of ligand to extracellular receptors activates kinases that phosphorylate specific tyrosine residues on the intracellular portion of the dimerized receptors. Receptor phosphorylation creates docking sites for Stat recruitment via their Src-homology (SH2) domains, after which Stats are in turn tyrosine phosphorylated. These activated Stats form dimers in the cytoplasm and then translocate to the nucleus where they bind to DNA and mediate gene transcription. Seven Stats have been identified: Stat1–6, including Stat5A and Stat5B. Although Stats are primarily activated by JAK family tyrosine kinases, they are involved in many signaling pathways and can be phosphorylated by a variety of tyrosine kinases[5].

Stat5 is of particular importance in mast cell cytokine production, migration, survival, and proliferation[4,6,7]. Stat5A and Stat5B share greater than 90% amino acid homology and have overlapping but also distinct functions[8]. For example, Stat5A KO mice are unable to lactate after parturition[9], while Stat5B KO mice lose sexual dimorphism in growth rates[10]. The differences between these two genes extends to immunological effects. Stat5A is required for expression of IL-2 receptor alpha chain, indirectly affecting T cell populations[11], while Stat5B is required for natural killer function[12]. In mast cells these two genes can also mediate distinct functions. Stat5A regulates mast cell proliferation and survival[13], while we previously found that Stat5B knockdown with siRNA reduces cytokine production[4].

Given its role in IgE-induced cytokine secretion, we hypothesized that Stat5B is critical for *in vivo* mast cell function. The current work shows that Stat5B deficiency does not alter mast cell number or tissue distribution, but greatly reduces IgE-mediated systemic anaphylaxis. We also noted increased plasma IgE levels that were not associated with an intrinsic B cell defect. Although elevated IgE has been shown to mask antigen-induced passive anaphylaxis[14], we found that anti-IgE-mediated anaphylaxis was also inhibited in Stat5B KO mice, suggesting an FcεRI signaling defect. Further, the Stat5B KO response to histamine-induced shock was normal, indicating that Stat5B deficiency does not alter the

vascular response to mast cell activation. These data match *in vitro* findings that Stat5B KO mast cells have reduced IgE-mediated degranulation and cytokine secretion. Collectively, our findings demonstrate a critical role for the Stat5B isoform in IgE-induced mast cell function and suggest that Stat5B has an indirect negative effect on IgE production. These data emphasize the selective role of Stat5 isoforms and support the potential impact of targeting Stat5B to suppress in allergic disease.

2. Materials and Methods

2.1 Mice

C.129-Stat5B^{tm1Hwd}/J mice (Stat5B KO) on a BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). Colonies were maintained in a pathogen-free facility with a standard chow diet. Stat5B KO mice were obtained by breeding Stat5B KO homozygous males and Stat5B KO heterozygous females. Mice were genotyped via PCR with primer sequences from by The Jackson Laboratory (5'→ 3' Wildtype 1: CCC AAG AGT ACT TCA TCA TCC AG, Mutant: TGA CTA GGG GAG GAG TAG AAG GTG G, and Wildtype 2: GAG CTT GCT CCT ACG ACC TTA CT) and MyTaq™ Extract-PCR Kit from Bioline (London, UK). Briefly, tail snips were obtained from mouse pups at weaning and processed according to the manufacturer's instructions. PCR product was then separated by electrophoresis on a 1.8% agarose gel. Results were determined using a 100-bp DNA ladder from New England Biolabs (Ipswich, MA) (Mutant = 200 – 300bp, Heterozygote = 110bp and 200 – 300bp, Wildtype = 110bp). For all experiments, Stat5B KO female mice were age matched to homozygous wildtype female litter mates. Bone marrow was extracted from mice at a minimum of 10 weeks old. All studies were performed with approval from the Virginia Commonwealth University Institutional Animal Care and Use committee.

2.2 Reagents

Cell culture medium (RPMI 1640 medium) was purchased from Invitrogen (Carlsbad, CA), fetal bovine serum (FBS) from Quality Biological, Inc. (Gaithersburg, MD). Recombinant mouse IL-3 and SCF were purchased from Shenandoah Biotechnology, Inc (Warwick, PA). Purified IgE (clone c38–2, κ isotype) was purchased from BD Biosciences (San Diego, CA). Dinitrophenyl-coupled human serum albumin (DNP-HSA) and histamine were purchased from Sigma-Aldrich (St. Louis, MO). Dr. Daniel Conrad (VCU) generously provided mouse anti-DNP IgE for *in vivo* experiments. Antibodies against c-Kit (PE anti-mouse CD117) and FcεRI (APC anti-mouse FcεRIα) were purchased from Biolegend (San Diego, CA) and used at a dilution of 1:100.

2.3 Mast Cell Culture and proliferation assay

Mouse bone marrow was differentiated into bone marrow derived mast cells (BMMCs) by culture in complete RPMI (cRPMI) 1640 medium containing 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 1mM sodium pyruvate and 1mM HEPES. Media was supplemented with IL-3-containing supernatant from WEHI-3B cells and stem cell factor (SCF)-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF was adjusted to 10ng/ml as measured by ELISA. Bone marrow cells were cultured for 21–28 days to yield cells yield over 90% FcεRI$^+$ and cKit$^+$ bone-marrow

derived mast cells. To assess proliferative potential, BMMC were plated at 2×10^5 cells/ml in cRPMI with 0.5ng/ml IL-3 and increasing concentrations of SCF. Cells were counted in the presence of a trypan blue after 72 hours, using a Vi-Cell XR cell viability analyzer (Beckman Coulter Life Sciences, Indianapolis, IN).

2.4 Enzyme-linked immunosorbent assay (ELISA)

IL-6, MCP-1, and TNFα levels were assessed by ELISA using ELISA Max™ Standard set kits purchased from Biolegend, Inc. (San Diego, CA). ELISAs were performed with culture supernatants or plasma samples according to the manufacturer's protocols. IL-13 and MIP-1α levels were assessed with Murine IL-13 and MIP-1α Standard ABTS ELISA Development Kits purchased from PeproTech (Rocky Hill, NJ) as per the manufacturer's protocol.

2.5 Flow cytometry

For surface c-Kit and FcεRI expression, $1–2\times10^5$cells were resuspended in 10μl rat anti-mouse CD16/CD32 clone 2.4G2 (10μg/ml) for 10 minutes at 4ºC, then stained with anti FcεRI/α c-Kit or isotype control antibodies for 20 minutes at 4ºC, washed with PBS, and resuspended in FACS buffer (PBS, 3% FBS, 0.1% Sodium Azide), and analyzed with BD FACSCaliber or BD FACSCelesta.

2.6 Degranulation and histamine content

To detect degranulation using surface markers, activated cells were incubated in 10μl rat anti-mouse CD16/CD32 clone 2.4G2 (10μg/ml) then stained with anti-CD107a or isotype control antibodies for 20 minutes at 4°C, washed with PBS and resuspended in FACS buffer (PBS, 3% FBS, 0.1% sodium azide) and analyzed using FACSCaliber or BD FACSCelesta. To measure histamine content and release, BMMCs were sensitized with 0.5μg/ml DNP-specific IgE for 16 hours at 37ºC. Cultures were washed and activated with DNP-HSA (50ng/ml) for 10 minutes at 37°C in cRPMI. After centrifuging, supernatants were collected and cell pellets were lysed for 30 minutes on ice with PBS/1% NP-40 detergent. Histamine content in supernatants and lysates were measured using an ELISA (Neogen Diagnostics, Lexington, KY).

2.7 Cytokine production

Mature BMMCs were sensitized with 0.5μg/ml DNP-specific for at least 16 hours at 37ºC. Cultures were washed and activated with DNP-HSA (50ng/ml) for 16 hours. Supernatants were collected and cytokines levels assessed using ELISA.

2.8 Passive systemic anaphylaxis (PSA)

Age-and sex-matched groups of co-housed mice 8–16 weeks old were used. For IgE-induced PSA, mice received intra-peritoneal (IP) injections of DNP-specific IgE (50μg) 16 hours before IP injection of DNP-HSA (100μg). For anti-FcεRI-mediated PSA, mice were injected IP with DNP-specific IgE (50μg/ml) 16 hours before IP injection of 50μg/ml anti-FcεRI clone MAR-1 antibody. For histamine-induced PSA, mice were injected with 8mg of histamine by IP injection. The core body temperature of each mouse was measured with a

rectal probe. Mice were euthanized with CO_2 asphyxiation and blood was collected via cardiac puncture 120 minutes after DNP-HSA injection. ELISA was used to analyze plasma cytokine levels.

2.9 Mast cell numbers

Age- and sex-matched groups of co-housed mice 8–16 weeks old were used. For tissue resident mast cells, tissue samples were removed and stored in neutral-buffered formalin solution (Sigma-Aldrich, St. Louis, MO). Tissues were embedded in paraffin, sectioned and mounted on glass slides, then stained with pinacyanol erythrosinate by StageBio (Mount Jackson, VA). Mast cells found in the entire tissue section mounted on the slides were enumerated. For peritoneal mast cell numbers, 5mL of PBS-EDTA (PBS, 1% EDTA) was injected into the peritoneal cavity and the cavity massaged thoroughly. The peritoneal exudate was collected with a plastic Pasteur pipette and stained for FcεRI and c-Kit double-positive cells as described above.

2.10 B cell analysis and magnetic isolation

The spleens of age- and sex-matched mice were isolated and homogenized between two glass slides and passed through a 40μm filter into a 50mL conical to create a single cell suspension. Red blood cells were lysed with ACK lysis buffer (Quality Biological, Gaithersburg, MD) followed by washing and resuspension in MACS buffer (2% BSA in PBS, 2mM EDTA, pH 7.2). The spleen was divided for flow cytometry, isolation of naïve B cells, and isolation of total B cell populations. Naïve B cells were isolated using magnetic bead depletion (anti-CD43, Miltenyi Biotec, Gaithersburg, MD) per manufacturer's instructions. Total B cells were isolated using magnetic bead selection (anti-B220, Miltenyi Biotec) per manufacturer's instructions. B cell populations were plated at decreasing cell concentrations (25,000, 12,000 or 6,000 cells/mL) with anti-CD40 (2μg/mL) in the presence or absence of IL-4 (10ng/mL) for 9 days. For flow cytometry, briefly, cells were washed with PBS and stained with Zombie Aqua fixable Live/dead stain for 10 mins at room temperature in the dark. Then, staining was stopped with MACS buffer and cells were washed. Cells were Fc blocked (clone 2.4G2) and stained on ice in MACS buffer with Brilliant Violet Stain Buffer (BD) for 20 mins, washed and fixed for 10 mins at room temperature, washed and resuspended in PBS until ready to analyze. This all took place in the dark. Splenic B2 cells were gated as Live, $CD45^+B220^+CD23^+$ and extrapolated back to total number B2 cells. Antibodies are included in supplemental Table 1.

2.11 ELISA

Cell-free supernatants were collected and ELISA used to measure the amount of total antibody produced [15]. For IgE ELISA, briefly, plates were coated with rat anti-mouse IgE (clone B1E3) in borate buffered saline, blocked (PBS with 0.02% Tween20 and 2% FBS), detected with biotinylated rat anti-mouse IgE (clone R1E4) and streptavidin-alkaline phosphatase (Southern Biotech). Plates were developed with phosphate tablets (Millipore-Sigma, St. Louis, MO) dissolved in substrate buffer (0.1g $MgCl_2.6H_20$, 0.2 NaN_3, 50mL diethanolamine, pH to 9.8 per 500mL). Absorbance was measured at 405nm-650nm. Samples were run in duplicate and compared to an 11pt standard curve of purified mouse IgE anti-DNP antibody. For total immunoglobulin levels, plates were coated with anti-mouse

IgG or anti-mouse IgM (Southern Biotech, Birmingham, AL), and detected with alkaline phosphatase-conjugated anti-mouse IgM, IgG1, or IgG2a (Southern Biotech). All ELISAs were read using SoftMax Pro Data Acquisition and Analysis software (Molecular Devices, San Jose, CA) on a Molecular Devices Plate reader.

2.12 Cell Proliferation

For assessment of proliferation, B cells were resuspended at 50,000 cells per mL in the presence of anti-CD40 (2μg/mL) and IL-4 (10ng/mL) for 72 hours. After this period, [H3]-thymidine was pulsed at a concentration of 1μCi/well for the final 24 hours (Perkin Elmer). Plates were harvested using a Filtermate cell harvester onto GFC plates. Assays were read using a Topcount Plate Counter (Perkin Elmer).

2.13 FcεRI occupancy

Peritoneal lavage was performed as described in the *B cell analysis* section, washed, stained, and flow cytometry analysis was performed on peritoneal exudate cells (PEC). Mast cells were gated as live $CD45^{+}Fc\varepsilon RI^{+}cKit^{+}$ cells and assessed for anti-IgE staining to determine the occupancy of mast cell FcεRI *ex vivo* with data acquisition on a BDFortessaLSR and analysis on FlowJo software. Antibodies are included in supplemental table 1.

3. Results

3.1 Stat5B deficiency increases in vitro mast cell differentiation and is required for optimal IgE-mediated responses.

To determine if mast cells could be cultured *in vitro* in the absence of Stat5B, bone marrow was harvested from Stat5B KO mice and WT littermates and cultured in IL-3 (10ng/ml) and SCF (10ng/ml) for six weeks. Each week an aliquot of maturing mast cells was removed and probed for the expression of the FcεRI and c-Kit. At each time point there was a modest but significantly higher percentage of $Fc\varepsilon RI^{+}$/c-Kit^{+} Stat5B KO cells compared to WT cells (Figure 1A). By the end of the culture period the two cultures had reached a similar plateau. Additionally, there was no difference in the expression intensity of FcεRI or c-Kit after maturation (Figure 1B). Thus Stat5B appears to have a moderately negative effect on mast cell differentiation.

We noted no significant differences in the rate of expansion among developing WT and Stat5B KO mast cells, but studied this in more detail after BMMC reached maturity. As shown in Figure 1C, Stat5B KO BMMC proliferated in response to SCF at a rate commensurate with matched wild type cells. These data suggest that Stat5B has a no significant role in proliferation.

To determine the importance of Stat5B for IgE-induced mast cell degranulation and cytokine production, we sensitized WT or Stat5B KO BMMCs with DNP-specific IgE for 16 hours, followed by activation with DNP-HSA (hence referred to as IgE XL). Degranulation was first determined by histamine release 10 minutes after crosslinkage. As shown in Figure 2A, Stat5B KO BMMC exhibited significantly less histamine secretion in response to IgE XL. However, the percent histamine release was not different (Figure 2B). This incongruity was

explained by a reduced histamine content in Stat5B KO BMMC (Figure 2C). To further examine the importance of Stat5B in FcεRI-induced degranulation, we employed surface staining for the lysosomal marker CD107a/LAMP-1 after 10 minutes of treatment with DNP-HSA. Stat5B KO BMMC showed significantly less CD107a staining compared to WT BMMC at a range antigen concentrations, suggesting a reduced degranulation response (Figure 2D). Coupled with histamine measurements, these data indicate a role of for Stat5B in histamine synthesis or storage, granule number, or granule transport. These data warrant further study into the molecular mechanisms involved.

Finally, cytokine levels in culture supernatant were determined by ELISA 16 hours after treatment with DNP-HSA. Corroborating our previous work using siRNA to deplete Stat5B[4], Stat5B KO BMMCs demonstrated a >50% reduction in IL-6, IL-13 and TNFα secretion compared to WT BMMCs (Figure 2E). These data show that Stat5B expression is critical for histamine synthesis and for both the early and late responses of mast cell activation *in vitro.*

3.2 Stat5B is required for mast cell function in vivo

To assess the functional relevance of Stat5B *in vivo*, we performed IgE-induced passive systemic anaphylaxis (PSA). Stat5B KO mice and age-matched WT mice received intra-peritoneal (IP) injections of DNP-specific IgE (50μg) 16 hours before IP injection of DNP-HSA (100μg). Although the core body temperature of both Stat5B KO and WT mice decreased starting at 10 minutes before starting to recover at 45 minutes, Stat5B KO mice experienced significantly less hypothermia than WT mice (Figure 2A). After 90 minutes, the mice were euthanized and plasma was collected to determine cytokine levels by ELISA. We noted similarities with our *in vitro* work, as IL-13 and MIP-1α were reduced >50% in Stat5B KO mice compared to their WT counterparts, but IL-6 was unchanged (Figure 2B). Thus on the whole, Stat5B KO mice show blunted *in vivo* responses, suggesting an important role for the Stat5B isoform in IgE-induced inflammation.

Hypothermia in the PSA model largely reflects the effects of mast cell degranulation on the vasculature, as this response is absent in mast cell-deficient mice[16]. To determine if Stat5B deficiency affected the vascular response, hypothermia induced by IP injection of histamine was measured over 90 minutes. As shown in Figure 2C, there was no difference in histamine responses between Stat5B KO and WT mice. This suggests that reduced hypothermia in the IgE-induced PSA model do not seem to be caused by altered vascular responses but rather a mast cell defect. These data were also not explained by reduced mast cell numbers *in vivo*, since tissue distribution was unchanged in Stat5B KO mice (Figures 2D and 2E). Collectively, these data suggest that Stat5B deficiency results in defective IgE-induced mast cell responses *in vivo*.

3.3 Stat5B has an inhibitory effect on IgE production that is not intrinsic to B cells.

An additional aspect of interpreting PSA data is the baseline level of IgE. Excess IgE can mask the effects of injected antigen-specific IgE, likely by competing for FcεRI binding[14]. We were surprised to find that Stat5B KO mice have elevated plasma IgE levels in standard specific pathogen-free (SPF) housing conditions, averaging approximately 10μg/mL (Figure

3A). This resulted in a variable increase of surface-bound IgE on peritoneal mast cells that trended toward significance (Figures 3B and 3C).

Stat5 inhibits T follicular helper (Tfh) cell differentiation[17,18] and is required for regulatory T cell (Treg) development[16]. The resulting imbalance during Stat5 deficiency therefore favors dysregulated Tfh function that could logically enhance IgE production in response to environmental antigens found in standard SPF housing. In favor of this theory, we also noted increased IgG1 and IgG2A levels in Stat5B KO mice (Figure 3D).

It is also possible that Stat5B deficiency increases B cell numbers or yields B cells with a predisposition to IgE class switching. However, we found no differences in plasma IgM levels or splenic B2 cells in WT and Stat5B KO mice (Figure 3D&E). To determine their capacity for IgE production, splenic B cells were purified from naïve WT and Stat5B KO mice using anti-CD43 or anti-B220 beads. Naïve and total B-cells were cultured with anti-CD40 and in the absence and presence of IL-4. We found no significant difference in IgE production (Figure 4A). Similarly, *in vitro* proliferation was unchanged by Stat5B deficiency (Figure 4B). These data argue against an intrinsic role for Stat5B in B cell expansion or IgE production and suggest that the reported effects of Stat5 deficiency favoring Tfh function likely convey to Stat5B KO mice as well.

3.4 Stat5B is required for in vivo mast cell degranulation.

To circumvent the confounding effect of elevated IgE levels, we sought to directly activate mast cell FcεRI receptors *in vivo*. We first injected IgE into WT and Stat5B KO mice, to control for any effects of FcεRI occupancy. Mice were then challenged with anti-FcεRIα clone MAR-1 16 hours later. Consistent with antigen-induced PSA, Stat5B KO mice had a >50% reduction in hypothermia compared to WT mice (Figure 5). These data further support the importance of Stat5B in IgE-induced mast cell function *in vivo*.

4. Discussion

Stat5 is important for mast cell differentiation, survival, proliferation, and cytokine production[6,7,13,19]. However, Stat5 consists of two proteins, Stat5A and Stat5B, which mediate distinct functions in immune cells[8]. The unique roles of these two proteins remain elusive not only in mast cells but in the immune response in general. This difference matters, since selectively inhibiting one isoform may provide clinical efficacy with fewer side effects. In this study we show a distinct role of Stat5B in IgE-induced mast cell function.

We found that Stat5B KO BMMC acquired FcεRI and c-Kit expression, indicators of mast cell differentiation, more rapidly than WT cells during *in vitro* culture. While these differences were moderate, they were also unexpected. Stat5 binds regulatory elements in the GATA2 locus and positively controls its expression, which is required for mast cell development[19]. This suggests that perhaps Stat5A is crucial for GATA2 expression, while Stat5B exerts other negative effects. The inhibitory role for Stat5B seems to wane as mast cells mature. Not only were WT and Stat5B KO BMMC similar after 5–6 weeks in culture, but tissue mast cell numbers and histological appearance were indistinguishable *in vivo*. This differs from Stat5A KO mice, from which BMMC yield nearly 90% fewer cells in

culture and have significantly decreased tissue mast cell numbers[13]. On balance, our data along with those of Ikeda et al. and Li et al. suggest that Stat5A is a critical positive regulator of mast cell differentiation and survival, while Stat5B exerts some inhibitory effects on differentiation and no effect on survival[6,7,13,19]. Such unique properties can inform rational drug design for selectively suppressing mast cells in allergic and myeloproliferative disorders. In fact, successful production of Stat5B inhibitors was recently reported[20].

Previous studies from our lab showed that the Stat5B is required for IgE-induced mast cell cytokine production *in vitro*. We first found that BMMC from Stat5A/B double-KO mice exhibited poor histamine release and cytokine secretion in response to IgE XL[6]. Subsequently, we found that Stat5B knockdown with siRNA, but not Stat5A knockdown, decreased IgE-mediated cytokine production[4]. Our current work extends this observation to an *in vivo* model of anaphylaxis and supports a role for Stat5B in mast cell degranulation as well. The effects of Stat5B deficiency on IgE-induced degranulation appear to be manifold. Stat5B KO expressed considerably less cell surface CD107a after IgE XL than wild type cells, an indication of reduced granule fusion. They also stored 40% less histamine than their wild type counterparts, but percent histamine release was unchanged. This latter result was counterintuitive, since histamine release requires granule fusion. One possible explanation is reduced granule synthesis or transport in the absence of Stat5b. The mechanistic contributions of Stat5B to degranulation is a focus of current study. Using the mast cell-dependent PSA model, we found that Stat5B KO mice were less responsive to IgE/antigen- or anti-FcεRI-induced hypothermia, and that systemic cytokine production was also reduced. Changes in hypothermia were not due to an impaired vasculature response to histamine nor reduced tissue mast cells. These data support an important non-redundant role for Stat5B in mast cell function *in vivo*.

The elevated IgE, IgG1, and IgG2a we noted in Stat5B KO mice housed in SPF conditions suggested a possible role in B cell activation or antibody secretion. However, we found no evidence of a B cell-intrinsic defect when WT and Stat5B KO B cells were examined *in vitro*. These data support previous findings that Stat5 inhibits Tfh differentiation[17,18] and promotes Treg development[22]. Thus Stat5B deficiency could result in increased Tfh and decreased Treg numbers or function. A T cell imbalance favoring Tfh action is a logical explanation for the increased antibody levels we noted and mirrors elevated IgG1, and IgG2a noted by Nurieva et al. when studying Tfh cells[18]. While our data suggest a role for the Stat5B isoform in Tfh function, further work specifically studying germinal center responses are needed to test this hypothesis.

Our data show that Stat5B expression is required for IgE-mediated mast cell function. In this regard, it is interesting to note that Stat5B mutation or dysregulation is linked to several human disorders, supporting it as a possible drug target. The most consistent reports demonstrate that Stat5B hyperactivation via mutation or constitutive activation is a strong promoter of T cell leukemia[23,24]. This link to neoplastic expansion indicates that Stat5B has critical roles in survival and proliferation, unlike what we noted in mast cells. This supports lineage-restricted functions that warrant further probing. Separate from neoplasia, Stat5B dysregulation has been linked to allergic disease. Constitutive Stat5B activation in mouse

mast cells caused atopic dermatitis[25]. This finding was enhanced by the finding of a Stat5B gain-of-function mutation in two pediatric patients that resulted in atopic dermatitis and eosinophilic infiltration[26]. In an oddly symmetrical outcome, Stat5B deficiency in humans also results in atopic dermatitis in addition to other issues[27]. The latter seems to stem from loss of Treg function, while constitutive Stat5B activation likely promotes Th and/or mast cell function. Collectively, these findings support the conclusion that Stat5B is a central regulator of immunity whose dysregulation often results in allergic dermatitis as one manifeStation. Our data add to this understanding by demonstrating the essential role for Stat5B in mast cell function and support selectively suppressing this transcription factor in allergic disease, while cautioning against its disruption in T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Kiwanuka *et al.*, Highlights

- STAT5B deficiency inhibits IgE-mediated mast cell responses.
- STAT5B KO mice have no change in mast cell survival or distribution.
- STAT5B KO mice have increased basal IgE levels that are not inherent to B cells.

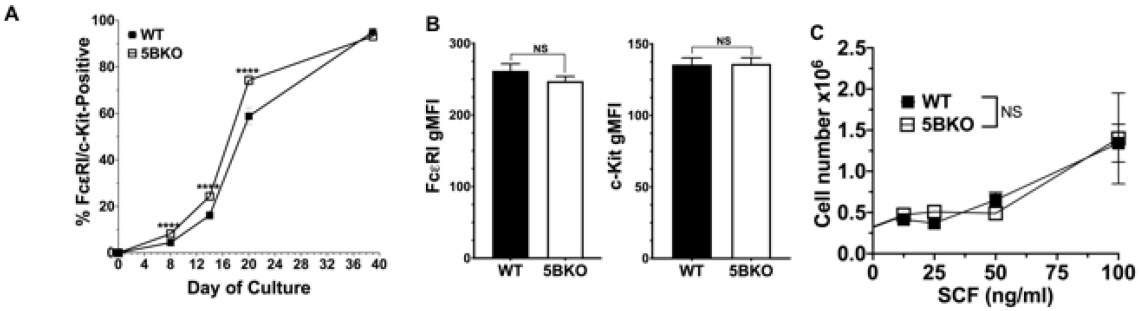

Figure 1. Stat5B deficiency increases mast cell differentiation *in vitro* and is necessary for IgE-mediated degranulation and cytokine production.

(**A**) Bone marrow cells were cultured in cRPMI with IL-3 and SCF. On the indicated days, aliquots were removed and tested for FcεRI and c-Kit expression by flow cytometry. Percentage of cells expressing both receptors is shown as mean +/− SEM from 11 samples. Note that error bars are smaller than icons. (**B**) Bone marrow cells were cultured as in (A) and analyzed for expression of FcεRI and c-Kit expression by flow cytometry on day 39 of culture. Data shown are mean +/− SEM from 6 samples. **(C)** Wild Type and Stat5B KO BMMC were cultured for 72 hours in IL-3(0.5ng/ml) with the indicated concentrations of SCF and viable cells were counted. Data shown are mean +/− SD from 5 samples.

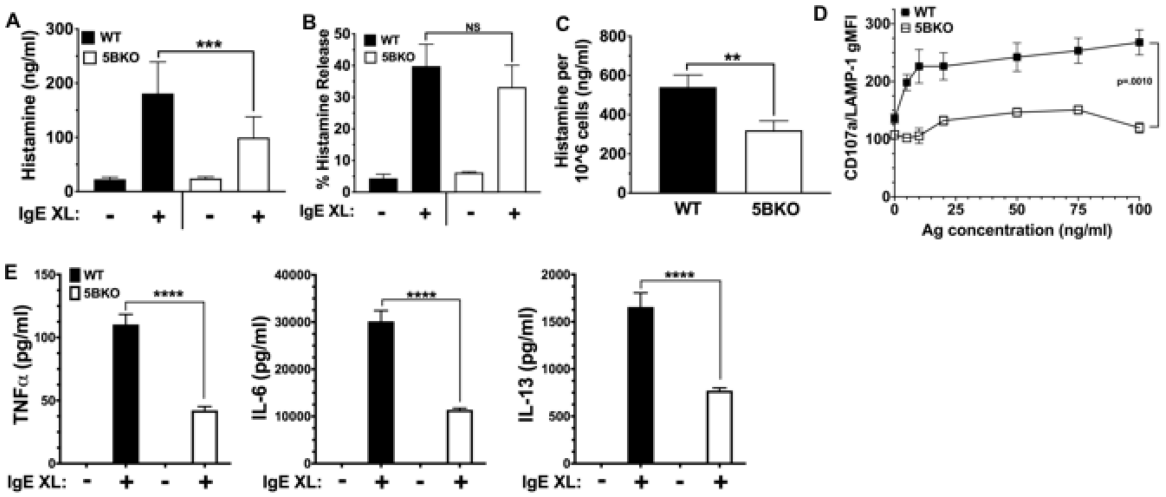

Figure 2. Stat5B deficiency reduces histamine content, degranulation, and cytokine secretion.

(A-C) Wild Type and Stat5B KO cells were stimulated with IgE XL for 10 minutes and histamine was measured in culture supernatants and cell pellet lysates. Percent histamine release was calculated by dividing histamine content in the supernatant by the total of histamine content in supernatant+cell pellet. Data are mean +/− SEM from 3 separate populations of each genotype. **(D)** BMMC were activated for 10 minutes by IgE XL using the indicated concentrations of antigen. Flow cytometry staining for CD107a/LAMP-1 was performed. 2-way ANOVA was used to determine p value between WT and Stat5B KO cells. # represents $p<0.005$ compared to unstimulated Stat5B KO cells. Data shown are mean +/− SEM from 8–15 samples. **(E)** BMMC were activated by IgE XL for 16 hours. Supernatants were analyzed by ELISA. Data are means ±SEM of 12 samples from three populations, with p values calculated using 1-way ANOVA.

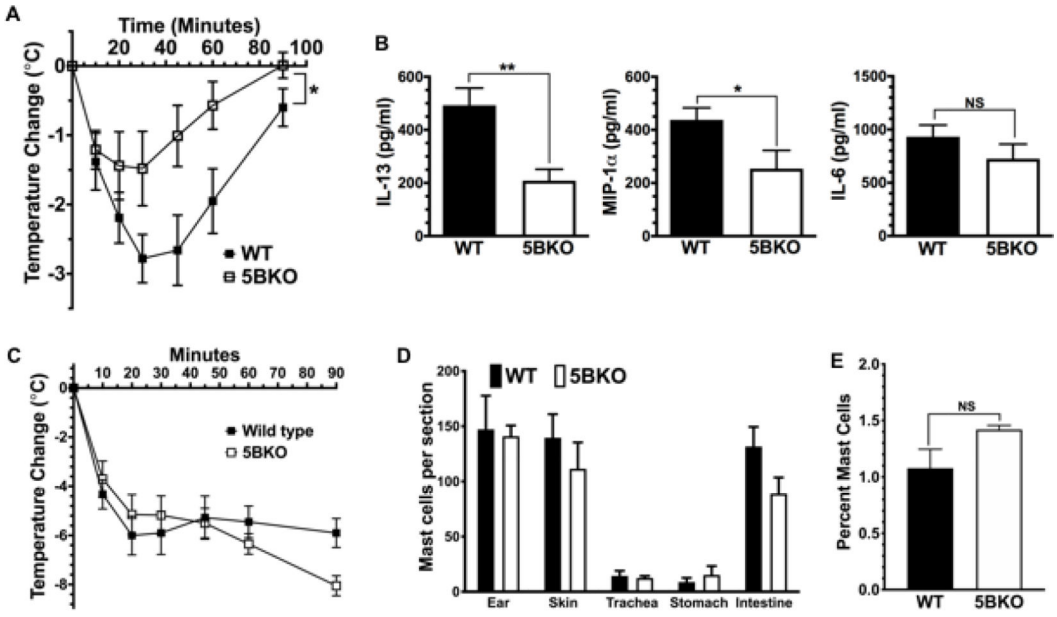

Figure 3. Stat5B is required for mast cell function *in vivo*.

(A) Stat5B KO or WT mice were injected with DNP-specific IgE, followed 16 hours later by DNP-HSA. Core body temperature was assessed over 90 minutes. p value was determined by comparing area under the curve (AUC) data using Student's t-test. AUC for WT mice was 166.7; Stat5B KO AUC was 80.2. (**B**) IL-13 and MIP-1α plasma levels from mice in (A) were determined using ELISA. p values were determined by Student's t-Test. **(C)** Mice were injected with 8mg of histamine. Core body temperature was assessed over 90 minutes. (**D**) Tissues were assessed for mast cells by staining with pinacyanol erythrosinate. **(E)** Peritoneal lavage was performed and the number of mast cells determined by flow cytometry. Data shown are mean +/− SEM from 10 mice per genotype. p values were determined by Student's t-Test.

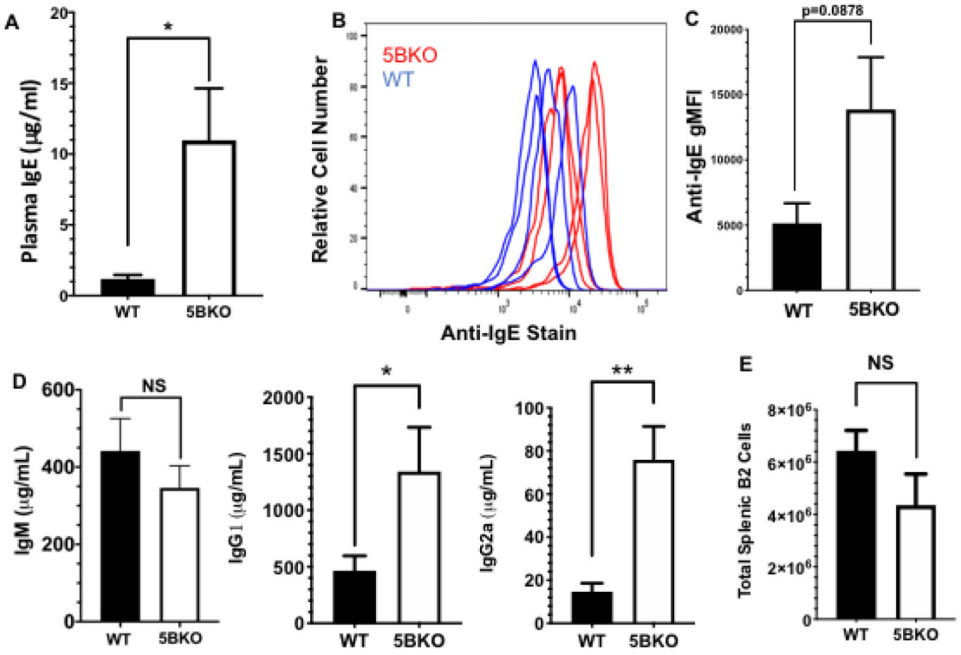

Figure 4: Stat5B deficiency increases IgE production under SPF housing conditions.

(A) IgE levels were determined from plasma of naive mice by ELISA. (**B**) Assessment of peritoneal mast cell FcεRI surface staining by flow cytometry. Histogram showing PE-positive cells. (**C**) Graph showing geometric mean fluorescence intensity (gMFI) of staining from (B). **(D)** Immunoglobulin levels determined from plasma of naive mice by ELISA. (**E**) Assessment of total splenic B2 cells from WT and 5bKO mice analyzed using flow cytometry. Data shown are mean +/− SEM from ten mice of each genotype. p values were determined by Student's t-Test.

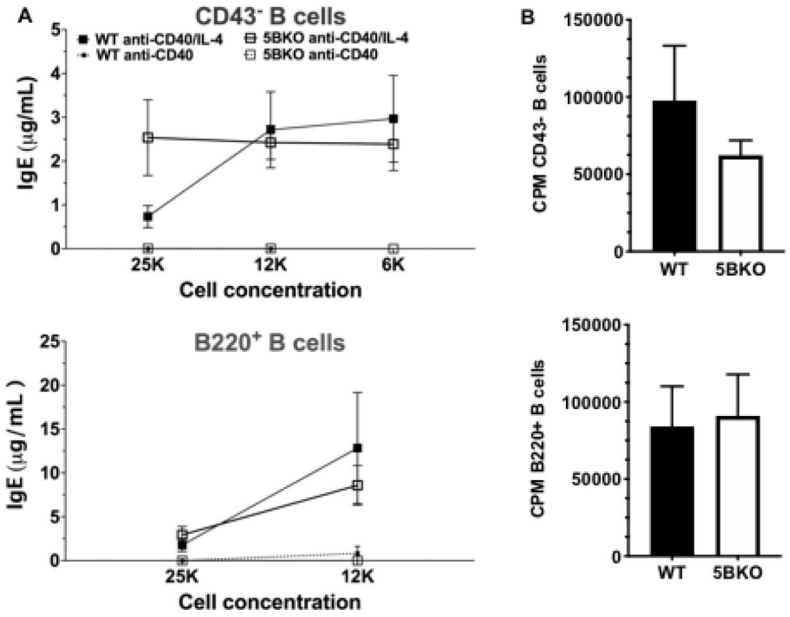

Figure 5: Stat5B KO B cells show no change in IgE production *in vitro*.

(A) Analysis of IgE production in B cells isolated from the spleens of WT and 5BKO mice. CD43-neg plasma B cells or B220+ naïve B cells were resuspended at the indicated concentrations and cultured in the presence or absence of anti-CD40 and IL-4. Supernatants were collected and ELISA was used to determine IgE production. (**B**) Analysis of B cell proliferation of CD43-neg plasma B cells or B220+ naïve B cells. Cells isolated as in (A) were suspended at 50,000 cells/ml in the presence of anti-CD40 and IL-4 and treated with titrated thymidine. Proliferation was determined after 4 days of culture using measurement of radioactivity, represented as counts per minute (CPM). Data shown are mean +/− SEM from 4 mice from 1 representative of two independent experiments.

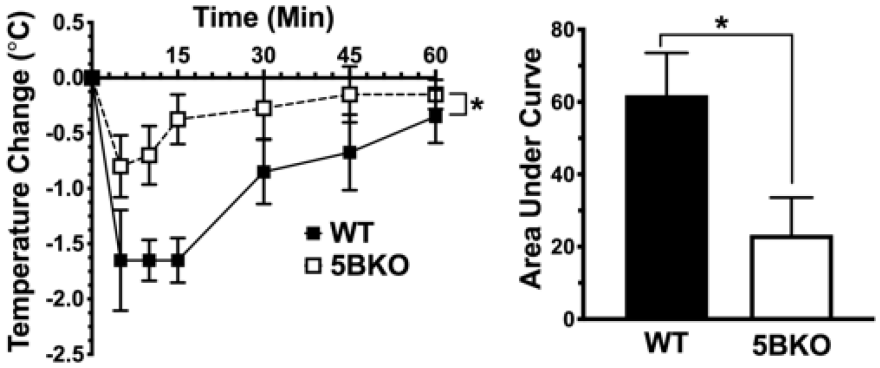

Figure 6. Stat5B is required for mast cell function *in vivo*.

(A) Stat5B KO or WT mice were injected with DNP-specific IgE then injected with anti-FcεRI-alpha clone MAR-1 16 hours later. Core body temperature was assessed over 60 minutes (**B**) Area under the curve determined from graph in (A). Data shown are mean +/− SEM from 8 mice of each genotype.