
Influence of FAS on murine mast cell maturation

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Background: FAS has been shown to be involved in the regulation of many immune processes by induction of cellular apoptosis. However, accumulated evidence shows that FAS signaling also exhibits nonapoptotic functions, such as induction of cell proliferation and differentiation. FAS is the only death receptor known to be expressed on murine mast cells (MCs).

Objective: To evaluate the role of FAS on murine MC maturation.

Methods: Mouse bone marrow–derived MCs (BMMCs) or peritoneal MCs were derived from FAS-deficient, FASlpr/lpr, and congenic wild-type strains. The MC degranulation and cytokine release after IgE activation was assessed by measuring β -hexosaminidase, interleukin 13, and tumor necrosis factor α release. Transmission electron microscopy analysis was performed to evaluate the level of BMMC maturation. The surface markers and intracellular preformed mediators were measured as well.

Results: Our data reveal that FAS deficiency has an impact on IgE-dependent activation of BMMCs, resulting in a significant decrease in β -hexosaminidase, interleukin 13, and tumor necrosis factor α release. The total content of preformed mediators (eg, tryptase and β -hexosaminidase) was reduced in BMMCs derived from FAS-deficient mice. We also found that the level of Fc ϵ RI in peritoneal mast cells from FAS-deficient mice was significantly diminished. FAS deficiency also influenced the kinetics of BMMC maturation as was revealed by transmission electron microscopy analysis.

Conclusion: Our data show that FAS has an impact on the regulation of mouse MC maturation in vitro.

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INTRODUCTION

Mast cells (MCs) not only play a pivotal role in allergic diseases but are also involved in many autoimmune and inflammatory disorders. Cross-linking of allergen specific IgE on the membrane of MCs leads to the release of mediators, which cause the allergy symptoms within minutes to hours. The following infiltration of eosinophilic granulocytes and T_H2 cells directs both the late-phase and chronic inflammatory response.¹

The number of tissue MCs in healthy individuals is stable but increases in inflamed tissues in allergic diseases, such as allergic rhinitis² and allergic asthma.³ The homeostasis of MCs can be influenced by many factors, such as maturation, migration, and viability.⁴ In the present study, we evaluated the role of FAS (CD95), the only death receptor known to be expressed on mouse MCs.⁵ FAS belongs to the tumor necro-

sis factor (TNF) superfamily of receptors. The best known function of FAS in signaling is the induction of apoptosis.⁶ FAS is a 45-kDa protein characterized by the presence of an 80–amino acid death domain in its cytoplasmic tail. This domain is essential for the recruitment of a number of signaling components on activation by either agonistic anti-CD95 antibody or the CD95 ligand that initiates apoptosis.⁶

The involvement of FAS in allergic disorders has been previously described. The elevated levels of soluble FAS and FAS ligand (FASL) were observed in the serum of patients with bronchial asthma and children with allergic rhinitis combined with asthma.^{7,8}

Although primary mouse MCs are resistant to FAS-induced apoptosis,⁵ FAS is known to induce apoptosis in other cells involved in allergic response (ie, neutrophils,⁹ eosinophils,¹⁰ and basophils¹¹). Recent studies show that FAS signaling also exhibits nonapoptotic functions, such as induction of cell proliferation and differentiation.^{12,13} Therefore, in this research, we examined the influence of FAS on mouse MC functions other than apoptosis, such as differentiation, degranulation, and cytokine synthesis.

METHODS

Mice

FASlpr/lpr (strain with mutated FAS, lpr) and C57BL/6 (B6) (congenic strain wild type [WT]) were bred and maintained under specific pathogen-free conditions at The Hebrew University of Jerusalem's Research Animal Facility. The lpr mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). The WT mice were obtained from Harlan

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Laboratories (Jerusalem, Israel). Mice at 8 to 12 weeks of age were used in the experiments. All animal experiments were performed after approval by the Institutional Committee for Care and Use of Laboratory Animals of The Hebrew University of Jerusalem.

BMMC Preparation

Bone marrow cells were isolated from female WT or lpr mice at 6 to 8 weeks of age. Bone marrow cells obtained from femurs of 6- to 9-week-old mice were placed in RPMI 1640 medium (Gibco BRL, Rockville, Maryland) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 U/mL of streptomycin, 25 mM HEPES, 1.0mM sodium pyruvate, nonessential amino acids (all purchased from Biological Industries, Beit Haemek, Israel), 20 ng/mL of recombinant mouse interleukin 3 (IL-3), and 20 ng/mL recombinant mouse stem cell factor (Peprotech, Rocky Hill, New Jersey). Cells were passaged once a week by replacing the cells in fresh medium. After 4 to 5 weeks of culture, more than 95% of the trypan blue-excluding viable cells were mature bone marrow-derived MCs (BMMCs), as assessed by staining with acidic toluidine blue. The c-kit and FcεRI expression levels were evaluated for every batch of BMMCs.

Isolation of MCs From Peritoneal Lavage

Peritoneal MCs (PMCs) were isolated as previously described¹⁴ with slight modifications. Briefly, the peritoneal cavity was washed with 5 mL of ice-cold phosphate-buffered saline. Peritoneal lavage exudates were centrifuged at 150 g for 5 minutes. Pellets were gently drained and resuspended in 72.5% isotonic Percoll (Amersham Pharmacia Biotech, Piscataway, New Jersey). Samples were centrifuged at room temperature for 15 minutes at 400 g. Pellets were collected and MC percentage was determined by toluidine blue staining. The PMCs comprised 70% to 80% of the resultant cell population. The number of PMCs after purification was approximately 10,000 cells per mouse.

Transmission Electron Microscopy

Samples of BMMCs derived from either 4 lpr or 4 WT mice after 4 and 8 weeks of culturing were pelleted and fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 minutes. The samples were washed 3 times in 0.1 M sodium cacodylate buffer (pH 7.4) and then postfixed with 1% osmium tetroxide for 1 hours at 4°C. Postfixed cells were dehydrated with an ascending ethanol series ending with 100% ethanol and then embedded in Dow epoxy resin (DER332; Unione Chimica Europea, Milan, Italy) and DER732 (Serva, Heidelberg, Germany), as previously described.¹⁵ Ultrathin sections were prepared with an Ultrathome III (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and were then double-stained with lead citrate and uranyl acetate and observed with a transmission electron microscope (EM208; Philips, Eindhoven, The Netherlands). Micrographs were taken with a Morada Camera (Olympus Soft Imaging Solutions, Munster, Germany). Detailed analysis of cell maturation was performed on at least 70 cell

sections and was based on the granule number and structure. Formed granules were compact and almost completely filled with electron dense material. The appearance of immature granules was characterized by the presence of granule chambers either completely empty or only partially filled with electron dense diffuse material. A distinction was also made between the cells with either large or small granule chambers. The cells were classified into 5 categories: group A, mature BMMCs with many complete formed granules; group B, mature BMMCs with few complete formed granules; group C, BMMCs undergoing maturation with a large number of granule chambers containing dense material; group D, BMMCs undergoing maturation with some large granule chambers containing diffuse granular material; and group E, BMMCs undergoing maturation with some large empty chambers. Taking into consideration that either A and B or C and D may represent sections of the same cell type, we report in Table 1 the sum of them.

Flow Cytometry

For the c-kit FcεRI staining, resting and activated BMMCs or PMCs were first blocked with 10 μg/mL of anti-Fcγ receptor II/III (2.4G2) (BD Biosciences Pharmingen, San Diego, California) at 4°C for 10 minutes, then incubated for another 30 minutes at 4°C with anti-mouse c-kit phycoerythrin-Cy5, anti-FcεRI fluorescein isothiocyanate conjugated (FITC) (purchased from eBioscience, San Diego, California), and anti-mouse CCR3-FITC (R&D Systems, Minneapolis, Minnesota) or appropriate isotype controls. Flow cytometric analysis of the stained cells was performed with FACSCalibur (BD Biosciences Pharmingen) equipped with CellQuest software.

Measurement of Degranulation, Cytokine Production, and Total Mediator Content

BMMCs (0.5×10^6 cells/100 μL) were sensitized overnight with IgE antidinitrophenyl (0.5 μg/mL; Sigma-Aldrich, Jeru-

Table 1. Quantitative Evaluation of Wild-Type (WT) and lpr Bone Marrow-Derived Mast Cell (BMMC) Maturation by Transmission Electron Microscopy^a

	Percentage of cells			No. of cells
	A and B	C	D and E	
WT 4 weeks	26.0	49.4	24.6	80
lpr 4 weeks	30.5	6.1	63.4	110
WT 8 weeks	55.9	40.0	4.1	70
lpr 8 weeks	49.1	6.0	44.9	80

^a BMMCs derived from either WT or lpr mice were cultured for the indicated time points, fixed, and analyzed by transmission electron microscopy. Five groups of cells were detectable and marked as A through E, each of them indicating a different stage in BMMC maturation: A, mature BMMCs with many complete formed granules; B, mature BMMCs with few complete formed granules; C, BMMCs undergoing maturation with a large number of granule chambers containing dense material; D, BMMCs undergoing maturation with some large granule chambers containing diffuse granular material; and E, BMMCs undergoing maturation with some large empty chambers.

salem, Israel). The IgE-sensitized cells were washed and stimulated with 10 ng/mL of dinitrophenyl-bovine serum albumin in Tyrode gelatin buffer at 37°C for 30 minutes, and supernatants were collected. Total mediator content was measured in cell lysates prepared by 10 seconds, 3 cycles of sonication on ice.

For β -hexosaminidase, 20 μ L of sample (supernatant or cell lysate) was mixed with 45 μ L of substrate solution (8 mM p-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 48 mM citric acid and 56 mM Na₂HPO₄ [pH 4.5]) and incubated for 2 hours at 37°C. The reaction was stopped by the addition of 120 μ L of ice-cold glycine (0.2 M) (pH 10.7), and the optical density (OD) was immediately read in a standard spectrophotometer at 410 nm.

Tryptase content in cell lysate was measured by mixing 50 μ L of sample with 2 μ L of substrate solution (25 mM *N*-p-tosyl-gly-pro-lys-p-nitroanilide in 100% dimethyl sulfoxide) and incubated at 37°C until chromogenesis and immediately read as above.

IL-13 and TNF- α release were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (eBioscience). The OD was measured at 405 nm in an ELISA microplate reader. The lower detection limit for both assays was 32 pg/mL.

All the mediator measurements were performed in BMMCs and not in PMCs. Because of the low recovered PMC numbers, their mediator levels could not be established because they were below the specific assay limit of detectability.

Statistical Analysis

Data are expressed as mean (SEM) of at least 3 independent experiments performed in triplicate. Parametric analysis (analysis of variance, followed by Tukey-Kramer post hoc test) was used to compare the effects. In both cases, $P < .05$ was considered statistically significant.

RESULTS

Effect of FAS Deficiency on IgE-Dependent Activation

The capability of BMMCs from *lpr* mice to degranulate after an IgE-dependent stimulus was evaluated. As measured by β -hexosaminidase release, BMMCs from *lpr* mice were found to have an impairment in degranulation. In fact, β -hexosaminidase release was 0.2 (0.06) OD vs 0.15 (0.03) OD

($P \leq .01$) from BMMCs from WT vs *lpr* mice (Fig 1A).

In addition, we evaluated TNF- α and IL-13 proinflammatory cytokine release as a paradigm of preformed and newly synthesized cytokines, respectively. The release of preformed TNF- α released from *lpr* BMMCs was significantly lower (27 [21] pg/mL) compared with WT- BMMCs (80 [28] pg/mL) ($P \leq .05$) (Fig 1B). A similar trend was found when TNF- α release was measured 24 hours after activation in BMMCs originated from *lpr* compared with WT mice (1,496 [181] [WT] and 1,167 [227] pg/mL [*lpr*], $P \leq .01$) (Fig 2B). We measured the release of an additional inflammatory cytokine known to be secreted during immunologic activation of BMMCs, IL-13.¹⁶ Similar to TNF- α , IL-13 release was significantly diminished in BMMCs

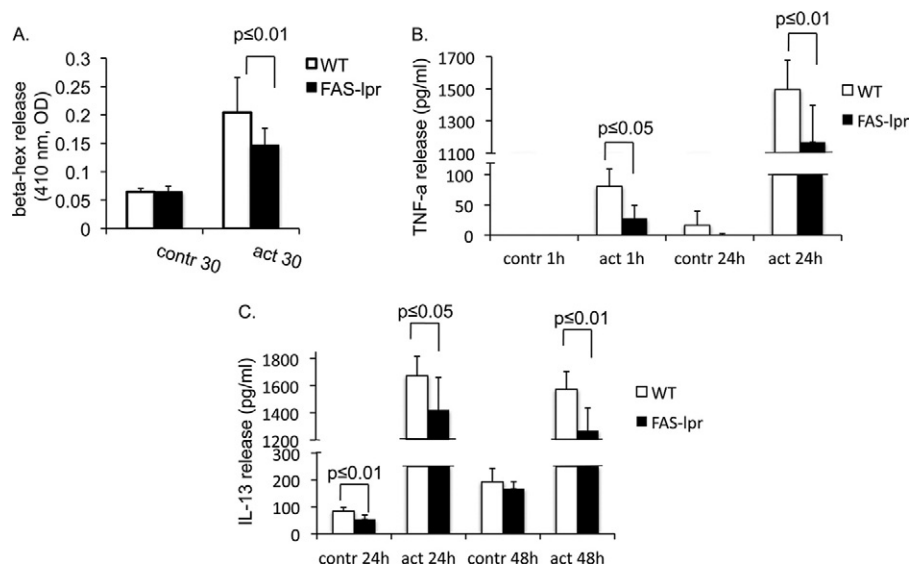


Figure 1. FAS deficiency impairs bone marrow-derived mast cell (BMMC) degranulation and cytokine release. BMMCs were sensitized overnight with IgE anti-dinitrophenyl and then activated with dinitrophenyl-bovine serum albumin. A, For β -hexosaminidase (β -hex) analysis supernatants were collected 1 hour after activation and analyzed by enzymatic-chromogenic assay ($n = 6$). B, For the release of preformed and newly synthesized tumor necrosis factor α , supernatants were collected 1 hour and 24 hours after activation, respectively. C, For the release of newly synthesized interleukin 13 (IL-13), supernatants were collected 24 hours and 48 hours after activation. The cytokines' release was evaluated by enzyme-linked immunosorbent assay and expressed in picograms per milliliter. Data are the mean (SD) of $n = 3$. WT indicates wild type.

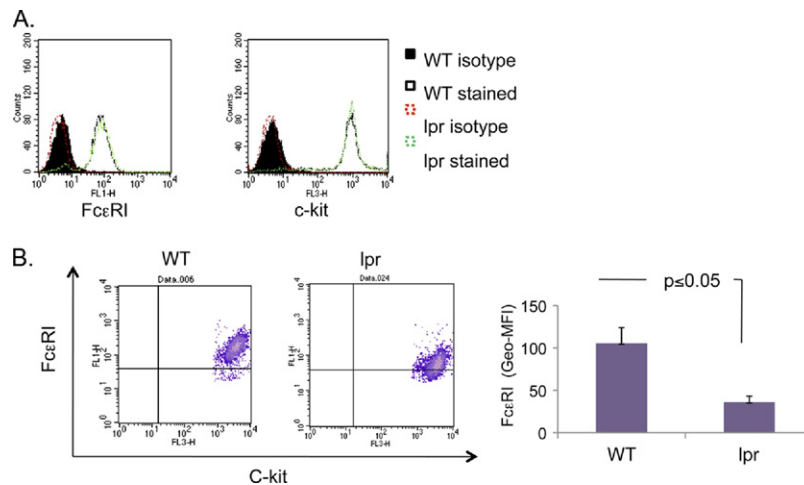


Figure 2. The expression of surface markers in bone marrow–derived mast cells (BMMCs) and peritoneal mast cells (PMCs) derived from *lpr* mice. BMMCs (A) or PMCs (B) derived from either *lpr* or wild-type (WT) mice were stained with anti-mouse c-kit phycoerythrin-Cy5 and anti-FcεRI fluorescein isothiocyanate conjugated and analyzed by fluocytometry. Data are the mean (SEM) of *n* = 3. Geo-MFI indicates geometric mean fluorescence intensity.

derived from mice with nonfunctional FAS both 24 and 48 hours after activation (Fig 1C).

Influence of FAS on MC Maturation

We assumed that one of the reasons for the lower degranulation and cytokines release of BMMCs from *lpr* mice could be the influence of FAS on MC maturation. We evaluated the expression level of BMMC surface markers (ie, c-kit, the marker of both differentiated BMMCs and BMMC progenitors, and FcεRI, the most reliable marker to estimate MC maturation, which appears on the surface of fully differentiated MCs).¹⁷ In addition, it was shown that there is a direct correlation between the level of FcεRI expression and differentiation level assessed by parameters such as histamine content and internal complexity.¹⁸

The expression of both markers was similar in BMMCs derived from WT and *lpr* mice (Fig 2A). However, when MCs derived from peritoneal lavage were analyzed, a significant decrease in FcεRI expression on the surface of *lpr* mice–derived PMCs compared with those derived from WT mice was found (geometric mean fluorescence intensity: 105 [18.6] [WT] vs. 36.2 [6.8] [*lpr*], *P* ≤ .05). No significant change in the c-kit expression on PMCs was observed (Fig 2B).

Next we examined the intracellular content of BMMC granule preformed mediators, such as the mouse counterpart of human tryptase, mouse MC protease 6, and β-hexosaminidase. The total content of both MC tryptase and β-hexosaminidase was lower, the latter significantly (*P* ≤ .01), in BMMCs derived from *lpr* compared with WT mice (Figure 3).

Transmission electron microscopy analysis of BMMCs derived from WT and *lpr* mice was performed to check whether the difference in mediator content is a result of impaired BMMC maturation. Cells were scored according to the level of maturation (Fig 4).^{19–21} The cells at levels A and B were considered to be the most mature BMMCs. It was

found that the percentage of such cells was similar between *lpr* and WT BMMCs after both 4 and 8 weeks of culturing (Table 1). The MCs at level C were scarcely represented in *lpr* mice, suggesting that few *lpr* mice–derived BMMCs continue to undergo maturation compared with BMMCs derived from WT mice.

In addition, there were much more *lpr* mice–derived BMMCs at levels D and E, with granule chambers either completely empty or containing diffuse granular material, suggesting that at these 2 levels the granulogenetic process is incomplete (Table 1).

DISCUSSION

FAS is expressed on a variety of normal cells, including activated T cells, B cells, monocytes, neutrophils,²² and eosinophils.¹¹ Human basophils have also been shown to express FAS.²³ The level of susceptibility of cells to FAS-induced apoptosis varies in different types of cells.²² We have previously shown that primary lung but not cord blood–

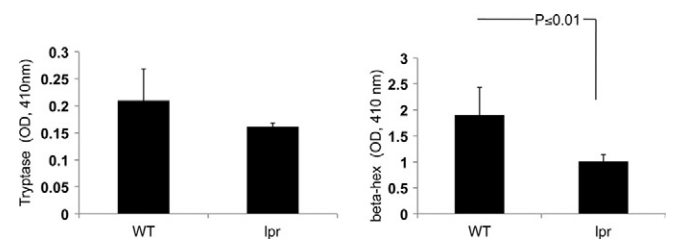


Figure 3. The expression of granule-associated mediators in bone marrow–derived mast cells (BMMCs) from *lpr* mice. BMMCs (1×10^6 cells/mL) from either *lpr* or wild-type (WT) mice were lysed by sonication and tryptase and β-hexosaminidase (β-hex) content was measured using chromogenic assay based on measuring of substrate degradation. Data are the mean (SD) of *n* = 3.

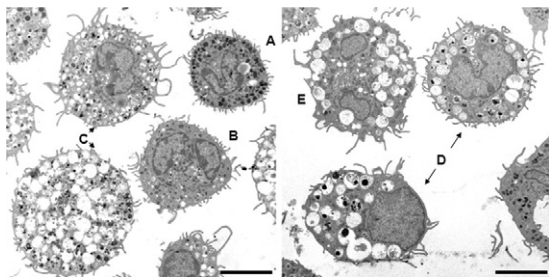


Figure 4. Transmission electron microscopy analysis of bone marrow-derived mast cells (BMMCs) from *lpr* mice. BMMCs derived from either wild-type (WT) or *lpr* mice were cultured for 4 weeks, fixed, and analyzed by transmission electron microscopy. The 5 stages in BMMC differentiation are marked by capital letters A through E. The data are representative of 1 of 2 experiments. The quantitative analysis of the BMMCs in each of the maturation steps is provided in Table 1.

derived MCs express FAS antigen.²⁴ In addition, the expression of FAS antigen was previously reported in murine BMMCs. In those cells, similarly to nonactivated lymphocytes, for example, FAS is nonfunctional and does not cause a significant increase in apoptosis.⁵

The role of FAS, other than in apoptosis induction, had been previously reported. FAS activation can cause cytokine synthesis. For example, FAS is important in the activation and cytokine production in murine T cells²⁵; its cross-linking mimics anti-CD3 stimulation on IL-3-induced histamine, IL-6, and IL-4 synthesis by murine hematopoietic precursor cells.²⁶

Besides cytokine production, FAS has been reported to have an impact on cell differentiation. FAS unfunctionality in *lpr* mice was shown to affect hematopoiesis during both adult and fetal life. These modifications were further described to occur independently from other diseases associated with the *lpr* phenotype.²⁷ Recently, it has been shown that FAS is involved in human erythroid maturation¹² and murine osteoblastic differentiation¹³ rather than having a role in cellular proliferation or triggering of apoptosis.

In murine MCs, FAS is not transducing an apoptotic signal due to a constitutive FLIP expression.²⁸ In the present study, we have demonstrated that the lack of FAS-FASL interaction in murine MCs had an impact on IgE-dependent degranulation and cytokine release. We also found that the reason for BMMC releasing fewer preformed mediators is the outcome of the lower level of total preformed mediator content. Some mediators, such as IL-9 and stem cell factor, are known to increase murine MC protease transcription.^{29,30} Whether additional triggers can reverse the phenotype of *lpr* mice-derived BMMCs still needs to be tested.

This in turn is probably a result of impaired BMMC maturation, which was observed by electron microscopy analysis. According to the score, there were no significant differences in the percentage of fully differentiated BMMCs. We can therefore assume that the differences are in the kinetics of differentiation because the percentage of cells

reaching the final differentiation is similar. The fact that there are significantly higher numbers of BMMCs derived from *lpr* mice in the early maturation stages D and E and very few in the intermediate (C) stage of maturation suggests that MC progenitors derived from *lpr* mice do not follow the same differentiation pattern as WT mice-derived MCs. We suppose that the observed effect was not due to the decrease in the overall number of MC precursors because, according to acidic toluidine blue staining and light microscopy examination, no difference in the number of mature cells was observed.

In addition, we did not find any differences in the expression of surface markers (ie, c-kit and FcεRI) in BMMCs derived from WT compared with *lpr* mice. This observation is in accordance with previous studies.³¹

Interestingly, the decrease in FcεRI expression was observed in MCs derived from the peritoneal cavity of *lpr* mice compared with WT mice, a finding that might point to impaired maturation as well. Moreover, it was reported that the ability of antigen presenting is reduced in the murine BMMC subset with low FcεRI expression.³² This finding suggests that FAS deficiency may attenuate MC capability to act as antigen-presenting cells.

The role of FAS in both phenotypical and functional maturation has been previously described in dendritic cells. It was demonstrated that FASL upregulated dendritic cell maturation markers and caused an increase in cytokine production.³³ There is a possibility that FAS is involved in functional maturation of MCs as well. This point still remains to be clarified.

The possible relevance of our findings to human diseases can relate to the autoimmune lymphoproliferative syndrome (ALPS) type Ia caused by the mutation of the FAS-encoding *NFRSF6* gene.³⁴ To the best of our knowledge, there are no reports about MC involvement in this disease. It would therefore be interesting to investigate whether in patients with ALPS type Ia there is any difference in MC numbers and/or distribution. It is feasible to speculate that if the murine FAS-deficient MC phenotype is present also in humans, allergy symptoms will be less severe in the ALPS patients. If this is the case, the possibility of a targeted anti-FAS therapy for allergic diseases can be considered.

In summary, to the best of our knowledge, this is the first report on the function of the FAS receptor in a role other than apoptosis on primary mouse MCs. Our data shed light on the possible role of FAS in the context of MC homeostasis.

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