

Modulation of Fc ϵ RI-dependent mast cell response by OX40L via Fyn, PI3K, and RhoA

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Background: The interaction of mast cells (MCs) with regulatory T cells through the OX40 ligand (OX40L):OX40 axis downregulates Fc ϵ RI-dependent immediate hypersensitivity responses both *in vitro* and *in vivo*. Little is known on OX40L-mediated intracellular signaling or on the mechanism by which OX40L engagement suppresses MC degranulation. **Objective:** We explored the role of OX40L engagement on IgE/antigen-triggered MCs both *in vitro* and *in vivo*.

Methods: The soluble form of OX40 molecule was used to selectively trigger OX40L on MCs *in vitro* and was used to dissect OX40L contribution in an *in vivo* model of systemic anaphylaxis.

Results: OX40L:OX40 interaction led to the recruitment of C-terminal src kinase into lipid rafts, causing a preferential suppression of Fyn kinase activity and subsequent reduction in the phosphorylation of Gab2, the phosphatidylinositol 3-OH kinase regulatory subunit p85, and Akt, without affecting the Lyn pathway. Dampening of Fyn kinase activity also inhibited RhoA activation and microtubule nucleation, key regulators of MC degranulation. The *in vivo* administration of a blocking antibody to OX40L in wild-type mice caused enhanced immediate hypersensitivity, whereas the administration of soluble OX40 to regulatory T-cell-depleted or OX40-deficient mice reduced MC degranulation.

Conclusions: The engagement of OX40L selectively suppresses Fyn-initiated signals required for MC degranulation and serves to limit immediate hypersensitivity. Our data suggest that soluble OX40 can restore the aberrant or absent regulatory

T-cell activity, revealing a previously unappreciated homeostatic role for OX40L in setting the basal threshold of MC response. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: OX40L, Fyn, PI3K, mast cell, degranulation, anaphylaxis

The induction of an allergic response requires the activation of mast cells (MCs) and is primarily a consequence of allergen-induced aggregation of the IgE-occupied Fc ϵ RI.¹⁻³ Engagement of Fc ϵ RI initiates the activation of the Src kinases Lyn and Fyn, which allows signal propagation through the phosphorylation and activation of several downstream proteins,^{1,2} many of which are recruited into lipid rafts microdomains.⁴ Lyn phosphorylates Syk and the adaptor LAT, which organizes and coordinates further signals, such as the activation of PLC- γ and calcium mobilization.⁵ Fyn initiates a complementary signaling pathway through the adaptor Gab2.^{6,7} This adaptor is essential for the activation of phosphatidylinositol 3-OH kinase (PI3K) and has been shown to be crucial for MC degranulation.⁸ The integration and regulation of the aforementioned signaling pathways is essential toward determining the type, duration, and extent of MC response. However, stimuli from the microenvironment and/or interaction with neighboring cells can impinge on IgE-dependent signaling and can selectively modulate MC responses.^{9,10} Indeed engagement of MC costimulatory molecules such as leukocyte-function-associated antigen 1,¹¹ CD226,¹² 4-1BB,¹³ and OX40 ligand (OX40L)¹⁴ may confer either positive or negative effects on MC effector responses. Our recent finding of a bidirectional functional interaction between regulatory T (Treg) cell-expressed OX40 (CD134) and MC-expressed OX40L (CD134L), which inhibited the extent of Fc ϵ RI-dependent MC degranulation both *in vitro* and *in vivo*,¹⁵ is an example of such regulatory control. While OX40 signaling in T cells has been extensively explored,^{16,17} there is little knowledge on signals generated by OX40L that promote its biological activity. This knowledge may be limited, because of potential signal cross-contamination caused by the binding of OX40L antibodies to the Fc γ receptors present on the surface of many immune and other cell types.¹⁸ In addition, OX40L-triggering antibodies are lacking. We recently described a soluble form of OX40 (sOX40) molecule, consisting of OX40 extracellular domain,¹⁹ that overcomes these limitations and mimics the Treg-cell-suppressive effect on MC degranulation. By using this unique reagent, we investigated whether OX40L engagement may influence IgE/Ag-dependent signaling events in bone marrow (BM)-derived MCs, focusing on both Lyn- and Fyn-mediated pathways. Since the loss of Treg cells

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Abbreviations used

BM:	Bone marrow
Cbp:	C-terminal Src kinase-binding protein
Csk:	C-terminal Src kinase
DNP:	Di-nitro-phenyl
MC:	Mast cell
MTOC:	Microtubule organizing center
NIH:	National Institutes of Health
OX40L:	OX40 ligand
PIP ₃ :	Phosphatidylinositol 3,4,5-trisphosphate
PI3K:	Phosphatidylinositol 3-OH kinase
RhoA KD:	RhoA knocked-down
sOX40:	Soluble OX40
<i>Tnfrsf4</i> -deficient:	OX40-deficient
Treg:	Regulatory T
WT:	Wild type

causes a marked enhancement in anaphylaxis, the effect of sOX40 was also tested in an *in vivo* model of passive systemic anaphylaxis. Our findings demonstrate that OX40L-mediated suppression of Fyn signaling underlies the dampening of FcεRI-dependent degranulation.

METHODS**Mice and reagents**

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Me) and were used in accordance with the animal study proposal A010-04-03 approved by the National Institutes of Health (NIH) and the National Institute of Arthritis, Musculoskeletal and Skin Diseases. C57BL/6 *Tnfrsf4*-deficient (OX40-deficient) mice were from Nigel Killeen (University of California, San Francisco). DNP-specific IgE was previously described.²⁰ Di-nitrophenyl (DNP)-human serum albumin (Ag) was purchased from Sigma Aldrich (St Louis, Mo).

sOX40 generation and purification

OX40-IgG₁ fusion protein was provided by Genentech (San Francisco, Calif), and the sOX40 was purified and used as previously described.¹⁹

BM-derived MC differentiation, activation, and degranulation assay

BM-derived MCs were obtained through *in vitro* differentiation of BM precursors as described in Frossi et al.²¹ Cell activation and degranulation assays were described in Gri et al.¹⁵

Antibodies, cell lysis, and immunoprecipitation

Antibodies to phospho-Syk (pY343), phospho-LAT (pY191), phospho-Gab2 (pY452), phospho-PI3K p85 (pY458), phospho-Akt (T308), phospho-SHIP-1 (pY1020), phospho-Src (pY416 and pY527), PI3K p85, phospho-tyrosine (pY100), and RhoA were purchased from Cell Signaling Technology (Danvers, Mass); antibodies to Syk, LAT, C-terminal Src kinase (Csk)-binding protein (Cbp), and Csk were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif); antibodies to Gab2 were purchased from Millipore (Billerica, Mass), whereas antibodies to Akt and actin were purchased from BD Biosciences (San Jose, Calif). Antibody to OX40L was provided by R&D Systems (Minneapolis, Minn), and horseradish peroxidase-conjugated Cholera toxin Subunit B (used for GM1 detection) was purchased from Sigma Aldrich. Antibody to Fyn was produced from the laboratory of J.R. as previously described.²² Antibody to FcεRI-γ (pY47) was obtained from mice immunized with the peptide DAVpYTGLNTRSQET. BM-derived MC lysates (20 × 10⁶

cells) were prepared as reported in Sibilano et al.¹⁹ For immunoprecipitation, antibodies were incubated with GammaBind Plus Sepharose (GE Healthcare, Waukesha, Wis) for 3 hours at 4°C and after washing the lysate was added for 3 hours at 4°C. Densitometric quantification was performed by using ImageJ software (NIH). Where indicated, numbers indicate fold-induction, normalized to total amount of protein or to the immunoprecipitated protein.

Assay for PI3K activity

PI3K activity was measured by ELISA (Echelon Biosciences, Salt Lake City, Utah). Lysates of activated BM-derived MCs (20 × 10⁶) were incubated with antibody (Cell Signaling) to the p85 subunit of PI3K for 2 hours at 4°C and thereafter with GammaBind Plus Sepharose (GE Healthcare) for 2 hours at 4°C. Immunoprecipitated PI3K was subjected to an *in vitro* kinase assay for 2 hours at 37°C, according to the manufacturer's instructions. Activity was measured as amount of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) produced by PI3K in 1 hour at 37°C.

Lipid raft isolation

BM-derived MCs (20 × 10⁶) were lysed with 1% Triton X-100 in TNE/P buffer (25 mM Tris/HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL pepstatin) for 20 minutes at 4°C. Lysates were mixed with ice-cold 80% sucrose in TNE/P buffer and then overlaid onto ice-cold 35% and 5% sucrose in TNE/P buffer and centrifuged at 180000g for 18 hours at 4°C. After centrifugation, ~300-μL aliquots were harvested and diluted in equal volume of SDS sample buffer. Western blot densitometric analysis was performed by using ImageJ software (NIH). Protein content was normalized to GM1. Fold induction of IgE/Ag + sOX40-stimulated BM-derived MCs over IgE/Ag-stimulated BM-derived MCs was calculated.

BM-derived MC fixation, α-tubulin staining, and microscopy

IgE-prensensitized BM-derived MCs (1 × 10⁶) were resuspended in Tyrode's buffer and plated on polylysine-coated microscopy borosilicate chambers (Nunc International, Penfield, NY) for 30 minutes at 37°C. At 2 and 5 minutes after Ag and sOX40 addition, attached cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. Chambers were washed with PBS, and 0.2% Triton X-100 was added for 30 minutes at room temperature. Mouse anti-α-tubulin-AlexaFluor488 (Invitrogen, Carlsbad, Calif) (5 μg/mL) was added to the permeabilized cells and incubated for 2 hours at room temperature. Excitation of AlexaFluor dye was by a 488-nm argon ion-laserline, and fluorescence was recorded through an emission filter (LP505; Carl Zeiss MicroImaging, Inc, Jena, Germany). ImageJ software (NIH) was used to generate pseudo-color images and to calculate fluorescence intensities in BM-derived MC cross-sections, identifying microtubule organizing centers (MTOCs) where present. The analysis was conducted for 3 representative cells in each field and represents at least 2 independent experiments.

RhoA activity assay

IgE-sensitized BM-derived MCs (20 × 10⁶) were stimulated with 50 ng/mL Ag with or without 20 μg/mL sOX40 for 2 minutes. Cells were lysed, and the amount of GTP-complexed RhoA was measured according to the manufacturer's instructions (Millipore).

Systemic anaphylaxis

Wild-type (WT) or OX40-deficient C57BL/6 mice were sensitized with 3 μg of DNP-specific mouse IgE and, when indicated, with 150 μg OX40L-blocking antibody (RM134L; BioXcell, West Lebanon, NH), isotype control (LTF-2, BioXcell) (n = 9), or sOX40 (n = 9) intravenously.

Twenty-four hours later, mice were challenged with PBS (n = 3, mock) or 0.3 mg Ag (n = 6).

One and a half minutes after Ag challenge, mice were euthanized and blood was collected by cardiac puncture for histamine ELISA quantitation (Immunotech, Prague, Czech Republic).

In some experiments, Treg cells were depleted by injection of 0.5 mg/0.2 mL of anti-CD25 (clone PC61, rat IgG₁, ATCC, LGC) 7 and 8 days prior to the induction of systemic anaphylaxis (*n* = 3 for mock; *n* = 5 for Ag).

Statistical analysis

Data are expressed as mean $\pm$ SEM and were analyzed with a Student *t* test for statistical significance (Prism; GraphPad Software, La Jolla, Calif).

RESULTS

OX40L engagement impairs Fc ϵ RI activation and Gab2/PI3K/Akt, but not Syk/LAT, phosphorylation

The effect of OX40L engagement on Fc ϵ RI-mediated signaling early events was investigated in IgE/Ag-activated BM-derived MCs, in the absence or presence of sOX40. Phosphorylation of pY47 in Fc ϵ RI- γ subunit was $\sim$ 50% reduced 2 and 5 minutes after Ag triggering in the presence of sOX40 (Fig 1, A). This was followed by no significant alterations in Lyn-supported phosphorylation of Syk (Y323) or LAT (Y191) on OX40L engagement (Fig 1, B). In contrast, Fyn-mediated phosphorylation of Gab2 (Y452), PI3K p85 regulatory subunit (Y458), and Akt (T308) revealed a 2.5- to 10-fold decrease within 5 minutes after Ag triggering in the presence of sOX40 (Fig 1, C, left and middle panels). Phosphorylation of the lipid phosphatase SHIP-1 (Y1020) was increased (>2 -fold) when cells were stimulated via Fc ϵ RI in the presence of sOX40 (Fig 1, C, left and middle panels). IgE-sensitized cells incubated with sOX40, but not activated by Ag, showed no induction or alterations in the phosphorylation of these proteins (Fig 1, C, right panel), indicating that signals derived from OX40L engagement were observed only on engagement of Fc ϵ RI.

Given that Fyn is essential for the activation of PI3K,⁷ we measured the production of PIP₃ by immunoprecipitated PI3K in Fc ϵ RI-triggered BM-derived MCs in the presence or absence of sOX40. As shown in Fig 1, D, at 5 minutes post-Fc ϵ RI stimulation, treatment with sOX40 caused a reduction in the amount of PIP₃ generated (from 7.1 ± 0.4 to 3.8 ± 0.2 nM; *P* = .020) when normalized to the amount of immunoprecipitated PI3K (Fig 1, E). At 10 minutes poststimulation, a further reduction from 9.3 ± 1.2 to 2.7 ± 0.7 nM (*P* = .041) was observed. Collectively, these findings showed that OX40L engagement preferentially dampens Fyn-initiated, but not Lyn-initiated, signals, causing a major impact on PIP₃ regulation as shown by the impairment of PI3K activation and by the concomitant increase in SHIP-1 (Y1020) phosphorylation, which is a surrogate marker of its activity.

The administration of sOX40 to IgE/Ag-activated BM-derived MCs significantly reduced the degranulation of WT and Lyn-deficient but did not significantly modify Fyn-deficient BM-derived MCs response (Fig 1, F). Consistently, the addition of sOX40 to cocultures of both WT and Lyn-deficient (but not Fyn-deficient) BM-derived MCs:OX40-deficient Treg cells restored Treg-cell inhibition of degranulation.

The here reported evidence on MC degranulation confirms that OX40L engagement selectively affects Fyn pathway activation, since Lyn-deficient (but not Fyn-deficient) MCs exhibited reduced response to Ag stimulation in the presence of WT Treg cells, sOX40, or OX40-deficient Treg cells together with sOX40.

sOX40 causes Fyn inactivation through enhanced Cbp phosphorylation, Csk recruitment, and direct OX40L:Csk interaction

The activity of Src kinases is regulated by intramolecular interactions depending on tyrosine phosphorylation and dephosphorylation at Y416 and Y527 (positive and negative regulatory tyrosines, respectively).²³ To evaluate the effect of OX40L engagement on the activation of Fyn, we immunoprecipitated Fyn at different time points after Ag addition in the absence or presence of sOX40 (Fig 2, A). The amount of phosphorylated Y416 and Y527, which jointly determines the amount of active Src kinase,¹ revealed that Fyn Y416 phosphorylation was decreased in the presence of sOX40. Concurrently, there was increased phosphorylation at the negative regulatory Y527 of Fyn (Fig 2, A and B).

Y527-Fyn phosphorylation is mediated by the recruitment of Csk to the plasma membrane through its interaction with the scaffolding adaptor Cbp following its phosphorylation.^{2,18} Thus, we explored whether OX40L-dependent signaling caused enhanced Cbp phosphorylation and Csk recruitment. Immunoprecipitation of Cbp revealed that its phosphorylation was enhanced in the presence of sOX40 (Fig 2, C). Interestingly, the amount of coimmunoprecipitated Csk was also enhanced by more than 2-fold compared with the levels of Csk coimmunoprecipitated with Cbp in the absence of sOX40 (Fig 2, C and D). On OX40L engagement, physical interaction between OX40L and Csk occurred. Two minutes after Ag/sOX40 costimulation, Csk associated with OX40L as shown in immunoprecipitation assay (Fig 2, E).

OX40L triggering modifies lipid raft organization and causes OX40L and Csk enrichment into raft fractions

Since Csk and Fyn have both been reported to be associated with lipid rafts on MC activation,^{24,25} we investigated whether combined OX40L and Fc ϵ RI stimulation of BM-derived MCs could cause changes in the molecular content of lipid rafts. Following Fc ϵ RI stimulation (2 minutes), a small amount of Csk was detected in lipid raft fractions; however, most Csk localized outside these domains (Fig 3, A). On the simultaneous engagement of OX40L and Fc ϵ RI (2 minutes), a 4-fold increase in Csk in the lipid raft fraction over activated BM-derived MCs in the absence of sOX40 was observed (Fig 3, A, histogram). This was mirrored by the presence of increased Y527 phosphorylation in the lipid raft fractions 2 minutes poststimulation, whereas reduced Y527 phosphorylation was present in lipid rafts in the absence of sOX40 (Fig 3, B). Consistent with the data shown in Fig 2, A, the band corresponding to phospho-Src Y527 (3-fold induction, Fig 3, B, histogram) was suggestive of Fyn, as we previously showed that Fyn is recruited into lipid rafts on Fc ϵ RI stimulation.²⁴

Moreover, OX40L engagement caused a marked relocation of OX40L itself into lipid rafts. As shown in Fig 3, C, and histogram, a $\sim$ 10-fold increase in OX40L into raft portions was observed following treatment with sOX40.

sOX40 impairs RhoA activation and microtubule nucleation and formation

Microtubule formation and nucleation are required for MC degranulation and depend on signals generated through

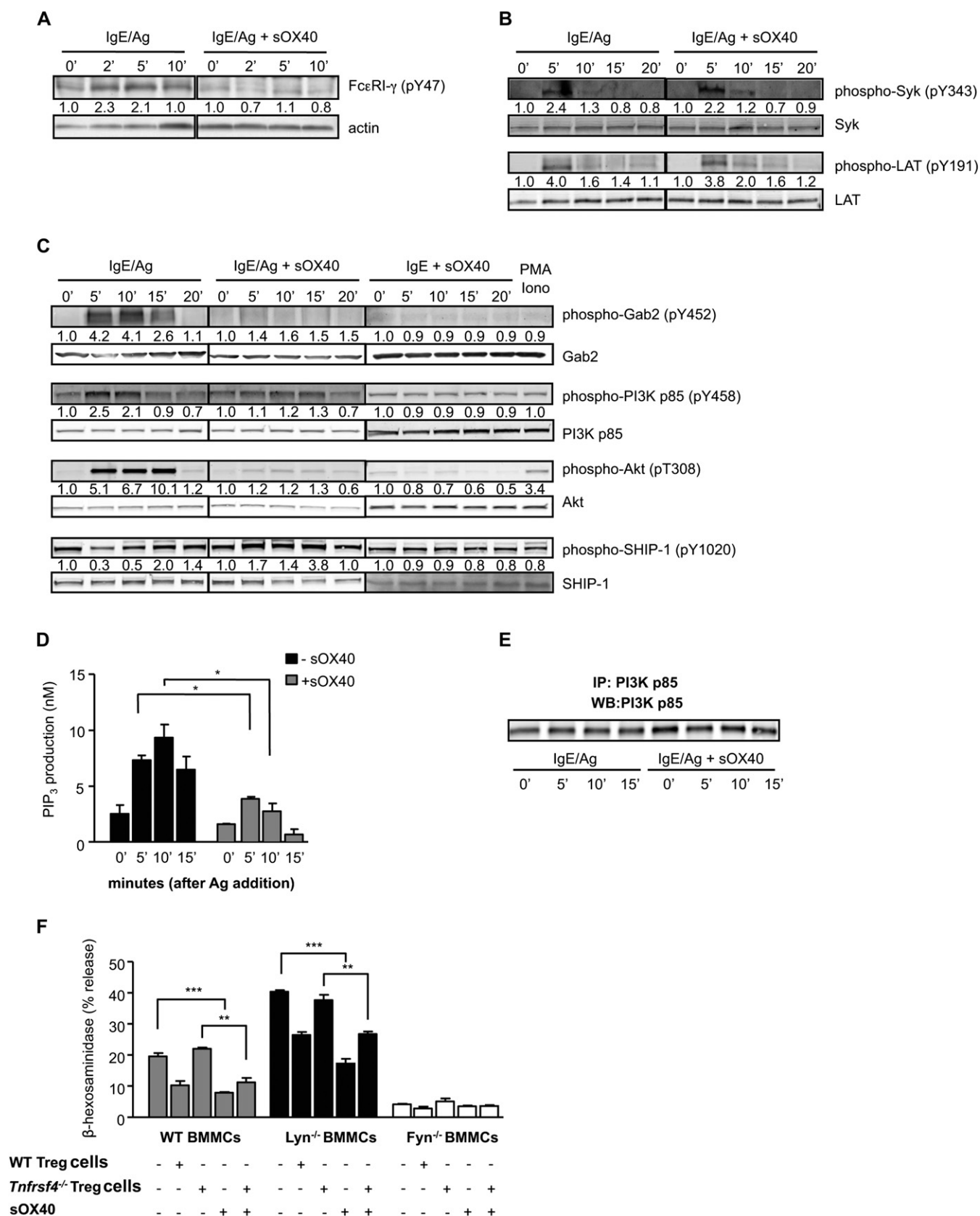


FIG 1. OX40L engagement selectively affects the Fyn pathway. Time-course analysis of FcεRI-γ (**A**); Syk and LAT (**B**); and Gab2, PI3K, Akt, and SHIP-1 (**C**) phosphorylation. Numbers indicate fold induction relative to 0 minute. One of the 3 experiments is shown. **D**, Time-course PIP₃ production. Data represent 1 of 3 independent experiments, each performed in duplicate. **E**, WB analysis of eluted PI3K for conditions shown in Fig 1, **D**.

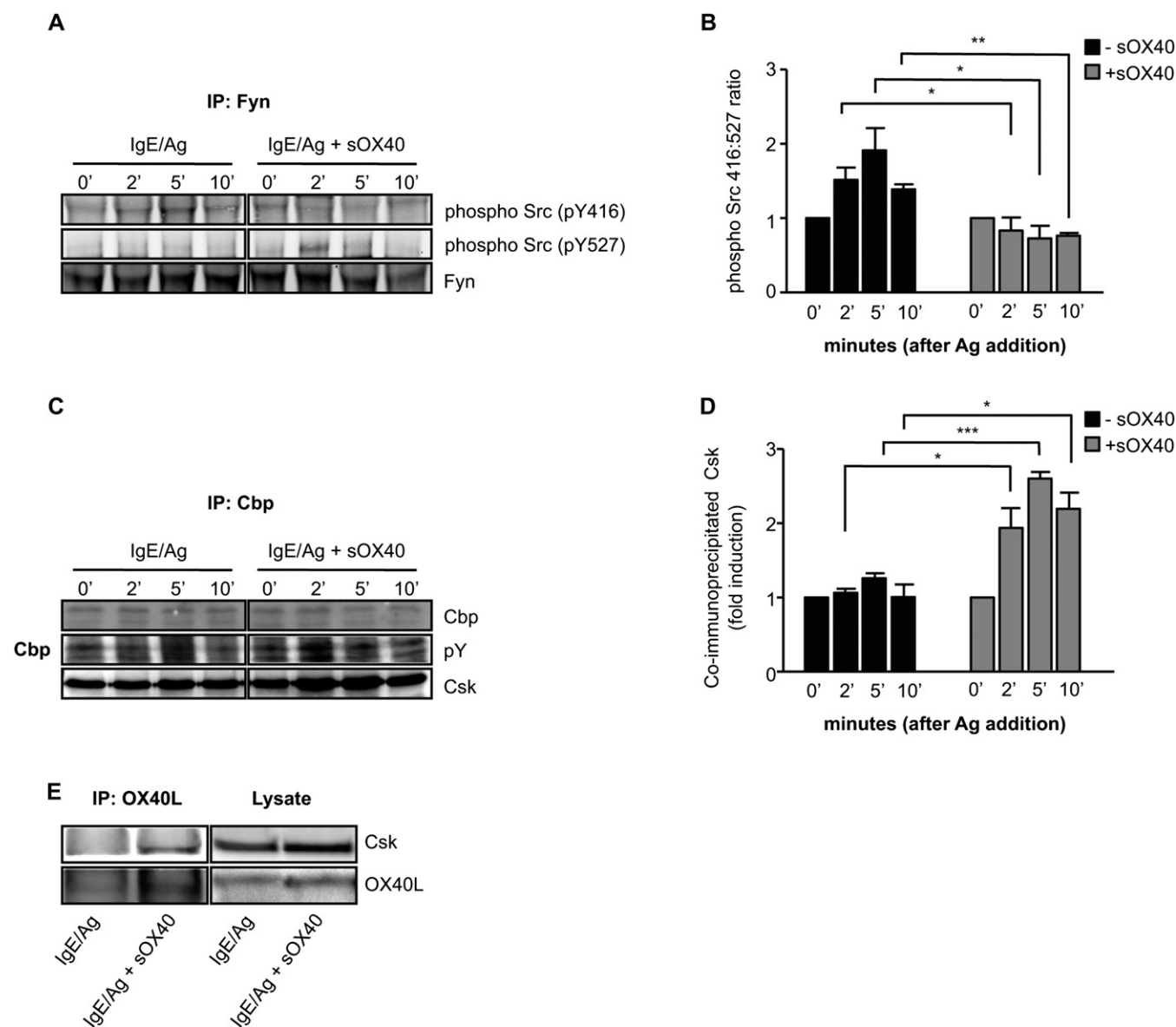


FIG 2. Effect of sOX40 on Fyn, Cbp, and Csk activation. **A**, Immunoprecipitated (IP) Fyn probed for Src pY416, pY527, and total Fyn. **B**, Densitometric quantification of SrcY416:Y527 ratio from 3 independent experiments. **C**, IP Cbp resolved and probed for tyrosine phosphorylation or for Csk. **D**, Densitometric quantification of coimmunoprecipitated Csk from 3 independent experiments. **E**, IP OX40L resolved and probed for Csk in lysed BM-derived MCs 2 minutes after Ag stimulation. * $P < .05$; ** $P < .01$; *** $P < .001$.

Fyn and Gab2.^{26,27} Thus, we explored the possibility that OX40L-generated signals might impinge on the formation of microtubules and MTOCs. Formation of microtubule and MTOC in BM-derived MCs challenged with Ag in the absence or presence of sOX40 is shown in Fig 4, A. In resting BM-derived MCs (Fig 4, A, upper left panel [0']), polarized microtubules and MTOCs were readily observed (measured as the fluorescence intensities in the cross-sections). While some microtubule reorganization was apparent after Ag challenge, MTOCs and microtubules were readily

recognizable (middle left [2'] and lower left [2'] images and graphs). On treatment of resting BM-derived MCs (upper right panel [0'] image and graph) with sOX40, no effect on the formation of microtubules and MTOCs was observed. In contrast, Ag stimulation in the presence of sOX40 led to the apparent disruption of the formation of microtubules and MTOCs as revealed by a less structured or polarized microtubule distribution and the absence of an apparent MTOC (middle right [2'] and lower right [5'] images and graphs). Indeed, most of the α -tubulin

F, β -hexosaminidase release in WT, Lyn-deficient, and Fyn-deficient BM-derived MCs. Data are from 3 independent experiments. IP, Immunoprecipitated; PMA, Phorbol 12-myristate 13-acetate. * $P < .05$; ** $P < .01$; *** $P < .001$.

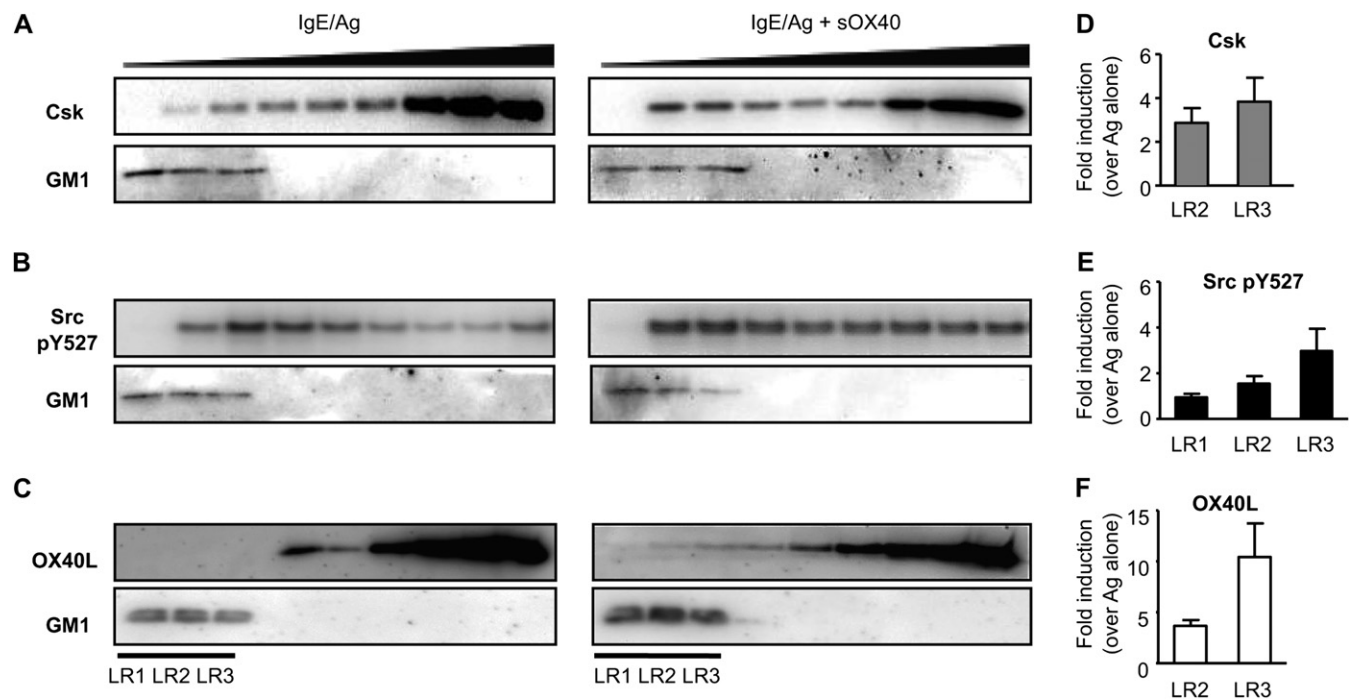


FIG 3. sOX40 treatment relocalizes Csk, Src pY527, and OX40L into lipid rafts. **A**, Fractions of sucrose gradient analyzed for Csk (**A**), pY527 (**B**), and OX40L (**C**). One of the 3 representative WB is shown. **D-F**, Fold induction of GM1-normalized proteins from IgE/Ag + sOX40-activated BM-derived MCs (2 minutes) over IgE/Ag-activated BM-derived MCs (2 minutes) in indicated lipid raft fractions (LR1, LR2, and LR3). Results were calculated from 3 independent experiments.

appeared to be scattered around the nucleus with apparently few microtubule tracks aligning toward the cell membrane.

To understand the mechanism underlying the impaired microtubule nucleation and formation, we investigated the effect of OX40L engagement on the activation of the small GTPase RhoA, known to drive tubulin polymerization in MCs.^{27,28} As shown in Fig 4, B, FcεRI-stimulated MCs in the presence of sOX40 revealed an approximate 2-fold decrease in the GTP-bound form of RhoA. To further confirm the role of RhoA in OX40L-mediated impairment of MC degranulation, RhoA was selectively silenced by lentiviral infection (see Fig E1 in this article's Online Repository at www.jacionline.org). RhoA knocked-down (RhoA KD) BM-derived MCs were tested for degranulation. A significant impairment in β-hexosaminidase release was observed in RhoA KD BM-derived MCs (WT: 52.5% ± 0.2%; RhoA KD: 37.2% ± 2.1%; *P* = .018) (Fig 4, C). Interestingly, while the treatment of FcεRI-stimulated WT BM-derived MCs with sOX40 caused a marked reduction in degranulation (from 52.5% ± 0.2% to 38.0% ± 1.9%; *P* = .017), the treatment of RhoA KD BM-derived MCs with sOX40 did not reveal a further significant decrease (36.0% ± 1.2%; *P* = .661).

Preventing OX40:OX40L axis functionality worsens anaphylaxis and sOX40 administration ameliorates the response when the OX40:OX40L axis is interrupted

To date, all available antibodies to OX40L act to inhibit OX40L signal transduction. Indeed, as shown in Fig 5, A, the use of anti-OX40L (clone RM134, BioXcell) prevented OX40:OX40L axis functionality, enhancing levels of circulating histamine compared

with controls (3317 ± 93 vs 2752 ± 148 ng/mL; *P* = .009). Thus, to investigate the *in vivo* consequence of OX40L stimulation during systemic anaphylaxis, we injected mice with sOX40, as we previously demonstrated that sOX40 is able to interact with MCs on *in vivo* injection.¹⁹ Unexpectedly, when compared with untreated controls, treating mice with sOX40 did not dampen the anaphylactic response (*P* = .447; Fig 5, B). We reasoned that the presence of other OX40-expressing cells (primarily Treg cells) might mask the effect of sOX40. Hence, we disrupted the *in vivo* OX40:OX40L cross-talk by injecting PC61 anti-CD25 Treg-cell-depleting antibody.^{29,30} On average, Treg cells were depleted to 40% of their normal level (see Fig E2, A, in this article's Online Repository at www.jacionline.org). When challenged, Treg-cell-depleted mice showed a more severe anaphylactic response as measured by circulating histamine levels (WT: 1976 ± 275 vs PC61-treated: 3221 ± 298 ng/mL; *P* = .015; Fig 5, B). Strikingly, the administration of sOX40 to Treg-cell-depleted mice fully restored the less severe anaphylactic response to the levels observed in WT mice (decreasing from 3221 ± 298 to 1680 ± 305 ng/mL; *P* = .007). To demonstrate that this effect was indeed a consequence of OX40 administration, we performed a systemic anaphylactic challenge of OX40-deficient mice with or without sOX40 treatment. As shown in Fig 5, C, sOX40 treatment of OX40-deficient mice caused a marked reduction in *in vivo* histamine release (from 2094 ± 286 to 1051 ± 112 ng/mL; *P* = .009). In these mice, sOX40 administration also reduced hypothermia following Ag exposure (Fig E2, B), mirroring the response observed in the experiments of *in vivo* histamine quantitation (Fig 5, C). Thus, the administration of sOX40 restores the suppressive effect on MC degranulation mediated by Treg-cell OX40-dependent engagement of MC-OX40L.

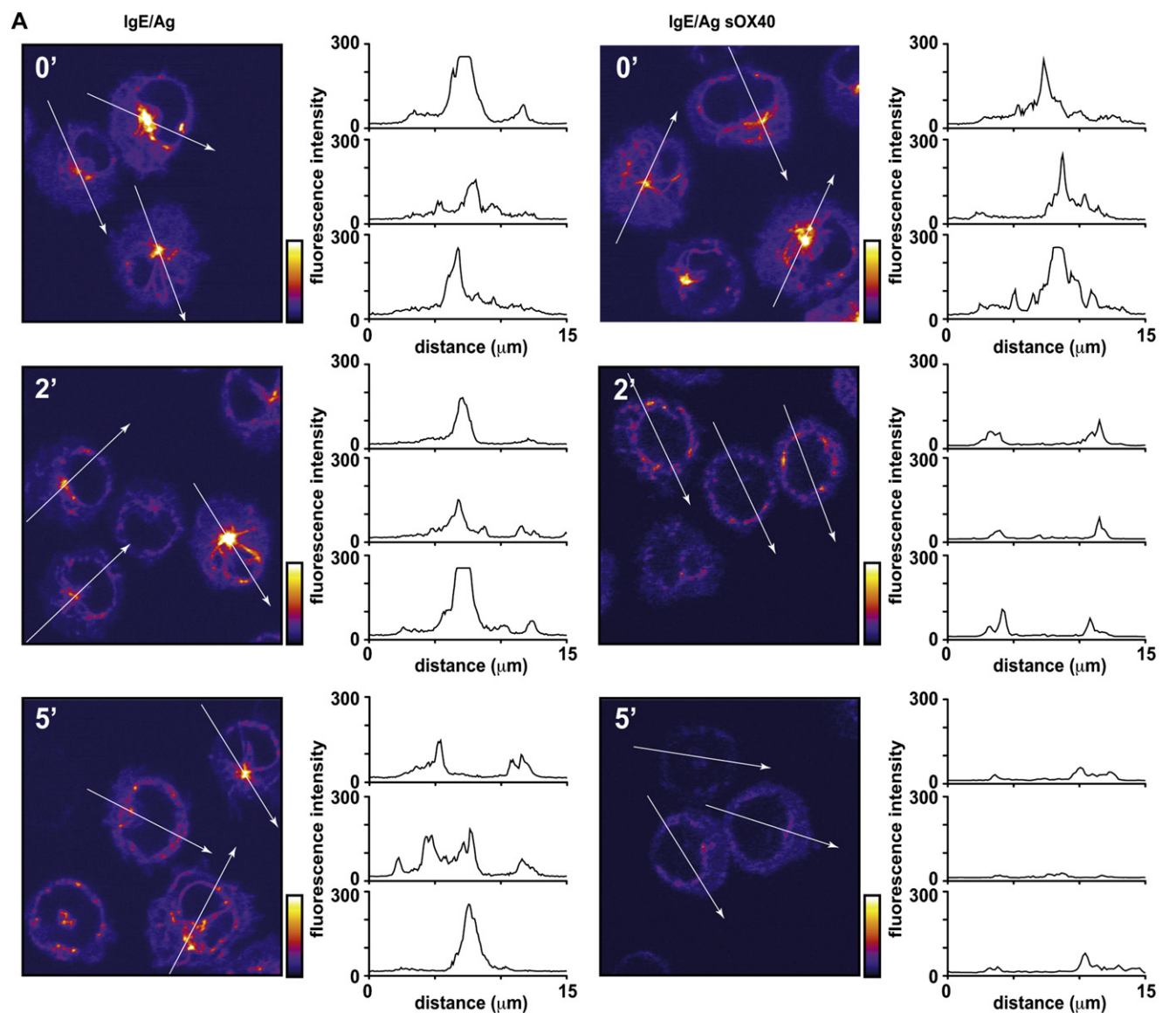


FIG 4. Microtubule organization is impaired on OX40L engagement. **A**, Pseudo-color images of BM-derived MCs stained for α -tubulin with reported fluorescence intensities in the cross-sections (arrows = 15 μ m). **B**, RhoA-pull-down in the presence or absence of sOX40. One of the 3 experiments is shown. Numbers indicate fold induction normalized to total RhoA. **C**, β -Hexosaminidase release in activated WT and RhoA KD BM-derived MCs from 2 independent experiments. * $P < .05$.

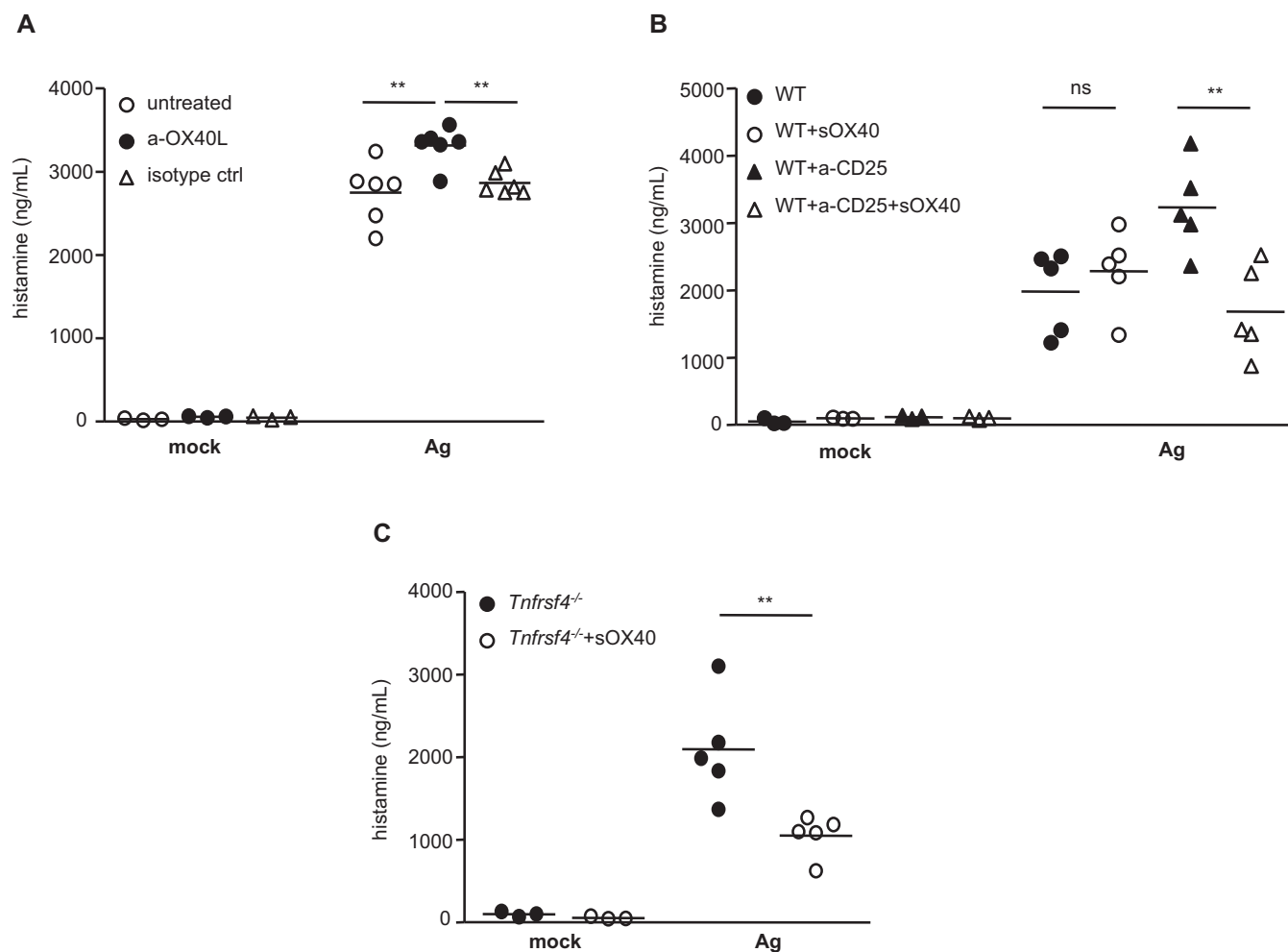


FIG 5. Consequences of OX40L engagement in passive systemic anaphylaxis. Plasma histamine levels measured in Ag-challenged (or PBS-treated, mock) WT mice pretreated with anti-OX40L antibody or isotype control (**A**); in WT mice and Treg-cell-depleted mice (anti-CD25), untreated or treated with sOX40 (**B**); in OX40-deficient mice, untreated or treated with sOX40 (**C**). *ctrl*, Control; *ns*, not significant. ** $P < .01$.

DISCUSSION

Evidence of the OX40:OX40L axis as a crucial element for the maintenance of immunological tolerance is rapidly accumulating. Interference with this molecular interplay has been connected to the development of a proinflammatory microenvironment characterized by MC-dependent hyperresponsiveness, atopy, and autoimmunity in the host.^{31,32}

In the present work, we demonstrate that OX40L engagement blunts FcεRI-dependent MC activation in a manner that has not previously been appreciated.

MC costimulation with IgE/Ag and sOX40 causes a down-activation of FcεRI and a deep reorganization of lipid rafts. We observe reduced phosphorylation of Tyr47 in the immunoreceptor tyrosine-based activation motif of FcεRI-γ subunit and the subsequent and selective modulation of the Fyn pathway, in the absence of a marked impairment of Lyn-dependent signals. It was previously shown that the phosphorylated γ chain at Tyr47 is a weak binding site for Syk and that phosphorylation of Tyr58 alone was sufficient to cause Syk activation in MCs.³³ In addition, both sOX40 and/or Treg-cell-reduced degranulation of Lyn-deficient

BM-derived MCs strengthens the evidence that OX40L signaling negatively regulates Fyn.

sOX40 promotes OX40L relocation in plasma membrane and its enrichment into lipid rafts. Concomitantly, OX40L triggering influences the Cbp:Csk complex, favoring the hyperphosphorylation of Cbp and the consequent enhancement of Csk recruitment. These results are enforced by the evidence of direct interaction between OX40L and Csk that occurs in the presence of sOX40 in activated BM-derived MCs (Fig 2, E, and Fig 6, A).

As previously described,¹⁸ the recruitment of Csk to the plasma membrane is required for the phosphorylation of Y527 on Fyn, which leads to the intramolecular interaction of the phosphorylated Y527 with the SH2 domain of Fyn (closed conformation), impairing its open or active conformation. The recruitment of Csk to lipid raft localized Cbp normally following FcεRI engagement and is likely required for the appropriate regulation of Fyn activity in MCs. This view is supported by previous findings^{24,25} in which *Lyn*-deficient MCs or selective removal of Lyn from lipid rafts led to deficiencies in Cbp phosphorylation, decreased Csk

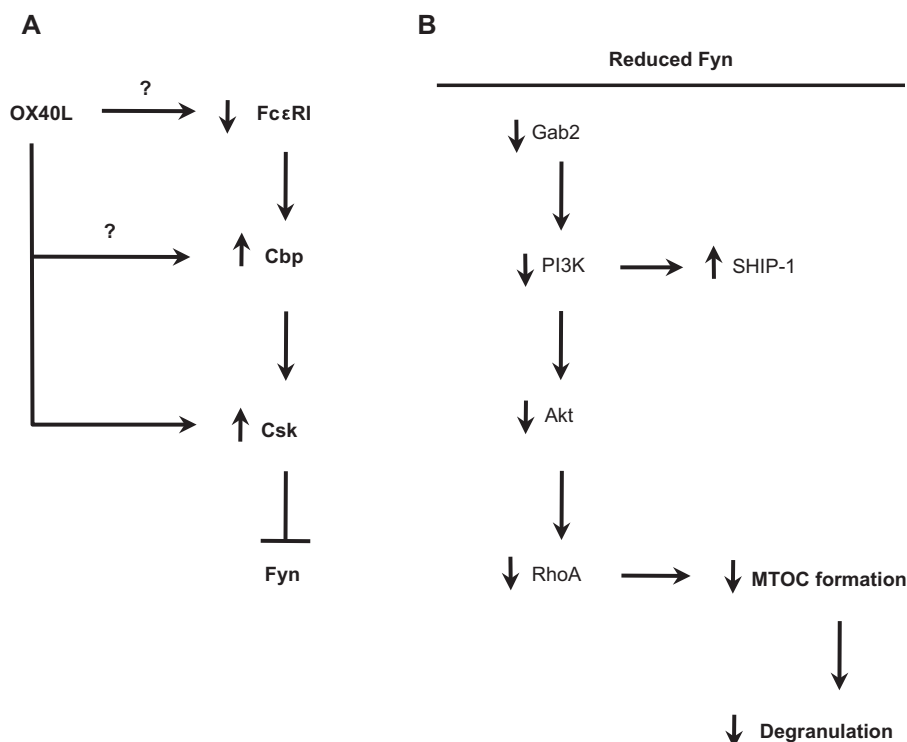


FIG 6. Model of OX40L-mediated signaling. **A**, OX40L engagement targets early FcεRI signaling by enhancing Cbp phosphorylation and Csk recruitment to the membrane where Csk phosphorylates Fyn, inducing its inactive conformation. **B**, Reduction in Fyn activity decreases Gab2, PI3K, and Akt phosphorylation but enhances SHIP-1 activation, causing a reduction in PIP₃ levels. Downstream of PIP₃ and Akt, MTOC dynamics are impaired, dampening IgE/Ag-induced degranulation.

recruitment, and enhanced Fyn activity. Herein, our results demonstrate the opposite effect on OX40L engagement, enhancing Cbp phosphorylation and Csk recruitment and causing a marked Fyn activity downregulation, which was previously demonstrated to cause impaired MC degranulation.⁷

Nonetheless, the contribution of other kinases, such as Lyn,^{24,25} or phosphatases, such as PTP³⁴ and/or Shp-2,³⁵ to decreased FcεRI activation and increased Csk activation cannot be excluded (Fig 6, A).

In our proposed model (Fig 6, B), OX40L engagement causes a considerable inactivation of downstream Fyn-dependent proteins Gab2, PI3K, Akt, and RhoA, all proteins whose activity has been shown to be dependent on Fyn.^{7,27}

Increased phosphorylation of SHIP-1 is ascribed to its interaction with the FcεRI β chain, and it is observed during supraoptimal Ag concentration, which preferentially involves Lyn.³⁶ Since sOX40 inhibited the Fyn pathway without affecting the Lyn pathway, we speculate that the OX40L signal provides a negative feedback through SHIP-1, concurring to the overall reduction of PI3K activity (Fig 6, B).

In MCs, the Fyn pathway plays a critical role in microtubule dynamics, which are dependent on RhoA activity and are required for full MC degranulation.^{27,28,37} Here, we found that OX40L triggering also impairs FcεRI-dependent MTOC dynamics, microtubule formation, and RhoA activation. While deficiencies in RhoA activity do not completely block MC degranulation, we show that RhoA is necessary for the “full” extent of the FcεRI-induced response.

Our previous study¹⁵ demonstrated that Treg:MC interaction resulted in increased cyclic adenosine monophosphate levels

that concur to inhibit MC degranulation. Current data of OX40L-mediated inhibition of Fyn is consistent with these findings, since the inhibition of Fyn activity leads to increased cyclic adenosine monophosphate production through decreased Akt activation, an upstream activator of phosphodiesterases that degrade cyclic adenosine monophosphate to adenosine monophosphate in T cells and oocytes.^{38,39} In addition, in a screening of gene expression profile of 12,000 genes, we found that the expression of phosphodiesterase 1c was reduced by 3-fold in Fyn-deficient MCs (not shown). Thus, phosphodiesterase function and/or expression is significantly reduced on the reduction of Fyn activity.

Previous work⁴⁰ demonstrated that Fyn activation is essential to achieve full MC responses and that low-affinity Ags can cause a preferential activation of Fyn, without the release of preformed MC mediators.⁷ Thus, it could be argued that OX40L signaling, which inhibits the Fyn pathway, acts to “fine-tune” rather than cause an *in toto* switch-off of MC degranulation. This role of “fine-tuning” is further evidenced by *in vivo* systemic anaphylaxis, in which treatment with blocking antibodies to OX40L enhanced the MC response. Moreover, the *in vivo* engagement of OX40L by the sOX40 in Treg-cell-depleted or OX40-deficient mice was able to restore the regulatory control as exerted by Treg cells, dampening the anaphylactic response to the levels seen in WT mice. While we demonstrate that OX40L may “fine-tune” FcεRI-stimulated MC degranulation, it is likely that its role extends beyond to MC homeostasis, since we were unable to reconstitute *Kit*^{W-sh/W-sh} mice with BM-derived MCs derived from OX40L-deficient mice (unpublished observations). This is similar to the findings reported for *PI3K*-deficient MCs,⁴¹

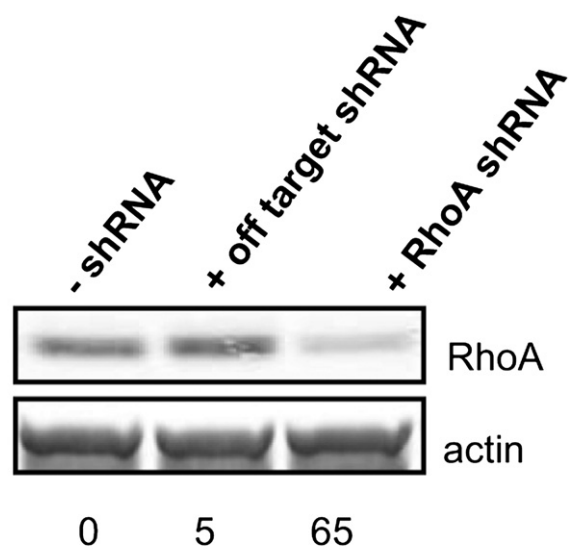
suggesting a potential role of the OX40L/PI3K axis that may involve fine regulation of MC migration and/or tissue homeostasis. The use of sOX40 *in vivo* could encourage the development of specific biologically active molecules that, by working on the interaction between paired costimulatory molecules, could transcend the treatment of abnormal MC responses and could be addressed to the treatment of pathologies ranging from autoimmune diseases to tumors.⁴² In these settings, such molecules would supply to the aberrant or lacking Treg-cell activity and restore the effect of normal Treg-cell functionality.⁴²

Key messages

- OX40L signaling downregulates the Fyn/Gab2/PI3K/RhoA pathway and prevents microtubule nucleation in mast cells.
- The use of sOX40 ameliorates the anaphylactic response, when the OX40:OX40L axis is interrupted, revealing a new homeostatic role for OX40L in regulating MC response.

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% silenced (relative to WT BMMC)

FIG E1. RhoA-specific short hairpin (*sh*) RNA infection of WT BM-derived MCs. WT BM-derived MCs were infected with lentivirus-expressing shRNA specific for RhoA or a control nontarget shRNA (Sigma Aldrich). Procedures for generation and infection with the virus were previously described.²² Endogenous RhoA silenced was normalized to total actin. Densitometric analysis was performed by using ImageJ software (NIH).

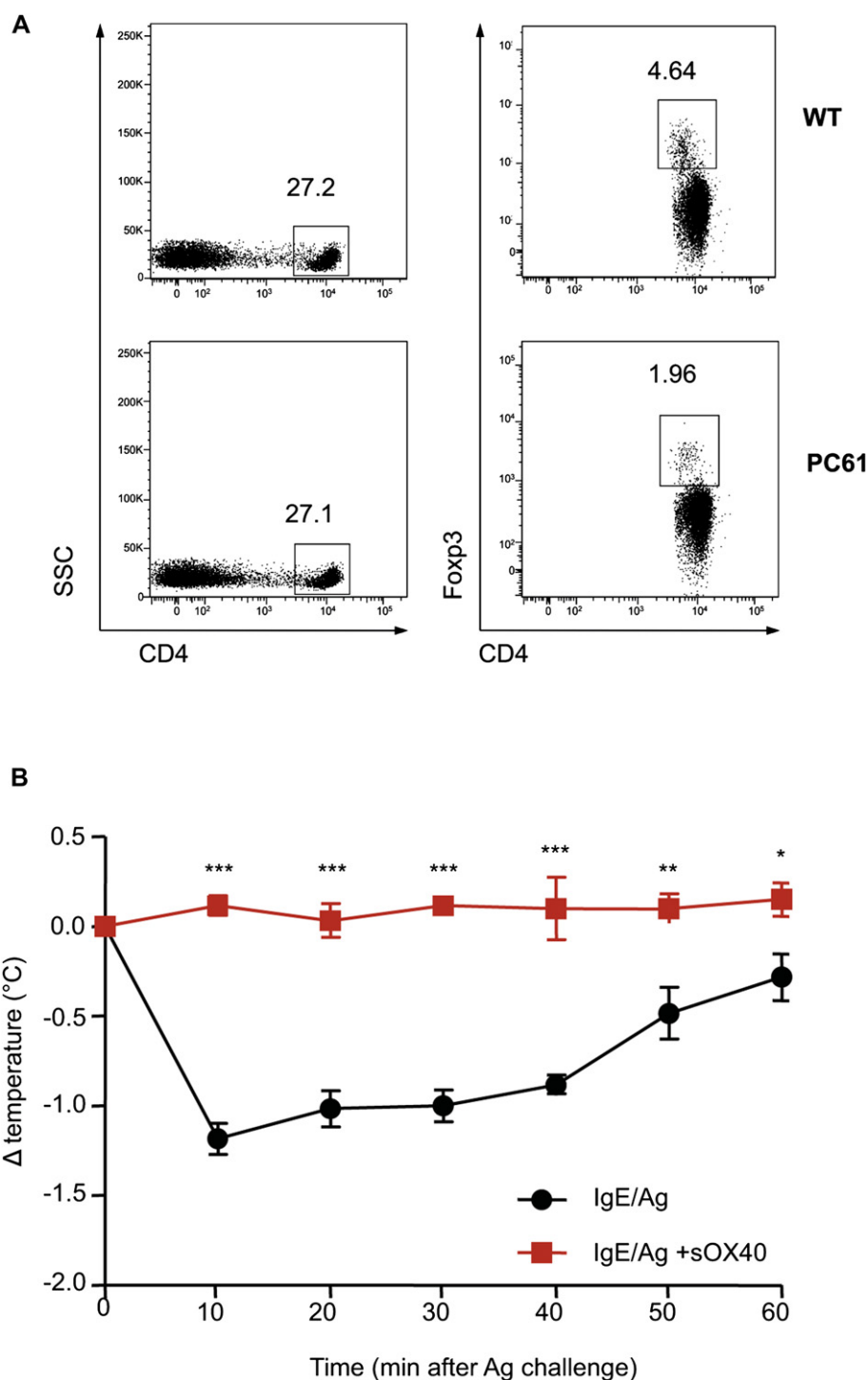


FIG E2. A, Treg-cell depletion is induced by anti-CD25 administration. Seven days after anti-CD25 antibody intravenous injection (clone PC61), CD4⁺ cells collected from blood were stained for Foxp3 to determine the extent of Treg-cell depletion. Data were acquired on a fluorescence-activated cell sorting Canto (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (TreeStar, Ashland, Ore). **B**, Passive systemic anaphylaxis in OX40-deficient mice. Mice were sensitized with 3 μ g IgE 24 hours before Ag challenge ($n = 12$). Some of these mice ($n = 6$) also received 150 μ g sOX40 at the same time as IgE. Before and at intervals after Ag administration, body temperature was measured with a rectal thermometer (Physitemp Instruments, Clifton, NJ). Changes in body temperature were measured at 10-minute intervals for a total of 60 minutes. * $P < .05$; ** $P < .01$; *** $P < .001$.