

The sphingosine-1-phosphate/sphingosine-1-phosphate receptor 2 axis regulates early airway T-cell infiltration in murine mast cell-dependent acute allergic responses

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Background: Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid produced by mast cells (MCs) on cross-linking of their high-affinity receptors for IgE by antigen that can amplify MC responses by binding to its S1P receptors. An acute MC-dependent allergic reaction can lead to systemic shock, but the early events of its development in lung tissues have not been investigated, and S1P functions in the onset of allergic processes remain to be examined.

Objective: We used a highly specific neutralizing anti-S1P antibody (mAb) and the sphingosine-1-phosphate receptor 2 (S1PR2) antagonist JTE-013 to study the signaling contributions of S1P and S1PR2 to MC- and IgE-dependent airway allergic responses in mice within minutes after antigen challenge.

Methods: Allergic reaction was triggered by a single intraperitoneal dose of antigen in sensitized mice pretreated intraperitoneally with anti-S1P, isotype control mAb, JTE-013, or vehicle before antigen challenge.

Results: Kinetics experiments revealed early pulmonary infiltration of mostly T cells around blood vessels of sensitized mice 20 minutes after antigen exposure. Pretreatment with anti-S1P mAb inhibited *in vitro* MC activation, as well as *in vivo* development of airway infiltration and MC activation, reducing serum levels of histamine, cytokines, and the chemokines

monocyte chemoattractant protein 1/CCL2, macrophage inflammatory protein 1 α /CCL3, and RANTES/CCL5. S1PR2 antagonism or deficiency or MC deficiency recapitulated these results. Both *in vitro* and *in vivo* experiments demonstrated MC S1PR2 dependency for chemokine release and the necessity for signal transducer and activator of transcription 3 activation. **Conclusion:** Activation of S1PR2 by S1P and downstream signal transducer and activator of transcription 3 signaling in MCs regulate early T-cell recruitment to antigen-challenged lungs through chemokine production. (J Allergy Clin Immunol 2014;■■■■:■■■■-■■■■.)

Key words: Airway inflammation, allergy, mast cells, recruitment, T cells, sphingosine-1-phosphate, sphingosine-1-phosphate receptor 2, signal transducer and activator of transcription 3

Mast cells (MCs) play a central role in both the development and exacerbation of allergic disorders.¹ Cross-linking of their high-affinity receptors for IgE (Fc ϵ RI) by antigen activates sphingosine kinases, which phosphorylate sphingosine to produce sphingosine-1-phosphate (S1P), a critical sphingolipid contributing to degranulation, the release of intragranular mediators.^{2,3} S1P can be exported from MCs to amplify their responses by binding to S1P receptors^{2,4,5} and to recruit other immune cells, including T_H2 lymphocytes.³ Thus S1P participates in cytokine/chemokine production and inflammatory cell trafficking.⁶ S1P and its small-molecule analogs are the subject of intense investigation in the pathogenesis of acute lung injury and asthma.⁷⁻¹¹

We previously reported that sphingosine-1-phosphate receptor 2 (S1PR2) plays a critical role in regulating human MC functions,¹² whereas S1PR1 is important for their migration to sites of inflammation.^{2,3} IgE-dependent systemic MC activation is responsible for increased circulating levels of histamine and associated pulmonary edema in mice,^{1,13} and both are attenuated after inhibition or deletion of S1PR2.¹² Although exacerbation of systemic allergic reactions has been mostly studied for its after-the-fact effects, the early steps of its development in lung tissues have not been investigated. Because our preclinical model of MC-dependent allergic reaction triggered respiratory distress in mice,¹² we hypothesized that early inflammatory events might be occurring in the acutely challenged airways that are S1PR2 dependent. S1PR2 signaling could also result in endothelial barrier dysfunction and increased permeability.¹⁴ However, the role of S1PR2 remains unclear because of its contrasting functions on various cell types.¹⁵

Here we evaluated IgE/antigen-induced pulmonary alterations after blockade of S1PR2 signaling through neutralization of S1P, the ligand of S1PR2, with Sphingomab (Lpath, San Diego, Calif), a specific anti-S1P mAb, and through inhibition of S1PR2 with

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Abbreviations used

BAL: Bronchoalveolar lavage
 BMMC: Bone marrow–derived mast cell
 DNP: Dinitrophenyl
 HSA: Human serum albumin
 MC: Mast cell
 MCP-1: Monocyte chemoattractant protein 1
 MIP-1 α : Macrophage inflammatory protein 1 α
 S1P: Sphingosine-1-phosphate
 S1PR2: Sphingosine-1-phosphate receptor 2
 STAT3: Signal transducer and activator of transcription 3
 WT: Wild-type

the specific antagonist JTE-013. We show that inflammatory infiltration of T cells occurs within minutes of systemic antigen challenge and is strongly mitigated in mice pretreated with either Sphingomab or JTE-013 in an MC-dependent model of acute allergic reaction. We further demonstrate that this effect is due in part to early production of chemokines. Moreover, signal transducer and activator of transcription 3 (STAT3) activation was induced in inflamed lungs and in MCs shortly after exposure to S1P in an S1PR2-dependent manner. Thus the acute onset of MC-dependent allergic responses features early lung T-cell recruitment in mice through the S1P/S1PR2/STAT3 axis and chemokine production.

METHODS**Mice**

C57BL/6J, C57BL/6x129/SVJ, and C57BL/6-*Kit*^{W-sh/W-sh} mice were purchased from the Jackson Laboratory (Bar Harbor, Me). S1PR2 knockout mice (Dr R. Proia, National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md) and corresponding wild-type (WT) mice were on a mixed C57BL/6x129/SVJ background. All mice were used at 8 to 12 weeks of age and maintained in a pathogen-free facility. Studies were performed in accordance with institutional animal care and use committee guidelines.

Reagents and antibodies

The murine mAb LT1002 (Sphingomab) directed against S1P and the LT1003 isotype-matched control (IgG₁, κ) antibody were provided by Lpath. Dinitrophenyl (DNP)–specific mouse IgE was a generous gift from Dr Daniel Conrad (VCU, Richmond, Va). p-STAT3 and STAT3 antibodies were from Cell Signaling Technology (Danvers, Mass). Hamster anti-mouse CD3 ϵ and IgG isotype control, Alexa Fluor 488 conjugates, rat anti-mouse CD14 and IgG_{2a}, κ isotype control, phycoerythrin conjugates, and rat anti-mouse CD16/CD32 (Fc block) were purchased from eBioscience (San Diego, Calif). JTE-013 was purchased from Tocris Bioscience (Minneapolis, Minn), and S1P was purchased from Enzo Life Sciences (Farmingdale, NY). DNP–human serum albumin (HSA) and ionomycin were obtained from Sigma-Aldrich (St Louis, Mo). Liberase TM and DNase I were obtained from Roche Diagnostics (Indianapolis, Ind).

Human skin and mouse bone marrow–derived mast cells

Human skin MCs and mouse bone marrow–derived mast cells (BMMCs) were isolated and cultured, as previously described,^{11,16} and were more than 98% pure. Human MCs and BMMCs were sensitized overnight with 1 or 0.5 μ g/mL DNP-specific mouse IgE, washed to remove excess unbound IgE, and stimulated with 30 or 20 ng/mL DNP-HSA (antigen), respectively.⁵

Ionomycin (1 μ mol/L) was used as a positive control. Degranulation was quantified by measuring β -hexosaminidase release.⁵

Cytokine and chemokine measurements

Human and mouse cytokines/chemokines were measured by means of ELISA, according to the manufacturer's instruction (R&D Systems, Minneapolis, Minn).

Acute allergic reaction

All injections were performed intraperitoneally in a final volume of 100 μ L, as previously described.¹² Briefly, mice were injected with DNP-specific IgE and 12 hours later with antigen in PBS. In some experiments blood samples were collected from a tail vein nick just before antigen challenge to conduct flow cytometric analysis of circulating T cells. A small nick was made over the lateral vein of restrained mice with a scalpel blade. Blood (150–250 μ L) was collected through heparinized capillary tubes into heparinized tubes. Core body temperature was measured immediately before antigen challenge and at indicated times with a rectal microprobe (Physitemp Instruments, Clifton, NJ). Mice were killed, and blood was collected by means of cardiac puncture for serum analysis. Bronchoalveolar lavage (BAL) fluid was obtained by lavaging lungs twice with 600 μ L of PBS, and samples were stored at -80°C after centrifugation. Lungs were removed for histologic examination or snap-frozen for Western blot analyses. Control mice injected with PBS showed no significant change in body temperature ($\pm 1^{\circ}\text{C}$) and had no detectable blood histamine.

Adoptive transfer of BMMCs into MC-deficient mice

Bone marrow cells from femurs of female WT mice were cultured for 6 weeks to generate BMMCs, as previously described.¹¹ For MC reconstitution, recipient (7- to 8-week-old) female *Kit*^{W-sh/W-sh} mice were injected intraperitoneally with 5×10^6 BMMCs in 200 μ L of PBS.¹⁷ Eight weeks later, MC-reconstituted *Kit*^{W-sh/W-sh} mice (Rec. *Kit*^{W-sh/W-sh}) were treated with vehicle or JTE-013 before antigen triggering, as previously described.¹² BAL fluid was collected 90 minutes after antigen challenge and BAL tryptase levels were determined to assess for appropriate reconstitution.

Lung histology

Lungs were prepared for histologic analysis, as previously described.¹² Briefly, after inflation, lungs were embedded in paraffin, serial sections were stained with hematoxylin and eosin, and perivascular areas were evaluated in 8 to 12 randomly selected sections for each sample. Investigators had no knowledge of the treatment group assignments at the time of analysis. A Nikon ECLIPSE E800M microscope (Nikon, Tokyo, Japan) equipped with a Diagnostic Instruments SPOT RT CCD camera (SPOT Imaging Solutions, Sterling Heights, Mich) was used to photograph the sections. Perivascular infiltration was scored semiquantitatively, as previously described,¹⁸ according to the following criteria: 0, normal; 1, few cells; 2, rings of inflammatory cells 1 cell layer deep; 3, rings of inflammatory cells 2 to 4 cells deep; and 4, rings of inflammatory cells 4 or more cells deep. Bars represent 100 μ m.

Lung enzymatic digestion and flow cytometric analysis

Lungs were removed and mechanically dispersed. Liberase TM solution (0.22 Wunch units/mL final concentration) supplemented with DNase I (0.01 U/ μ L final concentration) was added to each dispersed sample. After 1 hour at 37°C , digested tissues were filtered through 70- μ m cell strainers and centrifuged. RBCs were lysed with hypotonic salt solution. Cell suspensions were quickly filtered and centrifuged. Pellets were washed in ice-cold PBS/1% FCS for cell counting and staining. For blood samples, RBCs were first lysed with BD PharmLyse buffer (BD Biosciences, San Jose, Calif). Cells from individual mice were incubated before and during all antibody staining with Fc block. CD3 and CD14 expression analysis was performed with a standard flow

cytometric protocol, as described previously.¹⁹ Cells gated on forward and side scatter parameters to exclude dead cells and debris were analyzed on a Beckman Coulter FC500 Flow Cytometer (Beckman Coulter, Fullerton, Calif), and data were analyzed with CXP software.

Transwell T-cell migration assay

BMMC-conditioned cell-free supernatants were prepared with sensitized (0.5 μ g of mouse anti-DNP IgE/mL/2 $\times$ 10⁶ BMDCs for 12 hours at 37°C) BMDCs treated with JTE-013 (1 μ mol/L) or vehicle for 30 minutes at 37°C before adding antigen (DNP-HSA, 100 ng/mL/30 $\times$ 10⁶ BMDCs). After 24 hours, supernatants were collected and stored at -80°C. Splenic CD4⁺ and CD8⁺ T cells were isolated with the EasySep kit (STEMCELL Technologies, Vancouver, British Columbia, Canada) and used together for migration experiments. T-cell enrichment was verified by using flow cytometry. Assays were conducted with 5- μ m pore size polycarbonate filters in Costar 24-well Transwell chambers (Corning, Corning, NY) with 850 μ L of BMMC-conditioned medium in the lower chamber and 10⁵ T cells in 200 μ L in the upper chamber. Recombinant murine RANTES/CCL5 (100 ng/mL; PeproTech, Rocky Hill, NJ) and medium, dimethyl sulfoxide vehicle, or JTE-013 were used as positive and negative controls, respectively. After 24 hours at 37°C, T cells in the lower chambers were counted in quadruplicate by using flow cytometry after propidium iodide staining to exclude dead cells from analysis (FACSCalibur, BD Biosciences). Migration was expressed as the fold value of control (culture medium alone).

Histamine determination

Mouse serum histamine levels were determined with an ELISA kit (Neogen, Lexington, KY).

Tryptase determination

An ELISA kit was used to measure tryptase levels in the supernatants of centrifuged BAL fluid, according to the manufacturer's recommendations (EMD Millipore, Billerica, Mass).

Western blot analysis

Lungs or 5 $\times$ 10⁶ MCs were homogenized, and equal amounts of proteins were analyzed by using immunoblotting after transfer to nitrocellulose and blocking with 1:1000 dilutions of anti-p-STAT3 or anti-STAT3 overnight at +4°C. After washing in TBS-0.1% Tween 20, membranes were incubated with a 1:5000 dilution of species-appropriate horseradish peroxidase-linked anti-IgG (Cell Signaling Technology), and sizes of proteins of interest were determined by using molecular weight standards (Bio-Rad Laboratories, Hercules, Calif). Quantitation was carried out with ImageJ software (National Institutes of Health, Bethesda, Md). Integrated density numbers (in pixels) of p-STAT3 were normalized to total STAT3 and are means of 3 independent experiments.

Statistical analysis

Data are expressed as means $\pm$ SEMs and were analyzed by using the unpaired 2-tailed Student *t* test for comparison of 2 groups and 1-way ANOVA for analysis of 3 or more groups (Prism 6; GraphPad Software, La Jolla, Calif). Significance for all statistical tests is shown in figures. Experiments were repeated at least 3 times in triplicates with consistent results. *In vivo* experiments were repeated 3 times, and each experimental group consisted of 5 to 6 mice.

RESULTS

Sphingomab, a neutralizing anti-S1P mAb, significantly decreases human MC degranulation and cytokine/chemokine secretion

We investigated the effects of Sphingomab on *in vitro* MC activation. As shown in Fig 1, A through D, addition of Sphingomab

at concentrations ranging from 10 to 0.01 μ g/mL but not isotype-matched control mAb or Sphingomab at 0.001 μ g/mL at the time of antigen stimulation dose-dependently decreased IgE/antigen-induced degranulation, as measured based on β -hexosaminidase release, without altering either spontaneous or ionomycin-induced degranulation. Because the anti-S1P mAb inhibited degranulation by 50% at 0.1 μ g/mL, this concentration was selected to examine its effects on cytokine/chemokine secretion. Anti-S1P mAb treatment significantly decreased IgE/antigen-triggered IL-6 (Fig 1, F), CCL5 (Fig 1, G), CCL2 (Fig 1, H), TNF (Fig 1, I), and CCL3 (Fig 1, J) secretion without altering spontaneous or ionomycin-induced release. These results substantiate the notion that S1P released from activated MCs contributes to secretion of proinflammatory mediators and that this can be suppressed by neutralizing extracellular S1P.

Neutralization of S1P with a specific mAb mitigates IgE-dependent airway allergic reactions

Previous studies suggest that susceptibility to anaphylaxis in mice correlates with serum S1P levels.²⁰ Because Sphingomab neutralizes circulating levels of S1P,^{21,22} we sought to examine its effects in an *in vivo* MC- and IgE-dependent mouse acute model of allergic reaction. To this end, before IgE/antigen injections, anti-S1P mAb was administered intraperitoneally because it was previously demonstrated that more than 95% of the anti-S1P mAb appeared rapidly in the bloodstream after intraperitoneal injection of a bolus dose.²¹ The anti-S1P mAb-treated mice exhibited significantly reduced hypothermia compared with that seen in mice treated with an isotype-matched control mAb (Fig 2, A). Mice administered anti-S1P mAb also had markedly decreased levels of systemic histamine (Fig 2, B), monocyte chemoattractant protein 1 (MCP-1)/CCL2 (Fig 2, C), macrophage inflammatory protein 1 α (MIP-1 α)/CCL3 (Fig 2, D), RANTES/CCL5 (Fig 2, E), and thymus and activation-regulated chemokine/CCL17 (Fig 2, F) 2 hours after antigen administration. At this time point, histopathologic analysis showed extensive perivascular edema in mice pretreated with a mock mAb before antigen challenge (Fig 2, G), which was significantly attenuated in anti-S1P mAb-treated mice (Fig 2, H).

Neutralization of S1P decreases early allergic lung infiltration of T lymphocytes and macrophages

We further analyzed lung sections during the development of allergic reactions (Fig 3, A-F). Surprisingly, as early as 20 minutes after antigen challenge, cellular infiltrates were detected around blood vessels in antigen-challenged mice treated with mock mAb (Fig 3, D) and continued to intensify 30 to 60 minutes after challenge (Fig 3, E and F). By contrast, mice treated with the specific anti-S1P mAb exhibited delayed and markedly attenuated perivascular infiltrations after antigen challenge (Fig 3, G-L). Semiquantitative scoring confirmed that anti-S1P mAb significantly reduced infiltration (Fig 3, M). Flow cytometric analysis revealed that infiltrating cells were CD3⁺ T cells with fewer CD14⁺ monocytes/macrophages (Fig 3, N and O). No eosinophils or neutrophils were detected (data not shown). S1P neutralization mitigated perivascular infiltration of both cell types (Fig 3, N and O). Peripheral blood analysis before antigen triggering demonstrated that numbers of circulating CD3⁺ cells in anti-S1P-treated mice

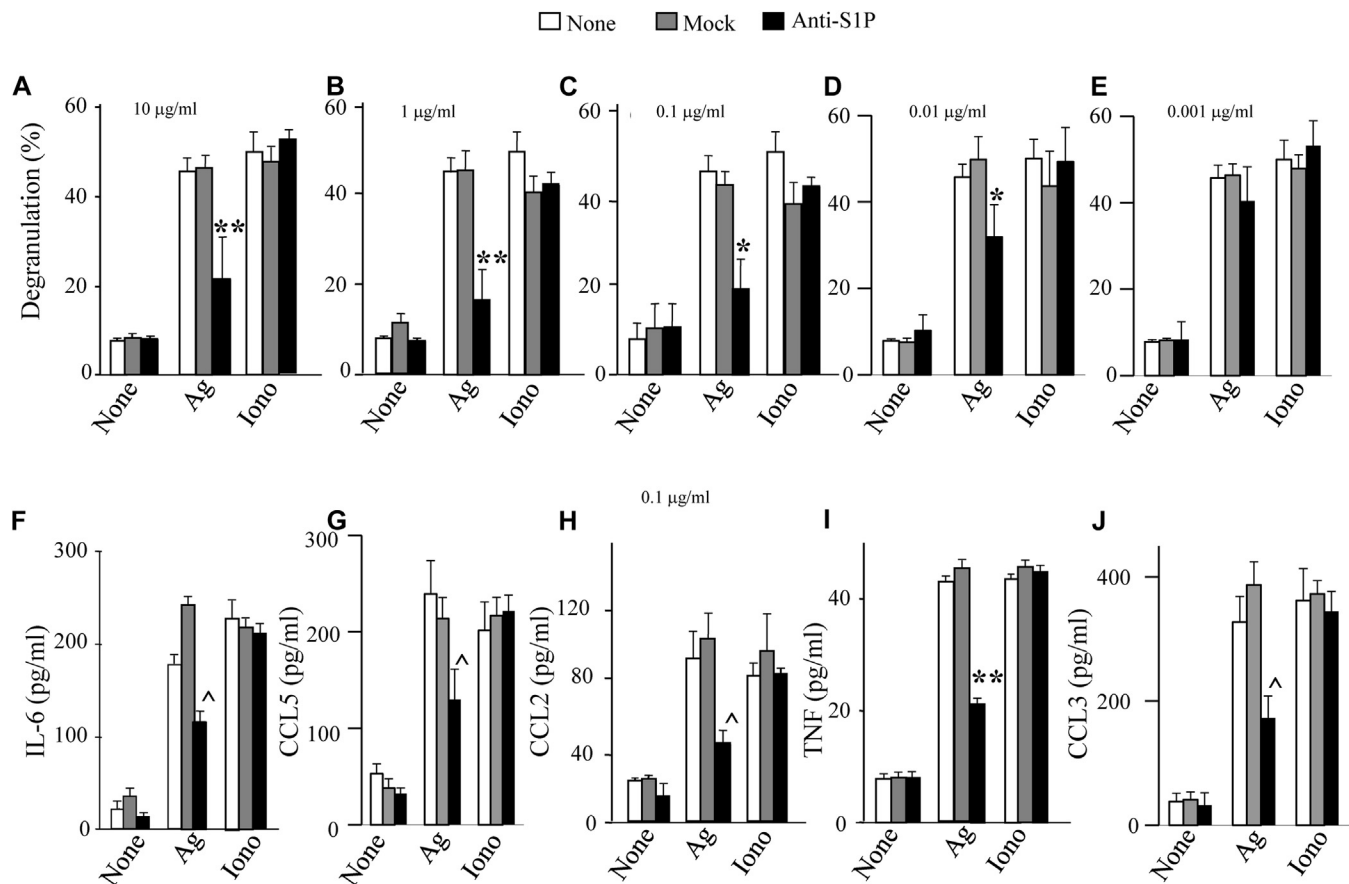


FIG 1. SpHINGOMAB, a specific anti-S1P mAb, reduces IgE/antigen-induced activation of human MCs. Human skin mast cells were pretreated with anti-S1P or control (*Mock*) before stimulation at the indicated concentration. **A-E**, Degranulation was measured by using a colorimetric assay. **F-J**, Secretion of IL-6 (Fig 1, F), RANTES/CCL5 (Fig 1, G), MCP-1/CCL2 (Fig 1, H), TNF (Fig 1, I) and MIP-1 α /CCL3 (Fig 1, J) was measured by using ELISA. * $P < .05$, ** $P < .01$, and *** $P < .0001$, 1-way ANOVA.

were not altered compared with those in mice treated with control mAb (Fig 3, P and Q).

Antigen-triggered lung perivascular infiltration is drastically decreased in MC-deficient *Kit^{W-sh/W-sh}* mice and restored in MC-reconstituted *Kit^{W-sh/W-sh}* mice

To investigate the role of MCs in early infiltration, we used C57BL/6-*Kit^{W-sh/W-sh}* MC-deficient mice.¹⁷ Significant perivascular cell infiltration was present in WT mice (Fig 4, A-C) but not in MC-deficient mice (Fig 4, D-F), which was confirmed by means of semiquantitative scoring of lung sections (Fig 4, G). MC involvement was further substantiated by using antigen-challenged MC-reconstituted *Kit^{W-sh/W-sh}* mice, which displayed histologic scores comparable with those of WT MC-sufficient mice (Fig 4, G). Flow cytometric analysis demonstrated that WT mouse lungs contained large numbers of infiltrating CD3⁺ T cells and CD14⁺ macrophages, which were dramatically decreased in MC-deficient mouse lungs and restored in MC-reconstituted *Kit^{W-sh/W-sh}* mice (Fig 4, H and I). These results suggest an important role for MCs in early infiltration of T lymphocytes and macrophages. As expected, blood samples before antigen challenge showed no significant differences in numbers

of circulating CD3⁺ cells between WT mice, MC-deficient mice, and MC-reconstituted *Kit^{W-sh/W-sh}* mice (Fig 4, J and K).

Extracellular S1P neutralization decreases *in vivo* MC activation and levels of circulating histamine and chemokines also observed in the absence of MCs

Although MCs account for the early airway responses to antigen challenge, it is still not clear how important these cells are for the development of the late-phase response and chronic inflammation.²³ MC activation was substantiated by increased tryptase levels in BAL fluid of control mAb-pretreated sensitized mice 2 hours after antigen challenge (Fig 5, A). In sharp contrast, tryptase was not detectable in BAL fluid from mice pretreated with anti-S1P mAb (Fig 5, A). As expected, tryptase was undetectable in BAL fluid from *Kit^{W-sh/W-sh}* mice, but levels were increased in MC-reconstituted *Kit^{W-sh/W-sh}* mice (Fig 5, B).

We have previously shown that acute allergic challenge leads to increased histamine and MCP-1/CCL2 and MIP-1 α /CCL3 levels, all of which could be ascribed to activation of MCs.¹² RANTES/CCL5, CCL2, and CCL3 also contribute to airway inflammation through their chemotactic effects on lymphocytes.²⁴⁻²⁷ Because MCs constitute a local source of T cell-recruiting CCL3 and

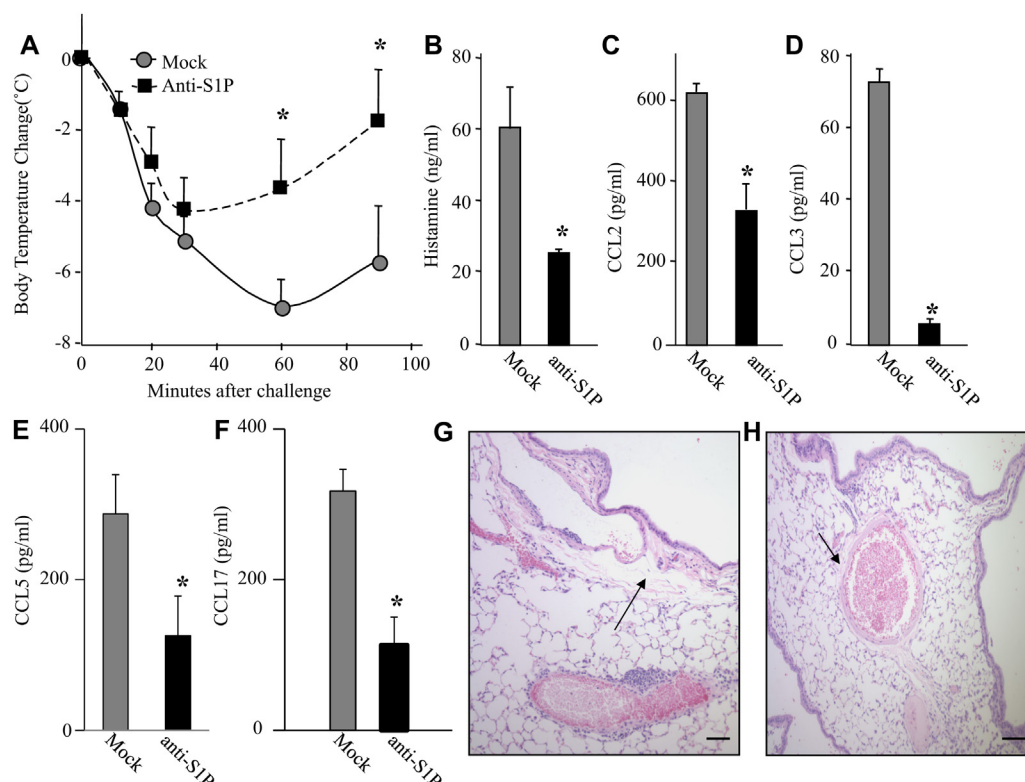


FIG 2. Sphingomab reduces passive systemic anaphylaxis. C57Bl/6 mice were injected intraperitoneally with anti-S1P or isotype-matched control (*Mock*) mAb (20 mg/kg). Twenty-four hours later, murine IgE anti-DNP mAb was administered. Mice were then reinjected intraperitoneally with mAbs at the same dose as above after 12 hours, and antigen challenge was performed 1 hour later by means of intraperitoneal injection of 100 μ g of DNP-HSA. **A**, Body temperature monitoring. **B-F**, Serum levels of histamine (Fig 2, B), MCP-1/CCL2 (Fig 2, C), MIP-1 α /CCL3 (Fig 2, D), RANTES/CCL5 (Fig 2, E), and thymus and activation-regulated chemokine/CCL17 (Fig 2, F) 2 hours after antigen challenge. **G** and **H**, Representative micrographs of hematoxylin and eosin-stained lung tissues of mice treated with mock mAb (Fig 2, G) or Sphingomab (Fig 2, H) collected 2 hours after antigen challenge. * P < .05, Student t test.

CCL5,²⁷ the kinetics of histamine (Fig 5, B), CCL2 (Fig 5, C), and CCL5 (Fig 5, D) release were monitored following antigen challenge. Levels of all 3 circulating mediators were increased time dependently up to 1 hour after antigen challenge. All were markedly reduced in sera of animals pretreated with anti-S1P mAb compared with nonspecific mAb, confirming the local and systemic anti-inflammatory effects of S1P neutralization in an MC-dependent acute model of allergic reaction (Fig 5, B-D). Moreover, as expected, tryptase was undetectable in BAL fluid from *Kit*^{W-sh/W-sh} mice, but levels were increased in MC-reconstituted *Kit*^{W-sh/W-sh} mice (Fig 5, E). Importantly, serum CCL2 (Fig 5, F) and CCL5 (Fig 5, G) levels were also decreased in MC-deficient *Kit*^{W-sh/W-sh} mice compared with those in WT or MC-reconstituted *Kit*^{W-sh/W-sh} mice 1 hour after antigen challenge.

The S1PR2 antagonist JTE-013 decreases inflammatory infiltration

Next, we examined the effects of JTE-013 on pulmonary infiltration in antigen-challenged mice (Fig 6). Antigen exposure triggered rapid and time-dependent cell infiltration in animals pretreated with vehicle (Fig 6, A-F). However, perivascular infiltration was greatly ameliorated by JTE-013 pretreatment (Fig 6, G-L). In agreement with this, immune cell infiltration (Fig 6, M,

and see Fig E1, B and C, in this article's Online Repository at www.jacionline.org) and activation of MCs, as demonstrated by increased BAL fluid tryptase levels (Fig 6, N), as well as RANTES/CCL5 (Fig 6, O, and see Fig E1, D) and CCL2 serum levels (see Fig E1, D, and Oskeritzian et al¹²), were reduced by JTE-013 treatment in WT and MC-reconstituted *Kit*^{W-sh/W-sh} mice (Fig 6, M and N). Of note, JTE-013 treatment of sensitized MC-reconstituted *Kit*^{W-sh/W-sh} mice also prevented the decrease in core body temperature observed after antigen challenge compared with that seen in vehicle-pretreated MC-reconstituted *Kit*^{W-sh/W-sh} mice (see Fig E1, A).

To substantiate the notion that activated MCs secrete chemokines capable of recruiting T cells in an S1PR2-dependent manner, we examined T-cell migration *in vitro* using a transwell assay.²⁸ Consistent with previous studies,^{27,28} T-cell migration was greatly enhanced by supernatants from IgE/antigen-activated BMMCs (Fig 6, P). T-cell chemotactic activity was significantly less in supernatants from activated BMMCs treated with JTE-013, providing further evidence of MC S1PR2-driven T-cell chemotaxis.

Taken together, the observations that neutralizing S1P and antagonism of S1PR2 reduce the secretion of potent chemokines that recruit T lymphocytes highlight the importance of intact MC S1PR2 signaling for adequate chemokine secretion and provide a

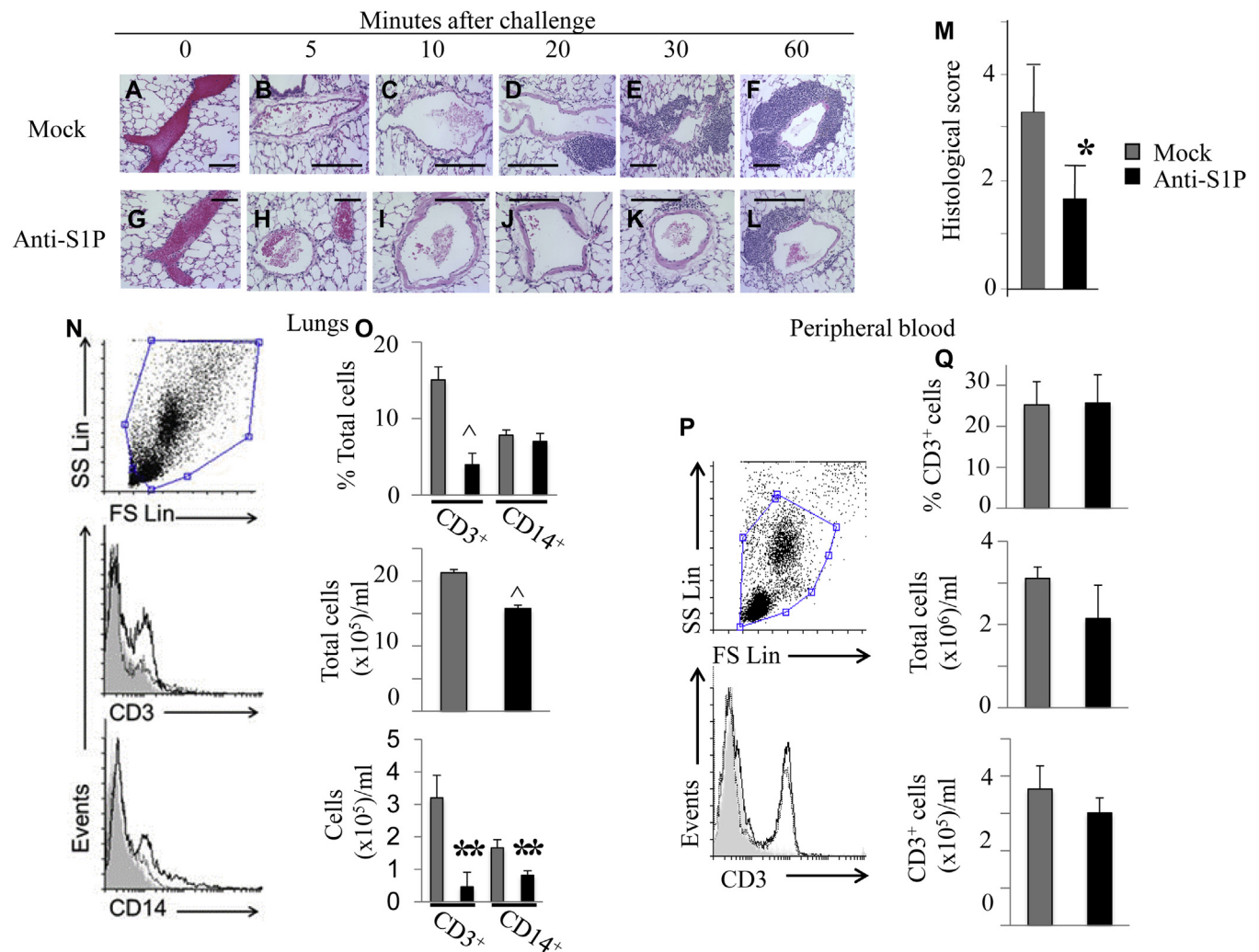


FIG 3. Early allergic lung infiltration is mitigated after Sphingomab pretreatment. **A–L**, Hematoxylin and eosin–stained lung sections of mice pretreated with a mock mAb (Fig 3, **A–F**) or Sphingomab (Fig 3, **G–L**), as described in Fig 2. **M**, Infiltration scoring 1 hour after antigen challenge. **N** and **O**, Flow cytometric analysis and quantification of lung CD3⁺ and CD14⁺ cells 2 hours after antigen challenge. **P** and **Q**, Flow cytometric analysis and quantification of circulating CD3⁺ cells before antigen challenge. Gray histograms represent isotype control staining, unfilled solid lines depict mock mAb–treated cell suspensions, and unfilled dotted lines depict the anti-S1P–treated cells. Graphs represent percentages of CD3⁺ or CD14⁺ cells, total cells, and CD3⁺ or CD14⁺ cell numbers. **P* < .05, ***P* < .01, and ****P* < .0001, Student *t* test. FS, Forward scatter; SS, side scatter.

mechanistic explanation for the early T-cell infiltration observed in pulmonary allergic reactions.

A functional S1P/S1PR2 axis on MCs triggers STAT3 activation and is required for chemokine release

We next sought to determine the signaling pathways downstream of S1PR2 that are involved in S1PR2-driven chemokine secretion from MCs using BMMCs prepared from WT and S1PR2-null mice. Phosphorylation of extracellular signal-regulated kinase 1/2 and Akt by S1P were not affected by S1PR2 deletion (data not shown). Remarkably, S1P exposure triggered STAT3 activation in WT BMMCs, as demonstrated by increased phosphorylation on Tyr705, which was almost completely absent in S1PR2-null BMMCs (Fig 7, **A**). Moreover, S1P-mediated STAT3 activation in both human MCs (Fig 7, **B**

and **C**) and mouse BMMCs (Fig 7, **D**) was eliminated by JTE-013 treatment, further supporting the role of MC S1PR2 in STAT3 activation. Consistent with these results, S1P- or IgE/antigen-induced secretion of the chemokines CCL3 (Fig 7, **E**) and CCL5 (Fig 7, **F**) was greatly attenuated in S1PR2-null BMMCs compared with WT BMMCs, whereas ionomycin-induced chemokine secretion was unaffected.

Importantly, *in vivo* STAT3 activation was observed 20 minutes after antigen challenge in the lungs of sensitized mice (Fig 7, **G** and **H**). Pretreatment with JTE-013, which reduced chemokine secretion (Fig 6, **O**, and see Oskeritzian et al¹²) and prevented early T-cell recruitment (Fig 6), also suppressed STAT3 activation (Fig 7, **G** and **H**). Moreover, increased STAT3 phosphorylation after antigen challenge was greatly reduced in mice treated with anti-S1P mAb but not affected by treatment with a nonspecific mock mAb (Fig 7, **I** and **J**). Therefore MC S1PR2 stimulation by S1P is important for activation of STAT3, a master

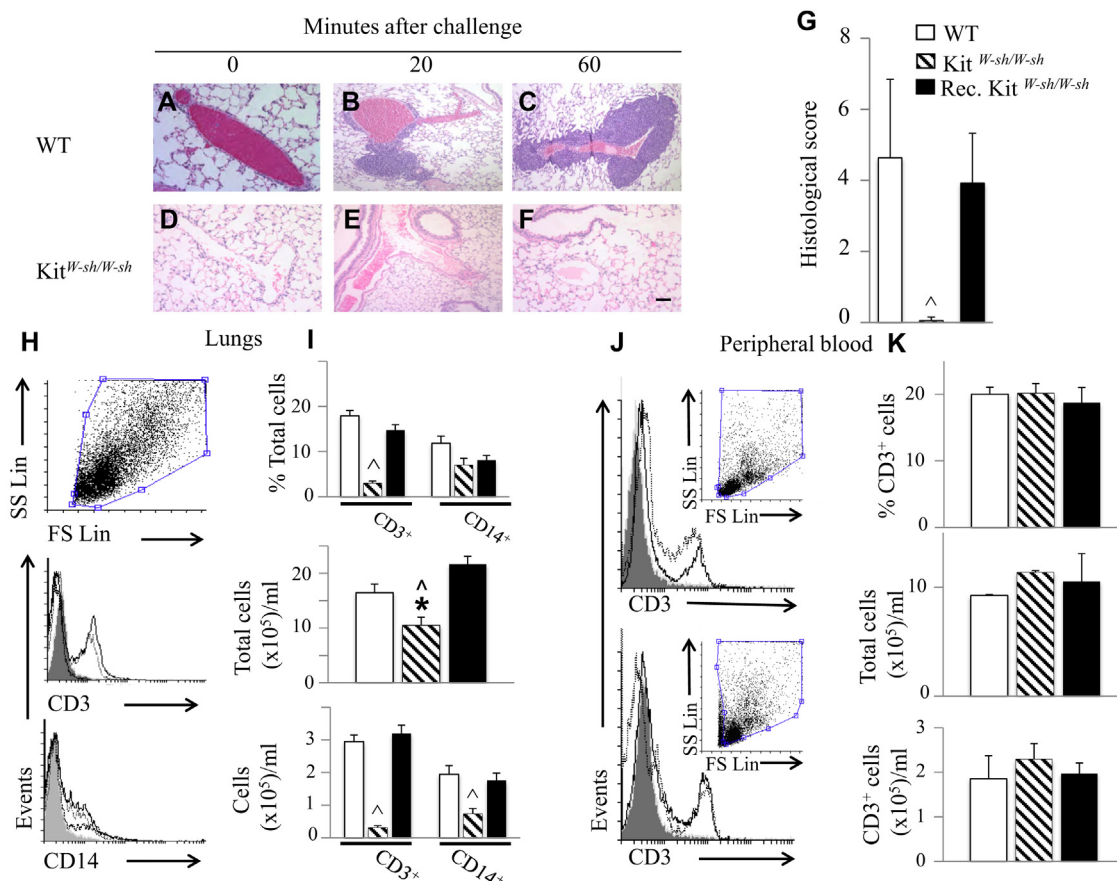


FIG 4. Lack of IgE/antigen-induced lung perivascular infiltration in MC-deficient *Kit*^{W-sh/W-sh} mice and its restoration in MC-reconstituted *Kit*^{W-sh/W-sh} mice (Rec. *Kit*^{W-sh/W-sh}). **A-F**, Hematoxylin and eosin-stained lung sections of MC-deficient and WT mice at the indicated times after antigen challenge. **G**, Infiltration quantification 1 hour after antigen challenge. **H** and **I**, Flow cytometric analysis and quantification of lung cells 1 hour after antigen challenge. Gray histograms represent isotype control staining, unfilled solid lines depict WT cell suspensions, unfilled dotted lines depict Rec. *Kit*^{W-sh/W-sh} cells, and unfilled dashed lines depict *Kit*^{W-sh/W-sh} cells. Graphs represent percentages of CD3⁺ or CD14⁺ cells, total cells, and CD3⁺ or CD14⁺ cell numbers. **J**, Flow cytometric analysis (upper panel compares WT [solid lines] with *Kit*^{W-sh/W-sh} cell suspensions; lower panel compares *Kit*^{W-sh/W-sh} [solid lines] with Rec. *Kit*^{W-sh/W-sh} [dotted lines] cells). **K**, Quantification of circulating CD3⁺ cells before antigen challenge. **P* < .05 and **P* < .0001, 1-way ANOVA. FS, Forward scatter; SS, side scatter.

transcription factor that regulates expression of many chemokines^{29,30} and contributes to T-cell recruitment in MC-dependent allergic responses.

DISCUSSION

The distinctive ability of MCs to produce a wide variety of preformed and *de novo*-synthesized bioactive products makes these tissue-dwelling cells first-line regulators of many immune functions.¹ S1P is produced by MCs and secreted on IgE/antigen activation. It can then activate its S1PR1 and S1PR2 receptors in an autocrine or paracrine manner to regulate degranulation, cytokine and chemokine production, and chemotaxis toward antigen.⁶ Investigations of S1P functions during a systemic allergic reaction uncovered an important role for S1PR2 on MCs and also on vascular endothelial cells, exerting seemingly contrasting effects at early versus late stages of anaphylaxis.^{12,15} We found that activation of S1PR2 on tissue-resident MCs was involved in the early development of pulmonary edema and labored

breathing in sensitized mice after systemic antigen challenge.¹² However, at later stages, high plasma S1P levels aid recovery from shock by counteracting hypotension and promoting plasma histamine clearance, an effect attributed to activation of S1PR2 in the vasculature.¹⁵ A recent publication by Olivera et al³¹ explains these apparent discrepancies. First, lack of S1PR2 reduced IgE/antigen-induced degranulation only in certain types of MC *in vitro*. Second, IgE from different sources differed in their ability to elicit MC signaling and effector responses (termed cytokinergic ability) and induced differential S1PR2-dependent anaphylactic responses. In contrast to the protective effects induced by moderately cytokinergic IgE, strongly cytokinergic IgE induced a more acute response, with increased lethality in WT mice compared with S1PR2-null mice. By using a mouse model of active anaphylaxis, a study by Cui et al³² also identified a new signaling pathway comprising endothelial S1PR2-driven Akt and nitric oxide synthase inhibition in vascular integrity. Of note, these previous studies focused on S1PR2 functions in vascular tone and histamine clearance impairment, whereas our

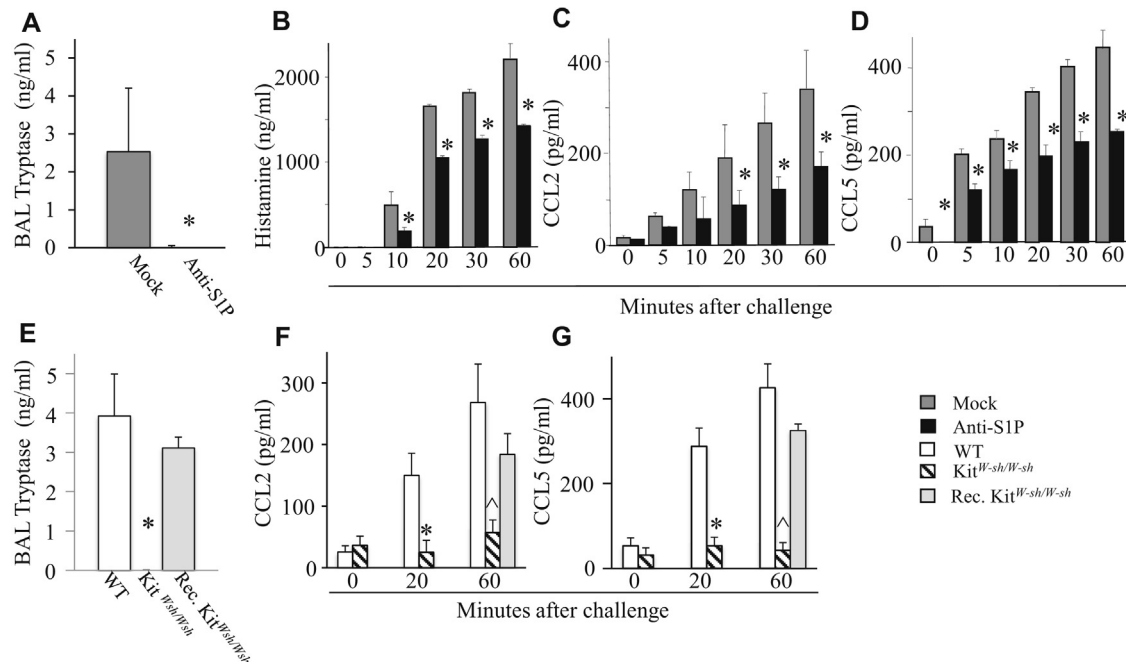


FIG 5. Effects of S1P neutralization, MC depletion, or reconstitution of MCs on circulating levels of proinflammatory mediators after antigen challenge. **A-D**, Mice were treated with anti-S1P or mock antibody, as described in Fig 2. BAL fluid tryptase levels were determined 2 hours after antigen challenge (Fig 5, A). Serum levels of histamine (Fig 5, B), MCP-1/CCL2 (Fig 5, C), and RANTES/CCL5 (Fig 5, C) are shown. **E-G**, Sensitized WT, *Kit^{W-sh/W-sh}*, or Rec. *Kit^{W-sh/W-sh}* mice were challenged with antigen. BAL tryptase levels are shown in Fig 5, E. Serum levels of MCP-1/CCL2 (Fig 5, F) and RANTES/CCL5 (Fig 5, G) are also shown. N = 5 to 6 mice per group repeated twice. **P* < .05, Student *t* test. ***P* < .01, WT versus *Kit^{W-sh/W-sh}* or Rec. *Kit^{W-sh/W-sh}* versus *Kit^{W-sh/W-sh}* mice, 1-way ANOVA. ****P* < .0001, WT or Rec. *Kit^{W-sh/W-sh}* versus *Kit^{W-sh/W-sh}* mice, 1-way ANOVA.

current study investigates the role of S1PR2-dependent MC-mediated cytokine/chemokine production and T-cell recruitment.

The importance of S1P production in murine asthma models is well established.^{7,11,33,34} In agreement, we found that a highly specific anti-S1P neutralizing mAb that is now in phase 2 clinical trials³⁵ significantly attenuated MC-dependent systemic allergic responses and decreased T-cell lung infiltration, likely because of the distinct ability of this antibody to predominantly block local tissue S1P.³⁶

Homeostatic S1P tissue levels are low compared with circulating concentrations, and thus augmented local S1P levels in inflamed tissues are likely to perturb the existing S1P gradient between tissues and the circulation, which controls lymphocyte trafficking.³⁷ Recently, we reported increased levels of lung S1P in an MC-dependent mouse model of asthma, which were normalized after administration of a specific SphK1 inhibitor.¹¹ Resident MCs could be a source of peripheral S1P at sites of inflammation. Because Sphingomab inhibits *in vitro* MC activation, systemic S1P neutralization *in vivo* likely suppresses MC-derived proinflammatory mediator release.

The relationship between airway dysfunction and inflammation is not completely understood. Our data underpin the occurrence of early-onset T-cell recruitment leading to massive infiltration within 1 hour after antigen challenge. Interestingly, few macrophages were also recruited, and almost no eosinophils or neutrophils were detected. Our results suggest that MC-derived mediators are involved in early T-cell recruitment into inflamed lungs because this is greatly diminished in *Kit^{W-sh/W-sh}* mice devoid

of MCs (but restored in MC-reconstituted *Kit^{W-sh/W-sh}* mice), which is consistent with reduced chronic inflammation in an asthma model.³⁸ Reconstitutions of *Kit^{W-sh/W-sh}* mice were conducted by using intraperitoneal rather than intravenous injections of WT BMDCs because it was reported that intravenous injection of BMDCs resulted in artificially increased numbers of MCs in the lung parenchyma of BMDC-engrafted *Kit^{W-sh/W-sh}* mice compared with *Kit^{+/+}* mice.¹⁷ Specifically, the absence of MCs correlated with significantly reduced levels of circulating histamine, CCL2, CCL3, and CCL5 after IgE/antigen triggering. It is striking that early T-cell recruitment to lung perivascular localization and MC-derived T-cell chemokine production were strongly mitigated after S1P neutralization, as well as S1PR2 antagonism or deficiency, making it likely that the S1P/S1PR2 axis serves as a dominant link between activated MCs and T-cell recruitment. These data are consistent with previous work showing that a humanized version of the anti-S1P mAb substantially reduced macrophage infiltration in a mouse model of choroidal neovascularization.³⁹

Several MC-derived mediators could contribute to lymphocyte recruitment.^{1,40} Many studies have highlighted the critical role of locally activated MCs in mediating T-cell recruitment^{41,42} and chemokine production.^{27,43} It is noteworthy that allergy-promoting T_H2 cell recruitment is mediated by CCR4 ligands, such as CCL2, CCL3, CCL5, and CCL17. Importantly, we found that the rapid increase in levels of all 4 chemokines in serum after antigen challenge was attenuated by neutralizing extracellular S1P and was dependent on the S1P/S1PR2 axis and the presence of MCs. Our results point

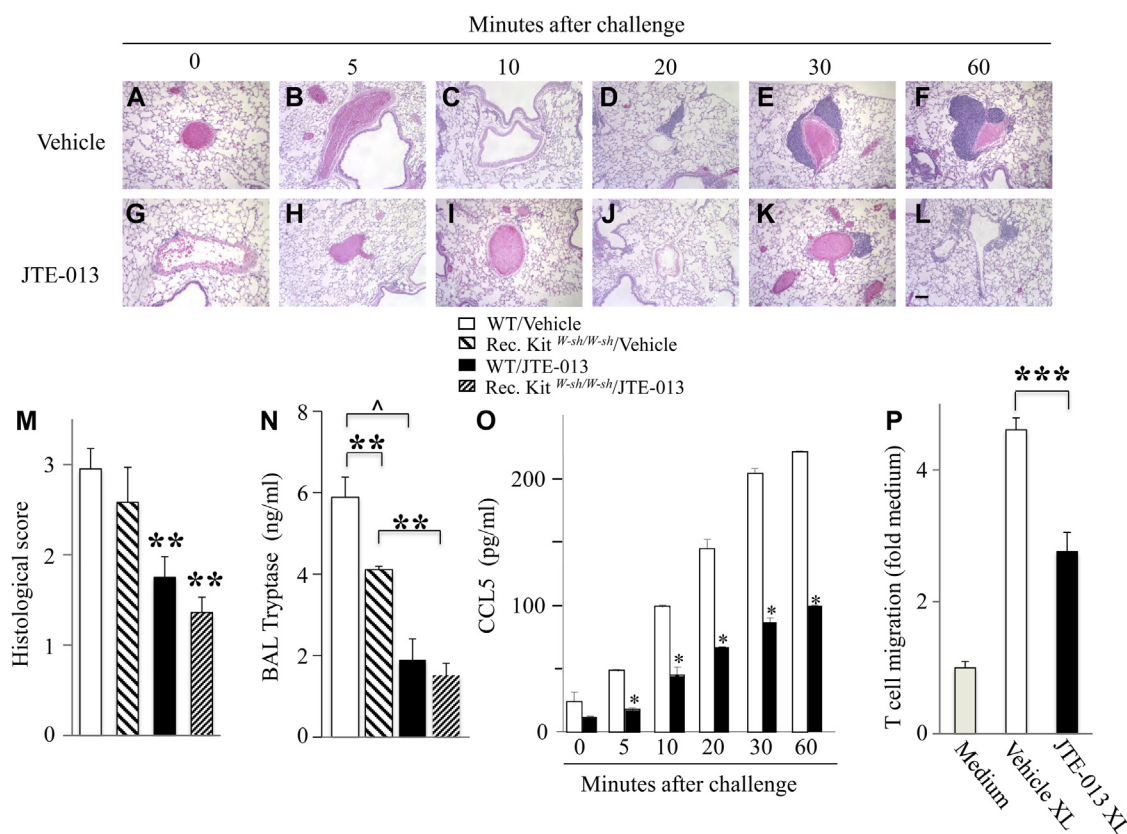


FIG 6. JTE-013, an S1PR2 antagonist, decreases allergic lung infiltration. **A-L**, Hematoxylin and eosin staining of lung sections from mice sensitized with IgE and pretreated with vehicle (Fig 6, A-F) or JTE-013 (Fig 6, G-L) 30 minutes before antigen challenge. **M**, Infiltration scoring 1 hour after antigen challenge. **N**, BAL fluid trypsinase levels. **O**, Serum RANTES/CCL5 levels. * $P < .0005$, Student *t* test. **P**, Transwell T-cell migration assay. Except in Fig 6, O: * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$, 1-way ANOVA.

to a unique signaling pathway in MCs linking S1PR2 to chemokine formation mediated by STAT3 activation. In this regard STAT3 is a critical regulator of CCL2 and CCL3 expression.^{29,44} Moreover, RANTES/CCL5 transcription is dependent on the binding of a p65/STAT3 complex to nuclear factor κ B-binding sites within its promoter.³⁰ Although the S1P/S1PR1 axis is involved in STAT3 activation in cancer cells,⁴⁵⁻⁴⁷ in MCs, as in muscle satellite cells⁴⁸ and cardiomyocytes,⁴⁹ S1P/S1PR2 signaling plays a critical function because S1PR2 deficiency abrogated STAT3 phosphorylation. Although activation of S1PR2 can directly activate STAT3 in a Rho-dependent manner,⁴⁸ the possibility cannot be excluded that IL-6 released from MCs could also contribute to STAT3 activation.⁵⁰ Interestingly, we previously demonstrated that secretion of IL-6 from MCs is also dependent on the S1P/S1PR2 axis.¹² Consistent with our findings, a recent study revealed compromised allergic reactions and MC degranulation in patients harboring *STAT3* mutations, as well as in human MCs transduced with small hairpin RNAs against *Stat3* and in a corresponding mouse model of *Stat3* mutation.⁵¹ Taken together, our data suggest that S1P/S1PR2 signaling in MCs leads to activation of STAT3 and chemokine production, which in turn plays an important role in early T-cell mobilization in acute pulmonary allergic responses.

Our study furthers the understanding of MCs as early inflammatory orchestrators and offers a new paradigm for the initiation of allergic T_H2 immunity that supports the importance of MC functions in inflammatory disease etiology.

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Key messages

- Kinetic analyses of acute MC-dependent allergic inflammatory events triggered after a single dose of antigen administered systemically revealed early T-cell recruitment in the lungs of sensitized mice, an event originally associated with the late-phase response.
- T-cell recruitment was driven by MC activation through S1PR2 signaling, leading to STAT3 activation and release of T-cell chemokines.
- Targeting early T-cell recruitment to inflamed lungs through a novel pathway linking MC S1PR2/STAT3 signaling to chemokine secretion might offer a relevant approach for treating patients with inflammatory diseases to prevent chronic inflammation.

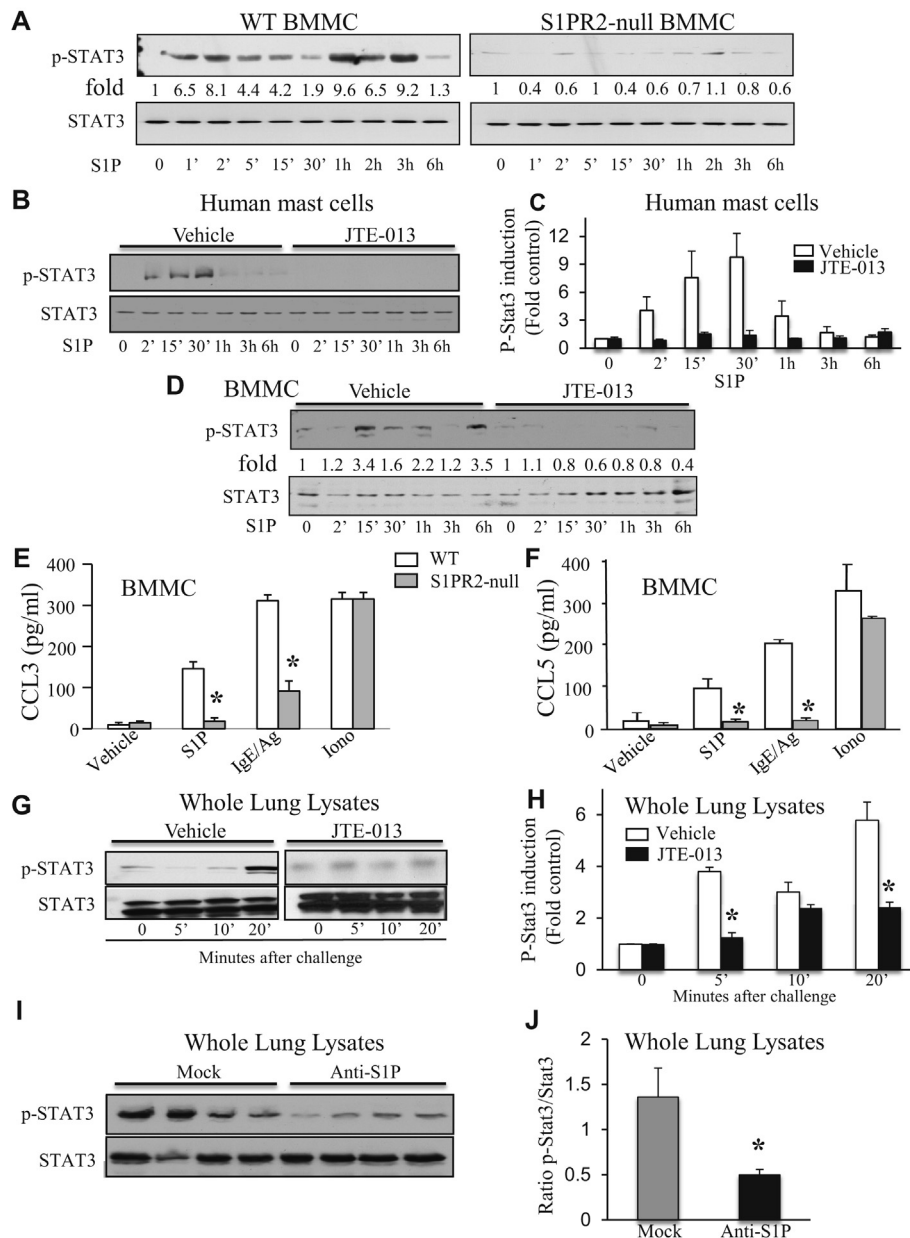


FIG 7. Activation of STAT3 by the S1P/S1PR2 axis in MCs *in vitro* and in lungs after antigen challenge. **A-D**, WT or S1PR2-null BMMCs (Fig 7, A) or vehicle- or JTE-013-pretreated human MCs (Fig 7, B and C) or WT BMMCs (Fig 7, D) were stimulated with S1P (100 nmol/L) for the indicated times. Cell lysates were immunoblotted with the indicated antibodies, and p-STAT3 was quantified. **E** and **F**, Activated BMMC supernatants were assayed for CCL3 (Fig 7, E) and CCL5 (Fig 7, F). **G** and **H**, Lung lysates from mice treated with vehicle or JTE-013 before IgE/antigen challenge were immunoblotted as above. **I** and **J**, Immunoblots of lung lysates from mice treated with anti-S1P or mock mAb before IgE/antigen challenge (Fig 7, I) and quantification of p-STAT3/STAT3 ratios (Fig 7, J). * $P < .04$, Student *t* test.

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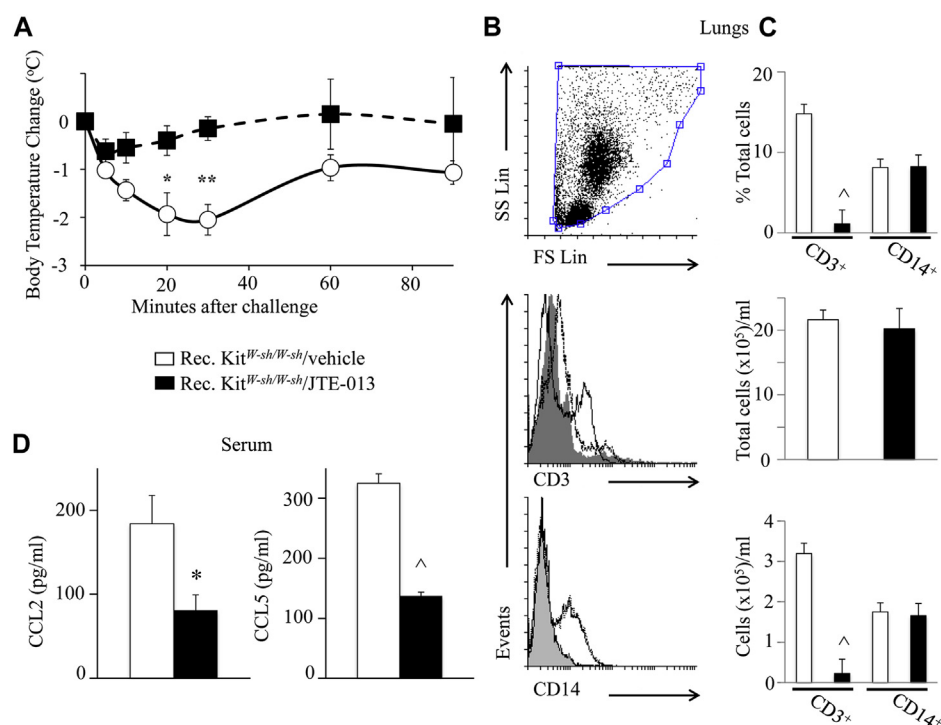


FIG E1. JTE-013, an S1PR2 antagonist, reduces passive systemic anaphylaxis and allergic lung infiltration in MC-reconstituted *Kit*^{W-sh/W-sh}. MC-reconstituted *Kit*^{W-sh/W-sh} mice were treated as described in Fig 6. **A**, Body temperature monitoring. **B**, Flow cytometric analysis of CD3⁺ and CD14⁺ cells in mouse lungs 90 minutes after antigen challenge. Gray histograms represent isotype control staining, unfilled solid lines depict Rec. *Kit*^{W-sh/W-sh} vehicle-treated lung cells, and unfilled dotted lines depict Rec. *Kit*^{W-sh/W-sh} JTE-013-treated lung cells. **C**, Graphs represent corresponding percentages of CD3⁺ or CD14⁺ cells, total cells, or CD3⁺ or CD14⁺ cell numbers in the lungs. **D**, Serum levels of MCP-1/CCL2 and RANTES/CCL5 90 minutes after antigen challenge. **P* < .05 and †*P* < .0001, Student *t* test.