The S1P/S1PR2 axis regulates early airway T cell infiltration in murine mast cell-dependent acute allergic responses

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Abstract

Background—Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid produced by mast cells (MC) upon cross-linking of their high affinity receptors for IgE by antigen (Ag) that can amplify MC responses by binding to its S1P receptors. 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 an S1P receptor 2 (S1PR2) antagonist, JTE-013, to study S1P and S1PR2 signaling contributions to MC- and IgE-dependent airway allergic responses in mice within minutes after Ag challenge.

Methods—Allergic reaction was triggered by a single intraperitoneal (i.p.) dose of Ag in sensitized mice pre-treated i.p. with anti-S1P or isotype control mAb, or JTE-013 or vehicle prior to Ag challenge.

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Results—Kinetics experiments revealed early pulmonary infiltration of mostly T cells around blood vessels of sensitized mice 20 minutes post-Ag exposure. Pre-treatment 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 MCP-1/CCL2, MIP-1α/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 (Stat3) activation.

Conclusion—Activation of S1PR2 by S1P and downstream Stat3 signaling in MC regulate early T cell recruitment to antigen-challenged lungs by chemokine production.

INTRODUCTION

Mast cells (MC) play a central role in both the development and exacerbation of allergic disorders [1]. Cross-linking of their high affinity receptors for immunoglobulin (Ig) E (FcεRI) by antigen (Ag) activates sphingosine kinases that phosphorylate sphingosine to produce S1P, a critical sphingolipid contributing to degranulation, the release of intragranular mediators [2, 3]. S1P can be exported from MC to amplify their responses by binding to S1P receptors [2, 4, 5] and recruit other immune cells, including Th2 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 S1PR2 plays a critical role in regulating human mast cell functions [12], whereas S1PR1 is important for their migration to sites of inflammation [2, 3]. IgE-dependent systemic MC activation is responsible for elevated circulating levels of histamine and associated pulmonary edema in mice [1, 13], and both are attenuated after inhibition or deletion of S1PR2 [12]. While 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 may 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].

We here evaluated IgE/Ag-induced pulmonary alterations upon blockade of S1PR2 signaling by neutralization of S1P, the ligand of S1PR2, with Sphingomab, a specific anti-S1P mAb and by inhibition of S1PR2 with the specific antagonist JTE-013. We show that inflammatory infiltration of T cells occurs within minutes of systemic Ag challenge and is strongly mitigated in mice pre-treated either with Sphingomab or JTE-013 in a MC-

dependent model of acute allergic reaction. We further demonstrate that this effect is due in part to early production of chemokines. Moreover, Stat3 activation was induced in inflamed lungs and in MC 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 via the S1P/S1PR2/Stat3 axis and chemokine production.

METHODS

Mice

C57BL6/J, C57BL/6x129/SVJ and C57BL/6-$Kit^{W-sh/W-sh}$ mice were purchased from The Jackson Laboratory. S1PR2 knockout mice (Dr. R. Proia, NIH/NIDDK, Bethesda, MD) and corresponding wild-type mice were on mixed C57BL/6×129/SVJ background. All mice were used at 8-12 weeks of age and maintained in a pathogen-free facility. Studies were performed in accordance with the institutional IACUC guidelines.

Reagents and antibodies

Murine monoclonal antibody, LT1002 (Sphingomab™) directed against S1P, and LT1003 isotype-matched control (IgG1, κ) antibody were provided by Lpath Inc., San Diego, CA. 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, MA). Hamster anti-mouse CD3ε and IgG isotype control, Alexa Fluor 488 conjugates, rat anti-mouse CD14 and IgG2a, κ isotype control, PE conjugates, and rat anti-mouse CD16/32 (Fc block) were purchased from eBioscience (San Diego, CA). JTE-013 was purchased from Tocris Bioscience (Minneapolis, MN) and S1P from Enzo Life Sciences (Farmingdale, NY). DNP-HSA (Ag) and ionomycin were obtained from Sigma-Aldrich (St. Louis, MO). Liberase TM and DNase I were obtained from Roche Diagnostics (Indianapolis, IN).

Human skin and mouse bone marrow-derived mast cells

Human skin mast cells (Sk-MC) and mouse bone marrow-derived mast cells (BMMC) were isolated and cultured as previously described [11, 16] and were more than 98% pure. Human MC and BMMC were sensitized overnight with 1 μg/ml or 0.5 μg/ml DNP-specific mouse IgE, washed to remove excess unbound IgE and stimulated with 30 or 20 ng/ml DNP-HSA (Ag), respectively [5]. Ionomycin (1 μM) was used as positive control. Degranulation was quantified by measuring β-hexosaminidase release [5].

Cytokine and chemokine measurements

Human and mouse cytokines/chemokines were measured by ELISA following manufacturer's instruction (R & D Systems, Minneapolis, MN).

Acute allergic reaction

All injections were performed i.p. in a final volume of 100 μl, as previously described [12]. Briefly, mice were injected with DNP-specific IgE and 12 h later, with Ag in PBS. In some experiments, blood samples were collected by tail vein nick just prior to Ag challenge to

conduct flow cytometric analysis of circulating T cells. A small nick was made over the lateral vein of restrained mice using a scalpel blade. Blood (150-250 μl) was collected via heparinized capillary tubes into heparinized tubes. Core body temperature was measured immediately before Ag challenge and at indicated times using a rectal microprobe (Physitemp Instruments, Clifton, NJ). Mice were euthanized and blood was collected by cardiac puncture for serum analysis. Bronchoalveolar lavage (BAL) fluids were obtained by lavaging lungs twice with 600 μl PBS and stored at −80°C after centrifugation. Lungs were removed for histological examination or snap-frozen for Western blot analyses. Control mice injected with PBS showed no significant change in body temperature (±1°C) and had no detectable blood histamine.

Adoptive transfer of BMMC into MC-deficient mice

Bone marrow cells from femurs of female wild-type mice were cultured for 6 weeks to generate BMMC, as described [11]. For MC reconstitution, recipient (7-8 weeks-old) female $Kit^{W-sh-/W-sh}$ mice were injected i.p. with 5×10^6 BMMC in 200 μl 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, prior to Ag triggering, as described [12]. To assess for appropriate reconstitution, BAL was collected 90 minutes after Ag challenge and BAL tryptase determined.

Lung histology

Lungs were prepared for histological analysis as previously described [12]. Briefly, after inflation, lungs were embedded in paraffin, serial sections stained with H&E, and perivascular areas were evaluated in 8-12 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, MI) was used to photograph the sections. Perivascular infiltration was scored semi-quantitatively, 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 μm.

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/μl final concentration) was added to each dispersed sample. After 1h at 37°C, digested tissues were filtered through 70 μm cell strainers and centrifuged. Red blood cells (RBC) were lysed using hypotonic salt solutions. Cell suspensions were quickly filtered and centrifuged. Pellets were washed in ice-cold PBS/1%FCS for cell counting and staining. For blood samples, RBC were first lysed with BD PharmLyse buffer (BD Biosciences, San Jose, CA). Cells from individual mice were incubated prior to and during all Ab staining with Fc block. CD3 and CD14 expression analysis was performed using a standard flow cytometry protocol 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 (Fullerton, CA) and data analyzed using the CXP software.

Transwell T cell migration assay

BMMC-conditioned cell-free supernatants were prepared using sensitized (0.5 μg mouse anti-DNP IgE/ml/2×10^6 BMMC, 12h, 37°C) BMMC treated with JTE-013 (1 μM) or vehicle for 30 min at 37°C prior to adding Ag (DNP-HSA, 100 ng/ml/30×10^6 BMMC). After 24h, supernatants were collected and stored at −80°C. Splenic $CD4^+$ and $CD8^+$ T cells were isolated using the EasySep kit (StemCell Technologies, Vancouver, BC, Canada) and used together for migration experiments. T cell enrichment was verified by flow cytometry. Assays were conducted using 5 μm pore size polycarbonate filters in Costar 24-well Transwell chambers (Corning, Corning, NY) with 850 μl BMMC-conditioned media in the lower chamber and 10^5 T cells in 200 μl in the upper chamber. Recombinant murine RANTES/CCL5 (100 ng/ml, Peprotech, Rocky Hill, NJ) and medium, DMSO vehicle or JTE-013 were used as positive and negative controls, respectfully. After 24h at 37°C, T cells in the lower chambers were counted in quadruplicate using flow cytometry after propidium iodide staining to exclude dead cells from analysis (FACScalibur, BD Biosciences). Migration was expressed as fold of control (culture medium alone).

Histamine determination

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

Tryptase determination

An ELISA kit was used to measure tryptase in the supernatants of centrifuged BAL fluids according to manufacturer's recommendation (EMD Millipore, Billerica, MA).

Western analysis

Lungs or 5×10^6 MC were homogenized and equal amounts of proteins were analyzed by immunoblotting after transfer to nitrocellulose and blocking with 1:1,000 dilutions of anti-p-Stat3 or anti-Stat3 overnight at +4°C. After washes in TBS-0.1%Tween 20, membranes were incubated with 1:5,000 dilution of species-appropriate HRP-linked anti-IgG (Cell Signaling) and sizes of proteins of interest were determined using molecular weight standards (BioRad, Hercules, CA). Quantitation was carried out using ImageJ (National Institutes of Health). Integrated density numbers (in pixels) of p-Stat3 were normalized to total Stat3 and are means of three independent experiments.

Statistical analysis

Data are expressed as means ± S. E. M. and were analyzed by unpaired two-tailed Student's *t*-test for comparison of two groups and one-way analysis of variance (ANOVA) for analysis of three or more groups (Prism 6; GraphPad software). Significance for all statistical tests is shown in figures for $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (^). Experiments were repeated at least three times in triplicates with consistent results. *In vivo* experiments were repeated three times and each experimental group consisted of five to six mice.

RESULTS

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

We investigated the effects of Sphingomab on *in vitro* MC activation. As shown in Fig 1A-D, addition of Sphingomab at concentrations ranging from 10 to 0.01 μg/ml, but not isotype-matched control mAb, or Sphingomab at 0.001 μg/ml, at time of Ag stimulation dose-dependently decreased IgE/Ag-induced degranulation as measured by beta-hexosaminidase release, without altering either spontaneous or ionomycin-induced degranulation. Since the anti S1P-mAb inhibited degranulation by 50% at 0.1 μg/ml, this concentration was selected to examine its effects on cytokine/chemokine secretion. Anti-S1P mAb treatment significantly decreased IgE/Ag-triggered IL-6 (Fig 1F), CCL5 (Fig 1G), CCL2 (Fig 1H), TNF (Fig 1I) and CCL3 (Fig 1J) secretion, without altering spontaneous or ionomycin-induced release. These results substantiate the notion that S1P released from activated MC contributes to secretion of proinflammatory mediators and this can be suppressed by neutralizing extracellular S1P.

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

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, prior to IgE/Ag injections, anti-S1P mAb was administered i.p., as it was previously demonstrated that over 95% of the anti-S1P mAb rapidly appeared in the bloodstream after i.p. injection of a bolus dose [21]. The anti-S1P mAb-treated mice exhibited significantly reduced hypothermia, compared to mice treated with an isotype-matched control mAb (Fig 2A). Mice administered anti-S1P mAb also had markedly decreased levels of systemic histamine (Fig 2B), MCP-1/CCL2 (Fig 2C), MIP1-alpha/CCL3 (Fig 2D), RANTES/CCL5 (Fig 2E) and TARC/CCL17 (Fig 2F) 2h after Ag administration. At this time point, histopathological analysis showed extensive perivascular edema in mice pretreated with a mock mAb prior to Ag challenge (Fig 2G), which was significantly attenuated in anti-S1P mAb-treated mice (Fig 2H).

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

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

triggering demonstrated that numbers of circulating $CD3^+$ cells in anti-S1P-treated mice were not altered compared to mice treated with control mAb (Fig 3P-Q).

Ag-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 MC in early infiltration, we utilized C57BL/6-$Kit^{W-sh/W-sh}$ MC-deficient mice [17]. Significant perivascular cell infiltration was present in WT (Fig 4A-C) but not in MC-deficient mice (Fig 4D-F), which was confirmed by semi-quantitative scoring of lung sections (Fig 4G). MC involvement was further substantiated using Ag-challenged MC-reconstituted $Kit^{W-sh/W-sh}$ mice which displayed histological scores comparable to WT MC-sufficient mice (Fig 4G). Flow cytometry 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-I). These results suggest an important role for MC in early infiltration of T lymphocytes and macrophages. As expected, blood samples prior to Ag challenge showed no significant differences in circulating $CD3^+$ cells between WT, MC-deficient mice and MC-reconstituted $Kit^{W-sh/W-sh}$ (Fig 4J-K).

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

While MC account for the early airway responses to Ag 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 levels of tryptase in the BAL fluids of control mAb-pretreated sensitized mice 2h after Ag challenge (Fig 5A). In sharp contrast, tryptase was not detectable in BAL fluids from mice pre-treated with anti-S1P mAb (Fig 5A). As expected, tryptase was undetectable in BAL fluid from $Kit^{W-sh/W-sh}$ mice, but was elevated in MC-reconstituted $Kit^{W-sh/W-sh}$ mice (Fig 5B).

We have previously shown that acute allergic challenge leads to elevated histamine and MCP-1/CCL2 and MIP-1α/CCL3, all of which could be ascribed to activation of MC [12]. RANTES/CCL5, CCL2, and CCL3 also contribute to airway inflammation through their chemotactic effects on lymphocytes [24-27]. Because MC constitute a local source of T cell-recruiting CCL3 and CCL5 [27], the kinetics of histamine (Fig 5B), CCL2 (Fig 5C) and CCL5 (Fig 5D) release were monitored upon Ag challenge. All three circulating mediators were increased time-dependently up to 1h after Ag challenge. All were markedly reduced in the serum of animals pretreated with anti-S1P mAb as compared to nonspecific mAb, confirming the local as well as systemic anti-inflammatory effects of S1P neutralization in a MC-dependent acute model of allergic reaction (Fig 5B-D). Moreover, as expected, tryptase was undetectable in BAL fluid from $Kit^{W-sh/W-sh}$ mice, but was elevated in MC-reconstituted $Kit^{W-sh/W-sh}$ mice (Fig 5E). Importantly, serum CCL2 (Fig 5F) and CCL5 (Fig 5G) were also decreased in MC-deficient $Kit^{W-sh/W-sh}$ mice, compared to WT or MC-reconstituted $Kit^{W-sh/W-sh}$ mice 1h after Ag challenge.

The S1PR2 antagonist JTE-013 decreases inflammatory infiltration

Next, we examined the effects of JTE-013 on pulmonary infiltration in Ag-challenged mice (Fig 6). Ag exposure triggered rapid and time-dependent cell infiltration in animals pretreated with vehicle (Fig 6A-F). However, perivascular infiltration was greatly ameliorated by JTE-013 pretreatment (Fig 6G-L). In agreement, immune cell infiltration (Fig 6M and Figs E1B-C), activation of MC as demonstrated by increased BAL tryptase (Fig 6N) as well as RANTES/CCL5 (Fig 6O and Fig E1D) and CCL2 serum levels (Fig E1D and [12]) were reduced by JTE-013 treatment, in WT and in MC-reconstituted $Kit^{W-sh/W-sh}$ mice (Figs 6M, 6N). Of note, JTE-013 treatment of sensitized MC-reconstituted $Kit^{W-sh/W-sh}$ mice also prevented the drop in core body temperature observed upon antigenic challenge, compared to vehicle pre-treated MC-reconstituted $Kit^{W-sh/W-sh}$ mice (Fig E1A).

To substantiate the notion that activated MC secrete chemokines capable of recruiting T cells in a 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/Ag-activated BMMC (Fig 6P). T cell chemotactic activity was significantly lower in supernatants from activated BMMC treated with JTE-013, providing further evidence of mast cell 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 mechanistic explanation for the early T cell infiltration observed in pulmonary allergic reaction.

Functional S1P/S1PR2 axis on MC 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 mast cells utilizing BMMC prepared from WT and S1PR2-null mice. Phosphorylation of ERK1/2 and Akt by S1P were not affected by S1PR2 deletion (data not shown). Remarkably, S1P exposure triggered Stat3 activation in WT BMMC, as demonstrated by increased phosphorylation on Tyr705, that was almost completely absent in S1PR2-null BMMC (Fig 7A). Moreover, S1P-mediated Stat3 activation in both human MC (Fig 7B-C) and mouse BMMC (Fig 7D) was eliminated by JTE-013 treatment, further supporting the role of MC S1PR2 in Stat3 activation. Consistent with these results, S1P- or IgE/Ag-induced secretion of the chemokines CCL3 (Fig 7E) and CCL5 Fig 7F) were greatly attenuated in S1PR2-null BMMC compared to WT, while ionomycin-induced chemokine secretion was unaffected.

Importantly, *in vivo* Stat3 activation was observed 20 min after Ag challenge in the lungs of sensitized mice (Fig 7G-H). Pretreatment with JTE-013, which reduced chemokine secretion (Fig 6O and [12]) and prevented early T cell recruitment (Fig 6), also suppressed Stat3 activation (Fig 7G-H). Moreover, increased Stat3 phosphorylation after Ag challenge was greatly reduced in mice treated with anti-S1P mAb but not affected by treatment with a nonspecific mock mAb (Fig 7I-J). Therefore, MC S1PR2 stimulation by S1P is important

for activation of Stat3, a master 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 MC 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 MC and secreted upon IgE/Ag 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 towards Ag [6]. Investigations of S1P functions during a systemic allergic reaction uncovered an important role for S1PR2 on MC 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 MC was involved in the early development of pulmonary edema and labored breathing in sensitized mice after systemic Ag 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 al. explains these apparent discrepancies [31]. First, lack of S1PR2 reduced IgE/Ag-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, a strongly cytokinergic IgE induced a more acute response with increased lethality in WT mice compared to S1PR2-null mice. Using a mouse model of active anaphylaxis, a study by Cui et al. also identified a new signaling pathway comprising endothelial S1PR2-driven Akt and nitric oxide synthase inhibition in vascular integrity [32]. Of note, these previous studies focused on S1PR2 functions in vascular tone and histamine clearance impairment whereas our 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 trials [35] significantly attenuated MC-dependent systemic allergic responses and decreased T cell lung infiltration likely due to the distinct ability of this Ab to predominantly block local tissue S1P [36].

Homeostatic S1P tissue levels are low compared to circulating concentrations, 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]. We recently reported increased levels of lung S1P in a MC-dependent mouse model of asthma, which were normalized after administration of a specific SphK1 inhibitor [11]. Resident MC could be a source of peripheral S1P at sites of inflammation. As Sphingomab inhibits *in vitro* MC activation, systemic S1P neutralization *in vivo* likely suppresses MC-derived pro-inflammatory mediator release.

The relationship between airway dysfunction and inflammation is not completely understood. Our data underpin the occurrence of an early onset of T cell recruitment, leading to massive infiltration within 1 h after Ag 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 as this is greatly diminished in $Kit^{W-sh/W-sh}$ mice devoid of MC (but restored in MC-reconstituted $Kit^{W-sh/W-sh}$ mice), consistent with reduced chronic inflammation in an asthma model [38]. Reconstitutions of $Kit^{W-sh/W-sh}$ mice were conducted employing intraperitoneal rather than intravenous injections of WTBMMC, as it was reported that intravenous injection of BMMC resulted in artificially increased number of MC in the lung parenchyma of BMMC-engrafted $Kit^{W-sh/W-sh}$ mice, compared to $Kit^{+/+}$ mice [17]. Specifically, the absence of MC correlated with significantly reduced levels of circulating histamine, CCL2, CCL3 and CCL5 after IgE/Ag triggering. It is striking that early T cell recruitment to lung perivascular localization and MC-derived T cell chemokine production were strongly mitigated upon S1P neutralization, as well as S1PR2 antagonism or deficiency, making it likely that the S1P/S1PR2 axis serves as a dominant link between activated MC 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 MC in mediating T cell recruitment [41, 42] and chemokine production [27, 43]. It is noteworthy that allergy-promoting Th2 cell recruitment is mediated by CCR4 ligands, such as CCL2, CCL3, CCL5 and CCL17. Importantly, we found that the rapid increase of all four chemokines in serum following Ag challenge was attenuated by neutralizing extracellular S1P, and was dependent on the S1P/S1PR2 axis and the presence of MC. Our results point to a unique signaling pathway in MC 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 NF-kappaB-binding sites within its promoter [30]. Although the S1P/S1PR1 axis is involved in Stat3 activation in cancer cells [45-47], in MC, as in muscle satellite cells [48] and in cardiomyocytes [49], S1P/S1PR2 signaling plays a critical function as S1PR2 deficiency abrogated Stat3 phosphorylation. Although activation of S1PR2 directly can activate Stat3 in a Rho-dependent manner [48], the possibility cannot be excluded that IL-6 released from MC could also contribute to Stat3 activation [50]. Interestingly, we previously demonstrated that secretion of IL-6 from MC 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 MC transduced with shRNAs against Stat3 and in a corresponding mouse model of Stat3 mutation [51]. Taken together, our data suggest that S1P/S1PR2 signaling in MC 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 understanding of MC as early inflammatory orchestrators and offers a new paradigm for the initiation of allergic Th2 immunity that supports the importance of MC functions in inflammatory disease etiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Abbreviations used

BMMC	Bone marrow-derived mast cell
HR	hyperresponsiveness
Sk-MC	human skin mast cells
MC	mast cell
MIP-1α	macrophage inflammatory protein 1 alpha
RANTES	regulated on activation, normal T cell expressed and secreted
S1P	sphingosine-1-phosphate
S1PR2	S1P receptor 2
Stat3	signal transduced and activator of transcription 3

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KEY MESSAGES

- Kinetic analyses of acute mast cell-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 mast cell activation via S1PR2 signaling, leading to Stat3 activation and release of T cell chemokines.

- Targeting early T cell recruitment to inflamed lungs via a novel pathway linking mast cell S1PR2/Stat3 signaling to chemokine secretion may offer a relevant approach for treating patients with inflammatory diseases to prevent chronic inflammation.

CAPSULE SUMMARY

Early T cell recruitment occurs in an acute mast cell-dependent preclinical model of allergic reaction via S1PR2/Stat3 signaling and chemokine production. S1P neutralization with Sphingomab™ or S1PR2 inactivation/deficiency suppresses local and systemic inflammation.
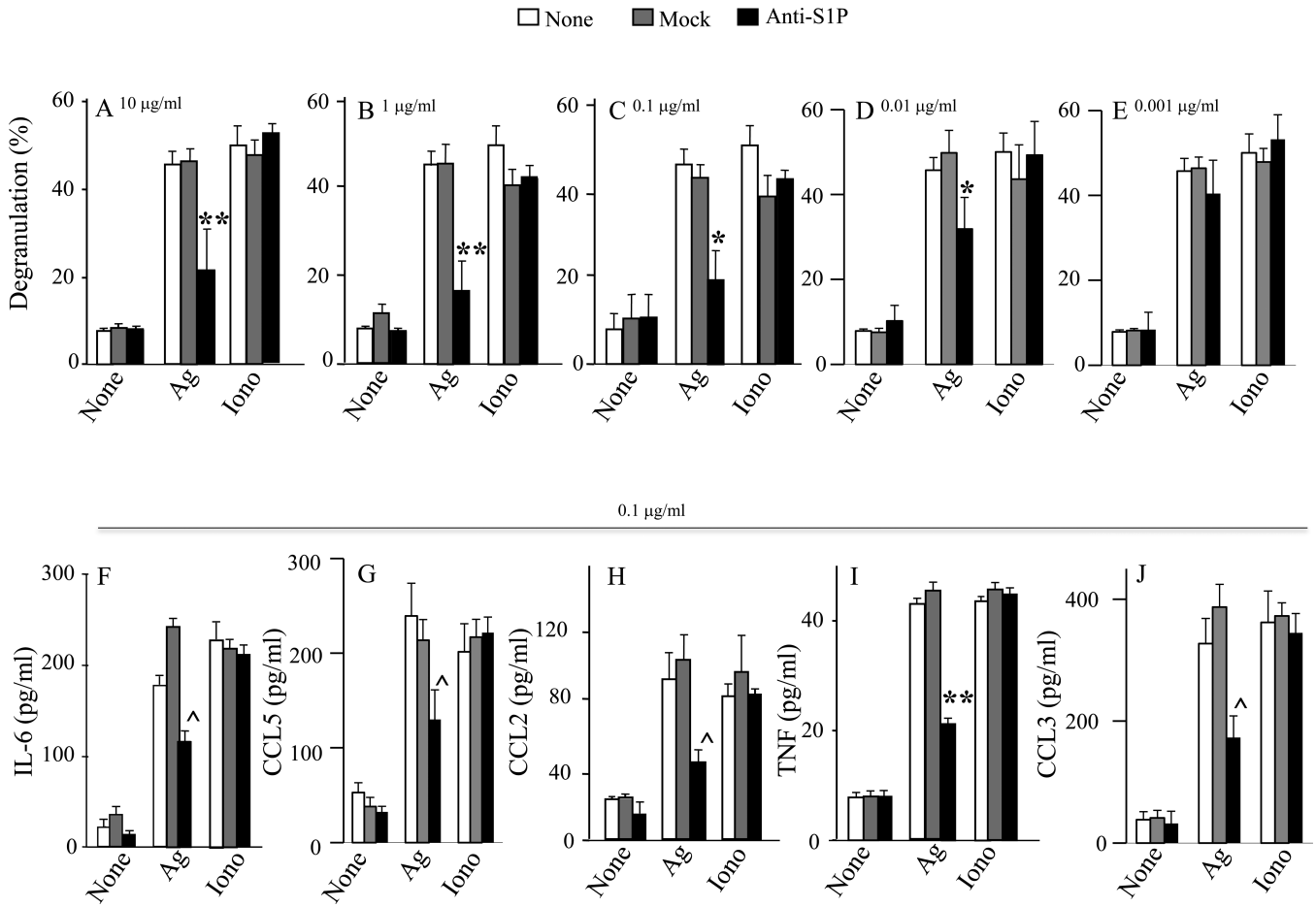
Fig. 1.
Sphingomab, a specific anti-S1P mAb, reduces IgE/Ag-induced activation of human mast cells. Sk-MC were pretreated with anti-S1P or control (mock) prior to stimulation, at the indicated concentration. Degranulation was measured by colorimetric assay (A-E). Secretion of IL-6 (F), RANTES/CCL5 (G), MCP-1/CCL2 (H), TNF (I) and MIP-1α/CCL3 (J) were measured by ELISA. (* $p < 0.05$; ** $p < 0.01$; ^ $p < 0.0001$, oneway ANOVA).
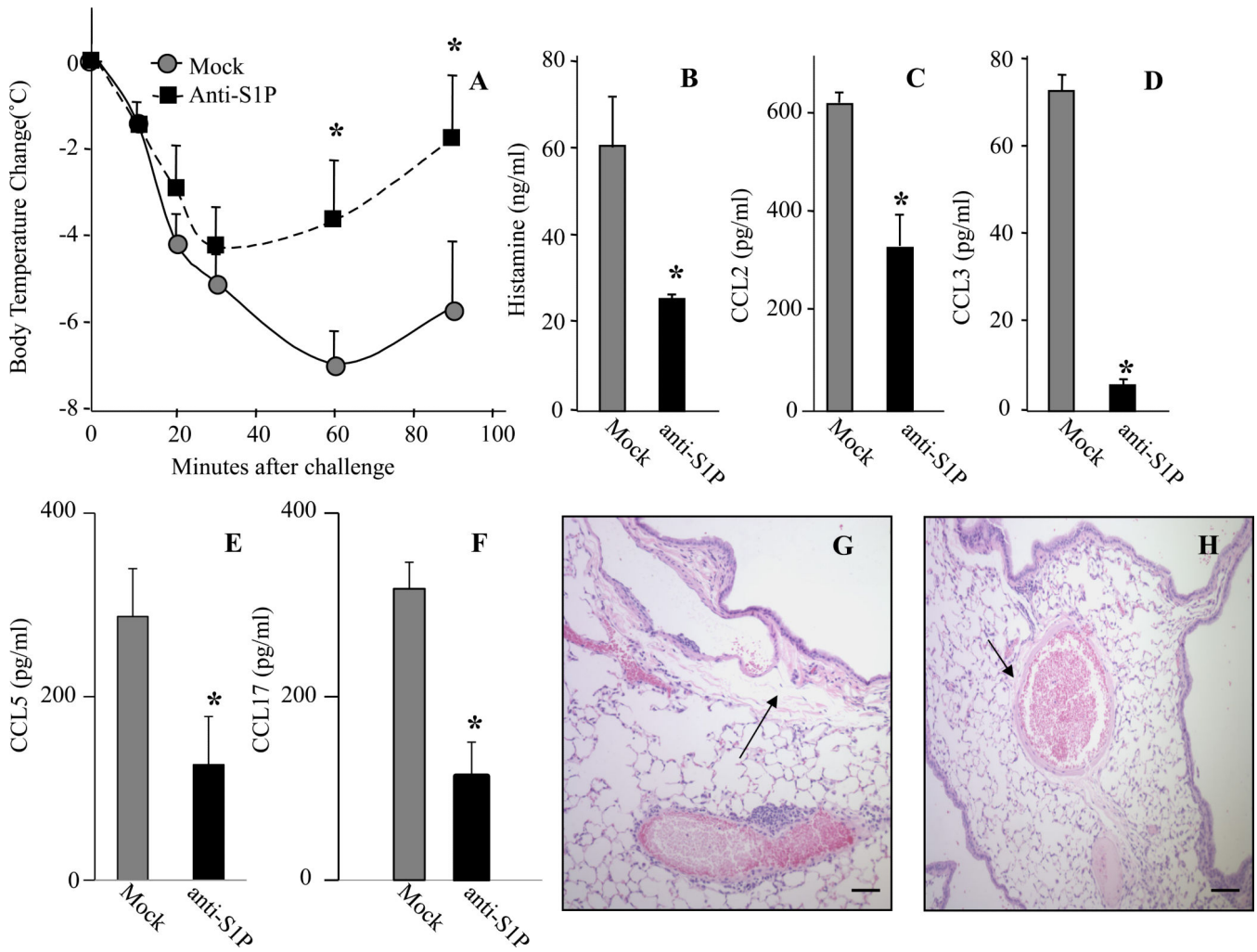
Fig. 2.
Sphingomab reduces passive systemic anaphylaxis. C57Bl/6 mice were injected i.p. with anti-S1P or isotype-matched control (mock) mAb (20 mg/kg). Twenty-four hours later, murine IgE anti-DNP mAb was administered. Mice were then re-injected i.p. with mAbs, same dose as above after 12h and antigenic challenge was performed 1h later by i.p. injection of 100 g DNP-HSA. Body temperature monitoring (A). Serum levels of histamine (B), MCP-1/CCL2 (C) MIP-1α/CCL3 (D), RANTES/CCL5 (E) and TARC/CCL17 (F) 2h after Ag challenge. Representative micrographs of H&E-stained lung tissues of mice treated with mock mAb (G) or Sphingomab (H) collected 2h after Ag challenge. (*$p < 0.05$; Student's *t*-test).
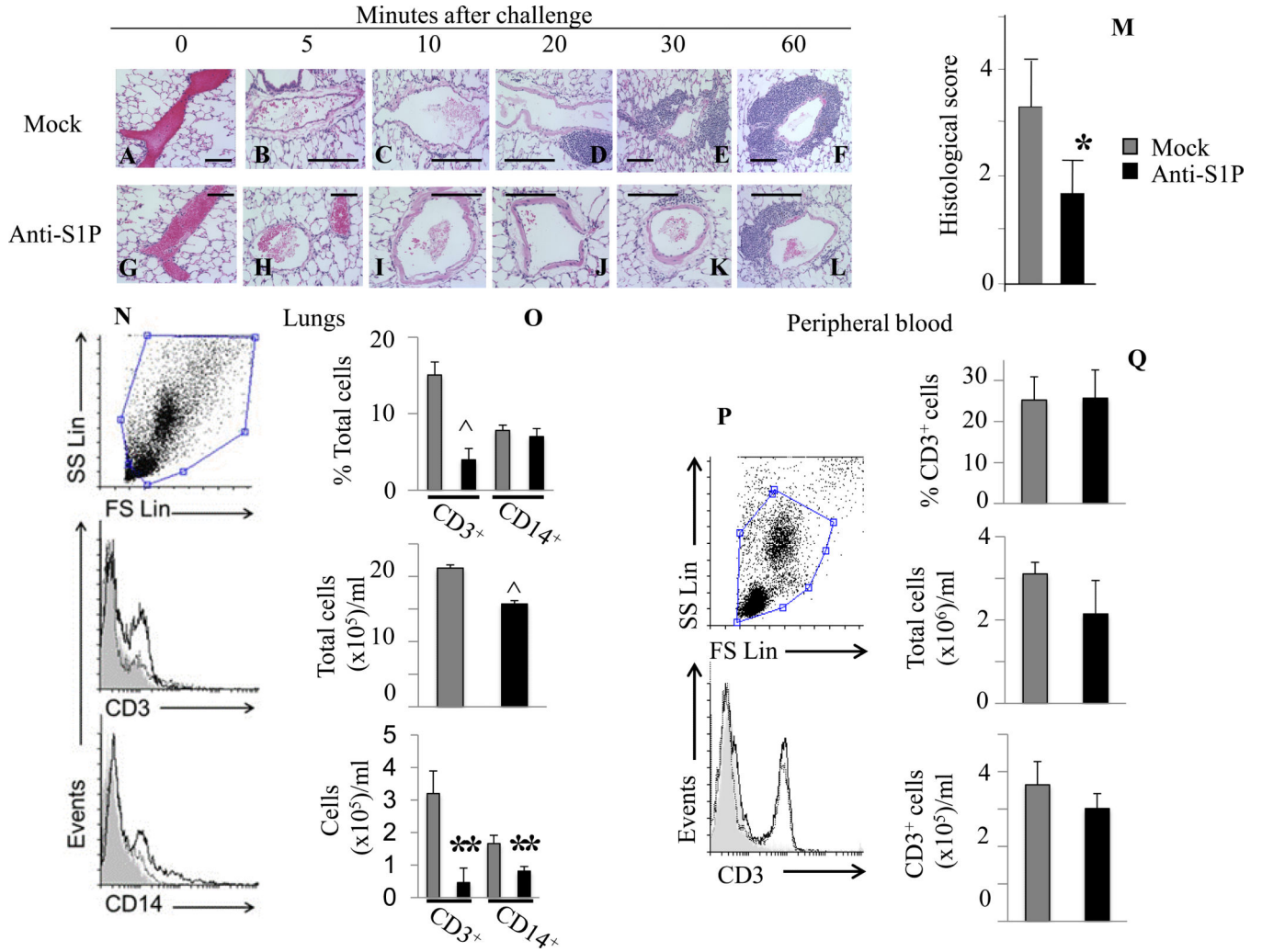
Fig. 3.
Early allergic lung infiltration is mitigated after Sphingomab pretreatment. H&E-stained lung sections of mice pretreated with a mock mAb (A-F) or with Sphingomab (G-L) as described in Fig 2. (M) Infiltration scoring 1h after Ag challenge. (N-O) Flow cytometric analysis and quantification of lung $CD3^+$ and $CD14^+$ cells 2h after Ag challenge. (P-Q) Flow cytometric analysis and quantification of circulating $CD3^+$ cells prior to Ag challenge. Gray histograms represent isotype controls staining, unfilled solid lines depict mock-mAb-treated cell suspensions, and unfilled dotted lines, the anti-S1P-treated cells. Graphs represent percentages of $CD3^+$ or $CD14^+$ cells, total cell, $CD3^+$ or $CD14^+$ cell numbers. (*$p < 0.05$, **$p < 0.01$, ^ $p < 0.0001$; Student's t-test).
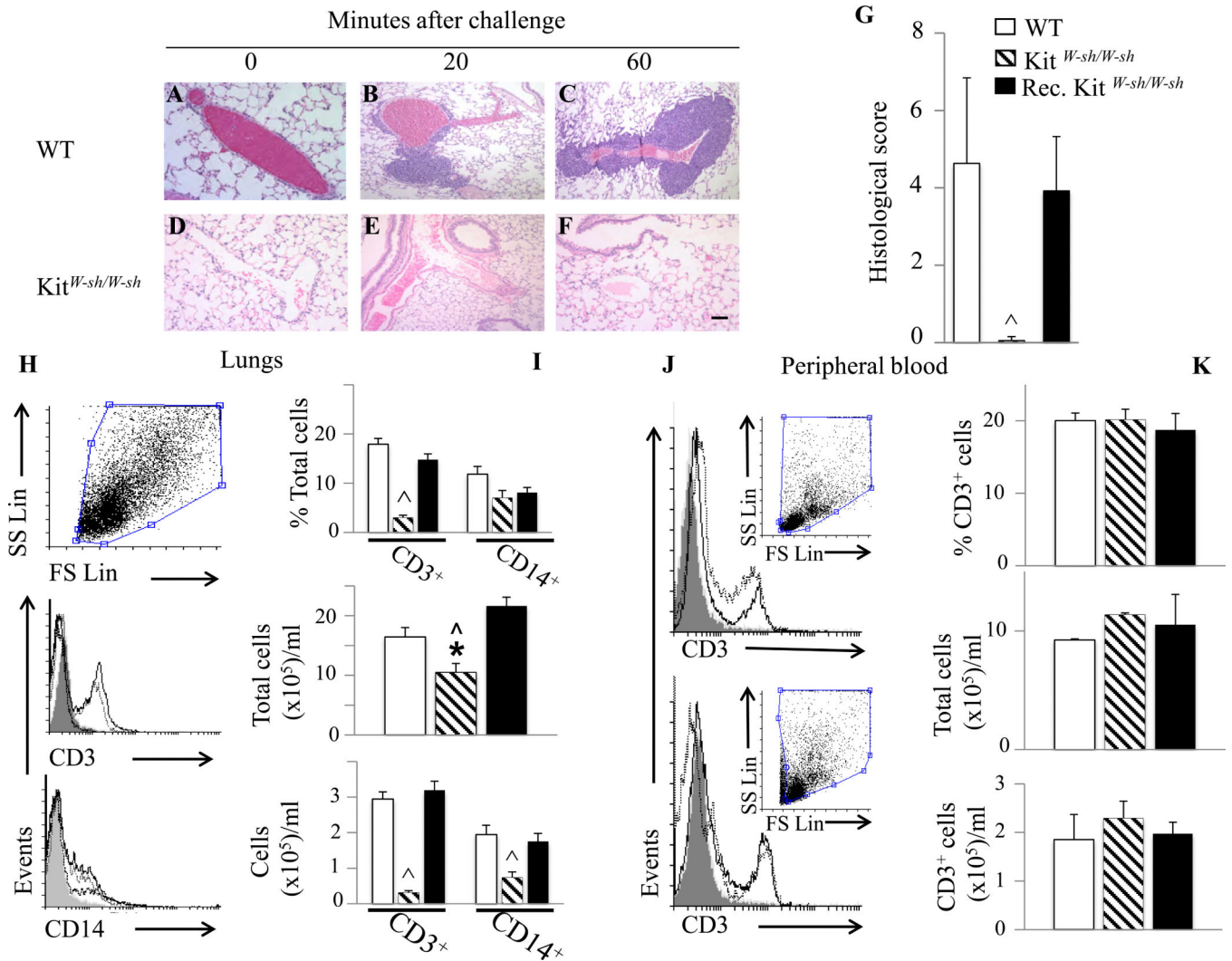
Fig. 4.
Lack of IgE/Ag-induced lung perivascular infiltration in MC-deficient $Kit^{W-sh/W-sh}$ mice and its restoration in MC-reconstituted $Kit^{W-sh/W-sh}$ mice (Rec. $Kit^{W-sh/W-sh}$). (A-F) H&E-stained lung sections of MC-deficient and WT mice at the indicated times after Ag challenge. (G) Infiltration quantification 1h after Ag challenge, (H-I) Flow cytometric analysis and quantification of lung cells 1h after Ag challenge. Gray histograms represent isotype controls staining, unfilled solid lines depict WT cell suspensions, unfilled dotted lines, Rec. $Kit^{W-sh/W-sh}$ cells and unfilled dashed lines $Kit^{W-sh/W-sh}$ cells. Graphs represent percentages of CD3$^+$ or CD14$^+$ cells, total cell, CD3$^+$ or CD14$^+$ cell numbers (J) Flow cytometric analysis (upper panel compares WT (solid lines) to $Kit^{W-sh/W-sh}$ cell suspensions, lower panel compares $Kit^{W-sh/W-sh}$ (solid lines) to Rec. $Kit^{W-sh/W-sh}$ (dotted lines) cells) and (K) quantification of circulating CD3$^+$ cells prior to Ag challenge. (*$p < 0.05$; ^ $p < 0.0001$; one-way ANOVA).
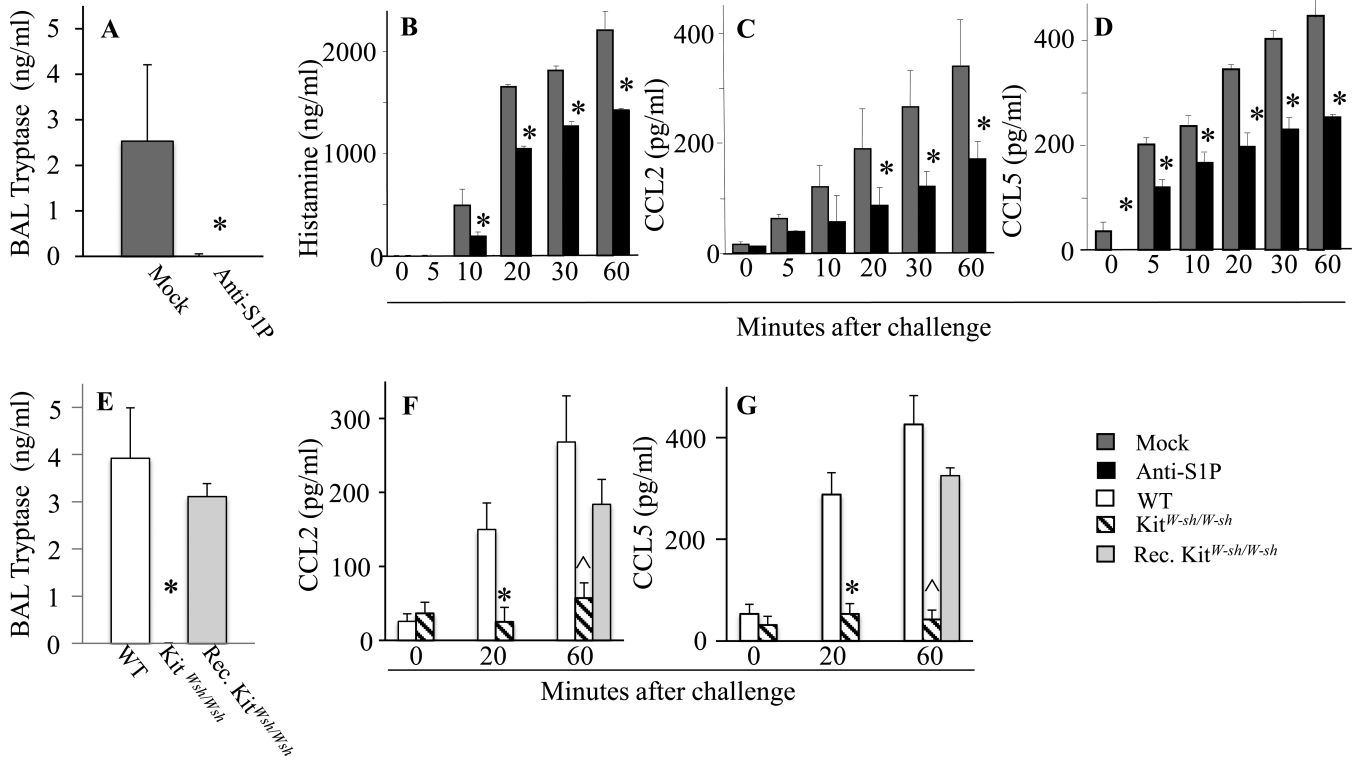
Fig. 5.
Effects of S1P neutralization, MC depletion or reconstitution of MC on circulating levels of proinflammatory mediators after Ag challenge. (A-D) Mice were treated with anti-S1P or mock antibody as described in Fig.2 and (A) BAL tryptase levels determined 2h after Ag challenge. Serum levels of (B) histamine, (C) MCP-1/CCL2, and (D) RANTES/CCL5. (E-G) Sensitized WT, $Kit^{W-sh/W-sh}$ mice or Rec. $Kit^{W-sh/W-sh}$ mice were challenged with Ag. (E) BAL tryptase levels. Serum levels of (F) MCP-1/CCL2 and (G) RANTES/CCL5. n =5-6 mice per group, repeated twice. (*$p < 0.05$, Student's t-test; ** $p < 0.01$, WT versus $Kit^{W-sh/W-sh}$ or Rec. $Kit^{W-sh/W-sh}$ versus $Kit^{W-sh/W-sh}$, one-way ANOVA; ^ $p < 0.0001$, WT or Rec. $Kit^{W-sh/W-sh}$ versus $Kit^{W-sh/W-sh}$, one-way ANOVA).
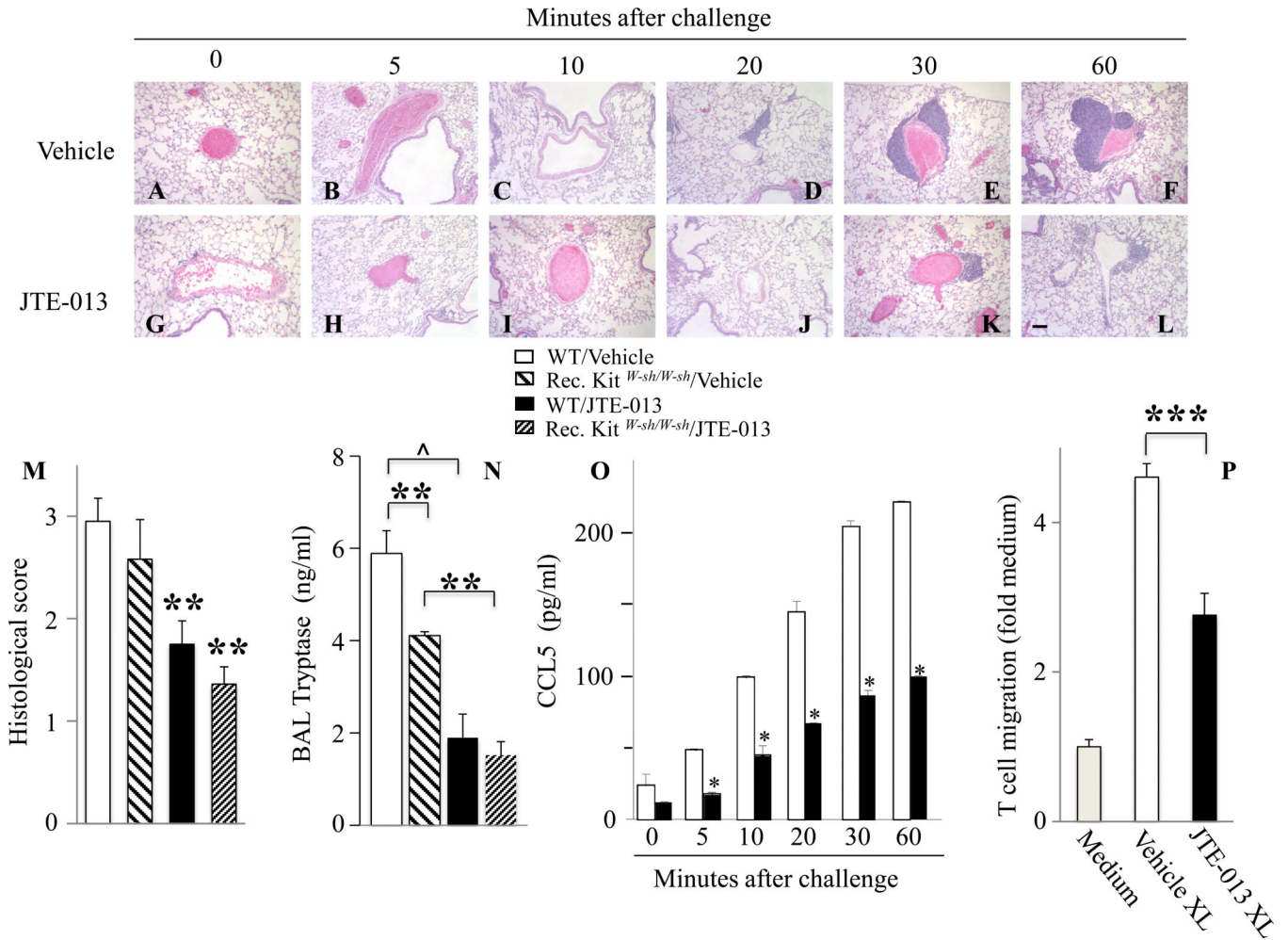
Fig. 6.
JTE-013, a S1PR2 antagonist, decreases allergic lung infiltration. H&E staining of lung sections from mice sensitized with IgE and pretreated with vehicle (A-F) or JTE-013 (G-L) 30 min prior to antigenic challenge. (M) Infiltration scoring 1h after Ag challenge. (N) BAL tryptase levels. (O) Serum RANTES/CCL5 levels (*$p < 0.0005$, Student's *t*-test). (P) Transwell T cell migration assay. (Except in Fig. 6O, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, ^ $p < 0.0001$, one-way ANOVA).
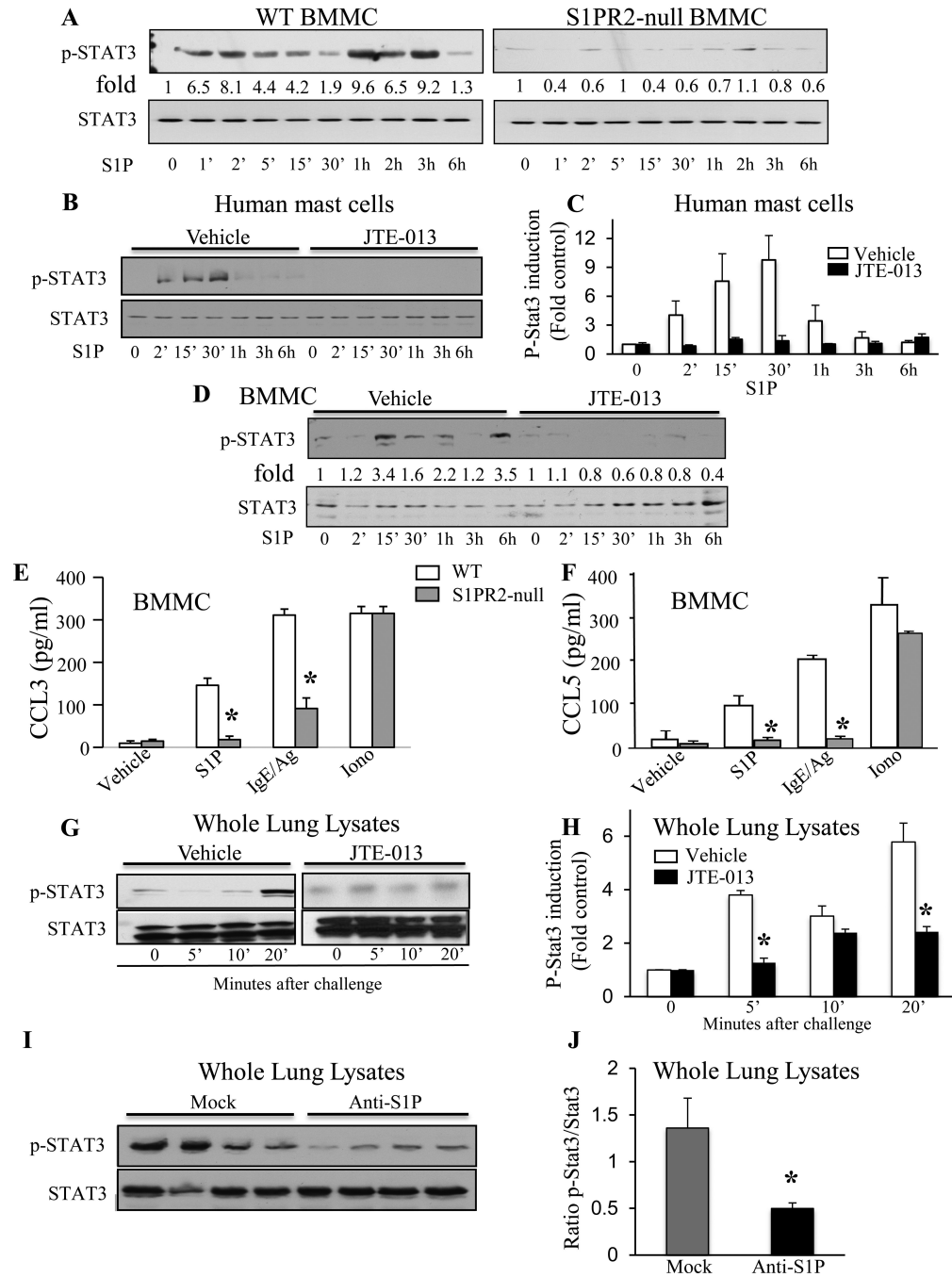
Fig. 7.
Activation of Stat3 by the S1P/S1PR2 axis in MC in vitro and in lungs after Ag challenge. (A) WT or S1PR2-null BMMC or (B-C) vehicle- or JTE-013-pretreated human MC or (D) WT BMMC were stimulated with S1P (100 nM) for the indicated times. Cell lysates were immunoblotted with the indicated Abs and p-Stat3 was quantified. Activated-BMMC supernatants were assayed for (E) CCL3 and (F) CCL5. (G-H) Lung lysates from mice treated with vehicle or JTE-013 prior to IgE/Ag challenge were immunoblotted as above. (I)

Immunoblots of lung lysates from mice treated with anti-S1P or mock mAb prior to IgE/Ag challenge and (J) quantification of pStat3/Stat3 ratios. (*p<0.04, Student's t-test).