The receptor tyrosine kinase c-Kit controls IL-33 receptor signaling in mast cells

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Members of the Toll/interleukin-1 receptor (TIR) family are of importance for host defense and inflammation. Here we report that the TIR-family member interleukin-33R (IL-33R) cross-activates the receptor tyrosine kinase c-Kit in human and murine mast cells. The IL-33R–induced activation of signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase 1/2 (Erk1/2), protein kinase B (PKB), and Jun NH_2-terminal kinase 1 (JNK1) depends on c-Kit and is required to elicit optimal effector functions. Costimulation with the c-Kit ligand stem cell factor (SCF) is necessary for IL-33–induced cytokine production in primary mast cells. The structural basis for this cross-activation is the complex formation between c-Kit, IL-33R, and IL-1R accessory protein (IL-1RAcP). We found that c-Kit and IL-1RAcP interact constitutively and that IL-33R joins this complex upon ligand binding. Our findings support a model in which signals from seemingly disparate receptors are integrated for full cellular responses. (*Blood*. 2010; 115(19):3899-3906)

Introduction

Members of the Toll/interleukin-1 (IL-1) receptor (TIR) superfamily are crucial for protective and pathogenic host responses to injury and infection. IL-33R (T1/ST2) is a member of the IL-1 receptor (IL-1R) family and occurs in membrane-bound or soluble (sIL-33R) isoforms.[1] Expression of membrane-bound IL-33R is restricted to hematopoietic cells, particularly T-helper 2 (Th2) lymphocytes, mast cells, eosinophils, and basophils.[2-7] Signaling via IL-33R induces the expression of IL-4, IL-5, and IL-13 in Th2 lymphocytes, independently of T-cell receptor triggering.[8,9] In mast cells, IL-33 induces the secretion of chemokines and cytokines such as IL-6, IL-8, and IL-13.[10,11] IL-33/IL-33R interactions have been implicated in murine models of asthma,[2,12,13] helminthic infections,[14,15] sepsis,[16] atherosclerosis,[17] and arthritis.[18] Genetic studies also suggest a pathogenic role for IL-33R signaling in human allergic inflammation.[19] Soluble IL-33R functions as a decoy receptor that can block IL-33/IL-33R interaction.[2,8,12,13] Elevated levels of sIL-33R have been reported in sera of patients with exacerbations of asthma, sepsis, myocardial infarction, or heart failure, and autoimmune diseases such as systemic lupus erythematodes or vasculitis.[1,20]

Despite the expanding knowledge on the biologic effects of IL-33, the signaling pathways emanating from IL-33R are only partially known. IL-33R signaling resembles that of other IL-1R family members, particularly IL-1βR and IL-18R. The IL-1R accessory protein (IL-1RAcP) associates with IL-33R in a ligand-dependent manner and serves as obligatory second subunit of IL-33R.[21,22] Similar to other members of the TIR superfamily, IL-33R's cytosolic TIR domain dimerizes with the TIR domain of the adaptor protein MyD88.[9,23] IL-1R–associated kinase 1, IL-1R–associated kinase 4, and tumor necrosis factor receptor–associated factor 6 (TRAF6) are subsequently recruited and activate mitogen-activated protein kinases and the transcription factor nuclear factor κB (NF-κB).[9,11,21,24,25] To date, it has not been elucidated how the common use of this canonical pathway by many different receptors results in highly diverse and often cell type–specific biologic effector functions. Recently, Funakoshi-Tago et al[26] reported that IL-33R–induced activation of extracellular signal-regulated kinase (Erk) is independent of TRAF6, whereas activation of p38, Jun NH_2-terminal kinase (JNK), and NF-κB depends on TRAF6, implying alternative IL-33R–induced activation pathways for Erk. The existence of additional signaling pathways emanating from IL-33R has already been suggested by Mitcham et al[25] who used chimeric constructs consisting of the extracellular domain of IL-1R and the intracellular domain of IL-33R (T1/ST2) to demonstrate that exposure to IL-1 induced both activation of NF-κB and phosphorylation of an epidermal growth factor receptor peptide.

Given the relevance of IL-33R for host defense, allergy, and inflammation, we sought to determine the signaling pathways emanating from IL-33R more closely. Mast cells are important for immune responses against nematodes, in allergy, asthma, and arthritis.[27] They express IL-33R constitutively and IL-33 stimulation induces or enhances the production of inflammatory mediators. Therefore, we chose to analyze IL-33R signaling in mast cells. We show here that IL-33R cross-activates the receptor tyrosine kinase c-Kit and that c-Kit cross-activation is required for IL-33–induced effector function in mast cells.

Methods

Mice

Mice were kept under specific pathogen–free conditions. All experiments were conducted in accordance with federal and institutional guidelines. Adult $Kit^{W\text{-}sh/W\text{-}sh}$ mice have mast cell deficiency, but c-Kit–deficient bone marrow–derived

Submitted October 5, 2009; accepted February 8, 2010. Prepublished online as *Blood* First Edition paper, March 3, 2010; DOI 10.1182/blood-2009-10-247411.

The online version of this article contains a data supplement.

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mast cells (BMMCs) can be generated.[28,29] The transgene for constitutively active c-Kit (kit^{D814V}) is expressed upon Cre-mediated excision of the lox*P*-flanked transcriptional stop element in adult mice. Thus, BMMCs generated from induced $kit^{D814Vflox}$ *deleter-Cre* double-transgenic mice express kit^{D814V} (the kit^{D814V} tg mice are characterized in A.G., C. Wickenhauser, J. Scholten, K. Peschke, S.D., H. P. Horny, T.K., R. Naumann, W. Müller, T. Krieg, K.H., and A.R., manuscript submitted, April 2009). As controls, we used sex- and age-matched C57BL/6 mice for $Kit^{W\text{-}sh/W\text{-}sh}$ and single transgenic mice ($kit^{D814Vflox}$) for kit^{D814V} tg.

Cell lines and BMMC generation

HMC-1.1 and HMC-1.2 cells (provided by Dr J. H. Butterfield, Mayo Clinic) were cultured in RPMI1640 supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% antibiotics (Biochrom), and 50μM mercaptoethanol.

BMMCs were generated from the femoral bone marrow by culture in complete Iscove modified Dulbecco medium (PAA) supplemented with 10 ng/mL recombinant murine IL-3 (PeproTech), 10% fetal calf serum, and 1% antibiotics. BMMCs were used after 4 weeks of culture and consisted of 95% mast cells as identified by the surface expression of FcεRI, c-Kit, IL-33R, and toluidine blue staining.

Cell stimulation and lysis

BMMCs or HMC-1 cells (10^6/mL) were serum-starved for 2 hours. Cells were preincubated with recombinant proteins (sIL-33R; R&D Systems) or inhibitors (imatinib; provided by E. Buchdunger, Novartis) for 30 minutes or methyl-β-cyclodextrin (MβCD; Sigma-Aldrich) for 2 hours as indicated before stimulation with IL-1β, IL-33, and/or stem cell factor (SCF; both from Peprotec). Cells were lysed with lysis buffer (20mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5; 10mM ethyleneglycoltetraacetic acid; 40mM β-glycerophosphate; 2.5mM $MgCl_2$; 2mM orthovanadate; 1mM dithiothreitol; 1mM phenylmethylsulphonyl fluoride; 20 μg/mL aprotinin; 20 μg/mL leupeptin supplemented with 1% Triton [for immunoblotting] or 0.5% Nonidet P40 [for immunoprecipitation]). For immunoprecipitation experiments, cell lysates were sonicated before centrifugation for cell debris removal.

For enzyme-linked immunosorbent assay, supernatants were analyzed for IL-4, IL-6, and IL-8 (Immunotools). Cytokine concentration is indicated as the mean of quadruple measurements (± SD).

Coimmunoprecipitation and immunoblotting

Lysates were subjected to coimmunoprecipitation with anti–c-Kit (M14; Santa Cruz Biotechnology) overnight. After adding protein-A sepharose for 6 hours and washing with lysis buffer (supplemented with 10mM MβCD as indicated), precipitated proteins were eluted by boiling in sample buffer. Protein extracts were separated on 10% sodium dodecyl sulfate (SDS)–Laemmli gels and transferred by electroblotting onto nitrocellulose membranes. Membranes were blocked with dry milk or bovine serum albumin and incubated with primary antibodies detecting phosphorylated or total proteins (Cell Signaling Technology), anti–IL-33R (Baf1004; R&D Systems), anti–IL-1RAcP (eBioscience), anti–c-Kit (M14; Santa Cruz Biotechnology), and anti-tubulin (as the loading control; Sigma-Aldrich). Membranes were washed in 0.1% Tween/tris(hydroxymethyl)aminomethane–buffered saline and incubated with the respective horseradish peroxidase–conjugated secondary antibodies: anti–rabbit-immunoglobulin (Ig), anti–goat-Ig (both from Santa Cruz Biotechnology), anti–mouse-Ig (Pierce), or horseradish peroxidase–conjugated streptavidin (eBioscience). Detection was performed using enhanced chemiluminescence reagent (Pierce).

Densitometric Western blot analysis

Pixel density of defined areas on immunoblot signals were calculated with ImageJ software (National Institutes of Health). The background values from the same blot were subtracted. The resulting values were divided by the corresponding tubulin value (normalized value). For each phosphorylated signaling molecule, the normalized value of the control was set as one. Data are represented as mean plus or minus standard deviation from at least 3 independent experiments.

Flow cytometry

Cells were washed with phosphate-buffered saline containing 0.25% bovine serum albumin and 0.02% natriumazide. Nonspecific binding of antibodies was blocked with anti-CD16/CD32 (clone 2.4G2) and rat-IgG (Jackson ImmunoResearch Laboratories). Staining was performed with the biotinylated antibody for IL-33R ($3E10^2$), and phycoerythrin-conjugated streptavidin or murine (phycoerythrin-conjugated) c-Kit (eBioscience). Data were acquired with LSRII flow cytometer (BD Biosciences) and evaluated with FlowJo 8.1.1 (TreeStar Inc).

Results

IL-33R signaling induces tyrosine phosphorylation of c-Kit and cytokine production in mast cells

HMC-1.1 cells express surface-IL-33R (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Confirming and extending earlier results,[9,11] we found that IL-33R signaling induced a time-dependent activation of Erk1/2, protein kinase B (PKB), JNK1/2, NF-κB, and p38, and degradation of IκB (supplemental Figure 1B). Preincubation with sIL-33R, but not with sIL-1R, blocked the IL-33–induced activation and cytokine production (supplemental Figure 1C-D). To investigate whether IL-33 induces activation of tyrosine kinases, we performed a pan-phosphotyrosine–specific immunoblot on IL-33–stimulated HMC-1.1 cells. A protein with an estimated molecular mass between 120 and 150 kDa was most strongly phosphorylated (data not shown). Suspecting that this was c-Kit (molecular weight: 145 kDa), we tested whether c-Kit was activated in response to IL-33.

Y721-c-Kit is autophosphorylated in response to stem cell factor (SCF). Therefore pY721 is commonly used to detect activation of c-Kit's tyrosine kinase activity. Stimulation of HMC-1.1 cells with either SCF or IL-33 induced Y721 phosphorylation with similar magnitude and kinetics (Figure 1A). To exclude IL-33 as a ligand for c-Kit, we used sc-Kit, a decoy receptor for SCF.[30] sc-Kit blocked SCF-induced signaling but did not impair IL-33–induced signaling and cytokine release (supplemental Figure 2A-C). Furthermore, IL-33 did not induce SCF release from HMC-1.1 cells (supplemental Figure 2D), indicating that IL-33 does not induce c-Kit activation indirectly via the induction of SCF release. These data suggest IL-33R–induced cross-activation of c-Kit as a novel mechanism of TIR-induced signal transduction.

c-Kit cross-activation is requisite for cytokine production in HMC-1 cells

To determine the relevance of IL-33–induced cross-activation of c-Kit for IL-33R signaling and effector functions, we used imatinib, an inhibitor of tyrosine kinases including c-Kit.[31] Prolonged culture in the presence of imatinib induces apoptosis in HMC-1.1 cells, which express the imatinib-sensitive c-Kit mutant V560G.[32] Therefore, we determined that apoptotic events in HMC-1.1 become detectable only after 10 hours of culture with 1μM imatinib (data not shown).

Imatinib blocked the basal tyrosine phosphorylation of c-Kit and Erk1/2 that is characteristic for HMC-1.1 cells and selectively inhibited the IL-33–induced activation of signal transducer and activator of transcription 3 (STAT3), Erk1/2, PKB, and JNK1 (Figure 1B), indicating that these pathways depend on c-Kit. Functionally, imatinib inhibited the IL-33–induced secretion of IL-4, IL-6, and IL-8 (Figure 1C). To confirm the requirement for

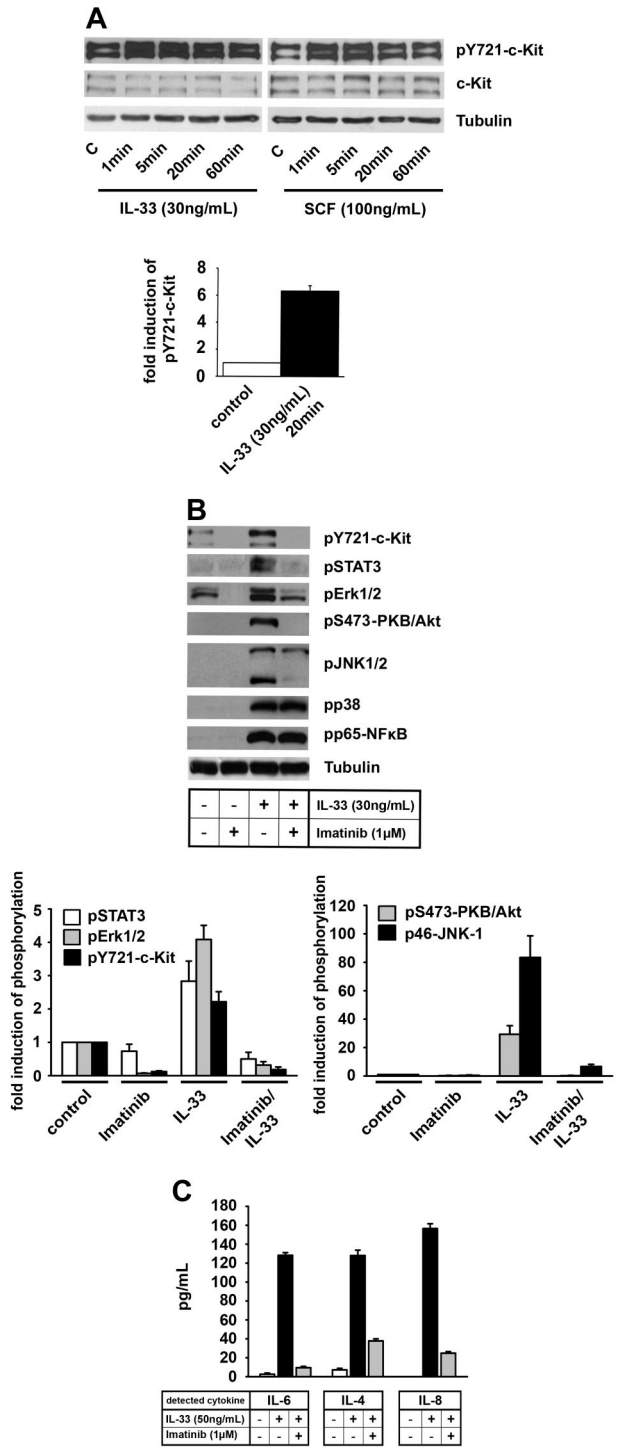

Figure 1. IL-33–induced signaling and cytokine release in mast cells. (A) HMC-1.1 cells were stimulated with IL-33 or SCF as indicated. Cell lysates were separated by SDS-PAGE, blotted, and probed with anti–pY721-c-Kit. Because of the high basal phosphorylation level, the exposure time was less than 5 seconds. (B-C) HMC-1.1 cells were preincubated with imatinib before stimulation with IL-33. Cell extracts were analyzed with the indicated antibodies (B). Phosphorylation was quantified in 3 independent experiments and results are shown as increase (mean ± SEM) relative to nonstimulated cells. Supernatants were analyzed for IL-4, IL-6, and IL-8 (C).

c-Kit, we used HMC-1.2 cells, which express the imatinib-insensitive, but PKC412-sensitive, c-Kit mutant V560G/D816V.[33] In contrast to imatinib, PKC412 blocked the IL-33–induced activation of c-Kit, STAT3, Erk1/2, PKB, and JNK1 as well as the IL-33–induced IL-6 production in HMC-1.2 cells (supplemental Figure 2E-F). Thus, IL-33R signaling in HMC cells branches into c-Kit–dependent and c-Kit–independent pathways. To analyze the functional relevance of IL-33–induced, c-Kit–dependent JNK and Erk1/2 activation, we used inhibitors for Erk (UO126) and JNK (SP600125). Both blocked the IL-33–induced cytokine release in HMC-1.1 cells (supplemental Figure 3).

Next we analyzed the mechanism leading to the IL-33–induced cross-activation of c-Kit in HMC-1.1 cells. Using pharmacologic inhibitors, we could exclude the involvement of reactive oxygen species, shedding of membrane-bound SCF precursors as well as Src kinases (data not shown).

c-Kit is necessary but not sufficient for IL-33R–induced signaling and cytokine production in bone marrow–derived murine mast cells

HMC-1 cells express constitutively active c-Kit mutants. To examine the role of wt c-Kit in IL-33R signaling, we used BMMCs from wild-type (wt) and $kit^{W\text{-}sh/W\text{-}sh}$ mice. W-sh is a mutation that blocks c-Kit expression and thus SCF responsiveness.[28] Surface expression of IL-33R was equal in wt and $kit^{W\text{-}sh/W\text{-}sh}$ BMMCs (Figure 2A). To determine the specificity of the IL-33–induced phosphorylations and cytokine production, we used BMMCs from $il\text{-}33r^{-/-}$ mice (supplemental Figure 4A-C). The constitutive phosphorylation of Erk1/2 and JNK1 was more noticeable in unstimulated wt than in $kit^{W\text{-}sh/W\text{-}sh}$ BMMCs. The IL-33–induced phosphorylation of JNK2, NF-κB, and p38 occurred with similar kinetic and magnitude in wt and $kit^{W\text{-}sh/W\text{-}sh}$ BMMCs (Figure 2B). However, the IL-33–induced phosphorylation of PKB and JNK1 was reduced in $kit^{W\text{-}sh/W\text{-}sh}$ BMMCs (Figure 2B). Importantly, IL-33 did not induce activation of c-Kit, STAT3, and Erk1/2 in wt BMMCs (Figure 2B). Although IL-33 did not cross-activate c-Kit in wt BMMCs, the presence of c-Kit was required for optimal IL-6 production (Figure 2C). Thus, c-Kit is necessary but not sufficient for optimal IL-33–induced effector functions.

c-Kit's tyrosine kinase activity is indispensable for IL-33–induced c-Kit cross-activation in BMMCs

To examine whether c-Kit's tyrosine kinase activity further increases IL-33R–mediated signaling, we used BMMCs from $kit^{D814Vflox}$ *deleter-Cre* double-transgenic mice (kit^{D814V} tg mice), which express the constitutively active c-Kit mutant D814V (A.G., C. Wickenhauser, J. Scholten, K. Peschke, S.D., H. P. Horny, T.K., R. Naumann, W. Müller, T. Krieg, K.H., and A.R., manuscript submitted, April 2009). IL-33R and c-Kit surface expression was equal in kit^{D814V} tg BMMCs compared with control BMMCs (Figure 3A).

As expected, the basal activity of c-Kit was markedly increased in kit^{D814V} tg BMMCs (Figure 3B). In kit^{D814V} tg BMMCs, IL-33 induced the activation of c-Kit, STAT3, Erk1/2, PKB, JNK1/2, p38, and NF-κB (Figure 3B) without any effect on the total levels of the investigated signaling proteins (supplemental Figure 5). This recapitulates in large part the situation in the HMC-1 mast cell lines.

SCF-induced activation of c-Kit enhances IL-33R signaling in wt BMMCs

We hypothesized that IL-33–induced cross-activation of c-Kit in wt BMMCs depends on SCF-induced preactivation of c-Kit. To test this, we stimulated wt BMMCs with SCF, IL-33, or both. IL-33 alone induced activation of JNK1/2, PKB, NF-κB, and p38 but did not induce activation of c-Kit, STAT3, or Erk1/2 (Figure 4A). SCF

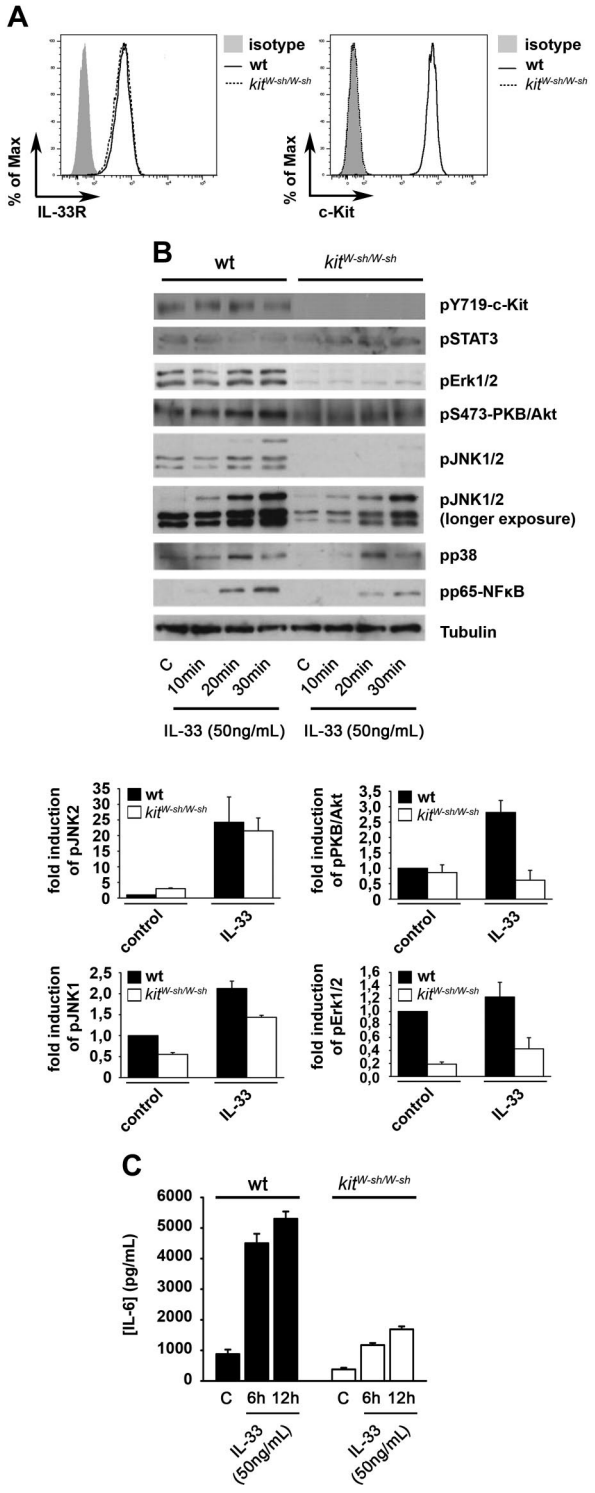

Figure 2. Presence of c-Kit is crucial for an effective IL-33–induced signaling. (A) Surface expression of IL-33R and c-Kit in wt (black line) or *kit*$^{W\text{-}sh/W\text{-}sh}$ (black dotted line) BMMCs. (B-C) Wt or *kit*$^{W\text{-}sh/W\text{-}sh}$ BMMCs were stimulated with IL-33 as indicated. Lysates were analyzed by immunoblot for phosphorylated signaling molecules as indicated (B). Longer exposure shows that IL-33–induced activation of JNK1 in *kit*$^{W\text{-}sh/W\text{-}sh}$ BMMCs is dramatically reduced but not completely blocked. Phosphorylation was quantified in 3 independent experiments and results are shown as increase (mean ± SEM) relative to nonstimulated wt BMMCs. Supernatants were analyzed for IL-6 (C).

alone induced the activation of c-Kit, STAT3, Erk1/2, JNK1, and PKB, which decreased after 60 minutes (Figure 4A). When BMMCs were cultured with SCF for 30 minutes prior to the addition of IL-33, this costimulation enhanced and prolonged the SCF-induced activation of c-Kit, Erk1/2, JNK1/2, and PKB. The c-Kit–independent IL-33–induced activation of p38 and NF-κB was only slightly increased (Figure 4A). IL-33 alone but not SCF alone induced the release of IL-6 (supplemental Figure 6A). In contrast to *kit*$^{W\text{-}sh/W\text{-}sh}$ BMMCs, costimulation with SCF plus IL-33 increased the amount of IL-6 released from wt BMMCs substantially (Figure 4B). Furthermore, we compared sequential costimulation (preincubation with SCF for 30 minutes before the addition of IL-33) with simultaneous costimulation. The increased IL-6 production was larger upon sequential costimulation than upon simultaneous costimulation with SCF and IL-33 (supplemental Figure 6B), indicating that preactivation of c-Kit is required for its responsiveness to IL-33R–induced cross-activation. This conclusion is further supported by the fact that preincubation with imatinib abolished the costimulatory effect of SCF on both the activation of c-Kit, Erk1/2, JNK1/2, PKB, p38, and NF-κB, and the IL-6 release (Figure 4C-D). The common finding on IL-33R–induced signaling in HMC-1.1 cells and BMMCs from wt or *kit*^{D814V} tg mice is, that preactivation of c-Kit is a prerequisite for IL-33R–induced cross-activation of c-Kit.

IL-1R signaling activates similar pathways as IL-33R and stabilizes transcripts of IL-6 in mast cells.[34] Hence, we investigated the IL-6 release after IL-1β or SCF/IL-1β stimulation. Stimulation of BMMCs with IL-1β, SCF, or both induced only a negligible release of IL-6 (Figure 4E). To exclude the possibility of an autocrine loop, we investigated whether stimulation with SCF leads to release of IL-1β. As shown in supplemental Figure 6C, SCF neither alone nor in combination with IL-33 induced the release of IL-1β. Furthermore, preincubation with a blocking antibody against IL-1β did not reduce the release of IL-6 (supplemental Figure 6D). These data confirm that IL-1β can neither induce IL-6 production in BMMCs nor synergize with SCF to enhance IL-6 production in BMMCs.

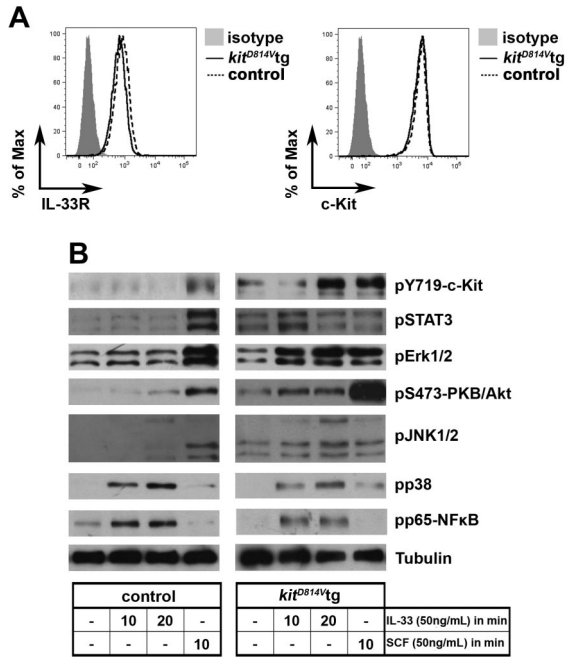

Figure 3. Constitutively active c-Kit augments IL-33R signaling in BMMCs. (A) Surface expression of IL-33R and c-Kit in *kit*^{D814Vflox} control (black dotted line) or *kit*^{D814V} tg (black line) BMMCs. (B) *kit*^{D814Vflox} control or *kit*^{D814V} tg BMMCs were stimulated with IL-33 or SCF as indicated. Lysates were analyzed by immunoblot for phosphorylated signaling molecules as indicated.

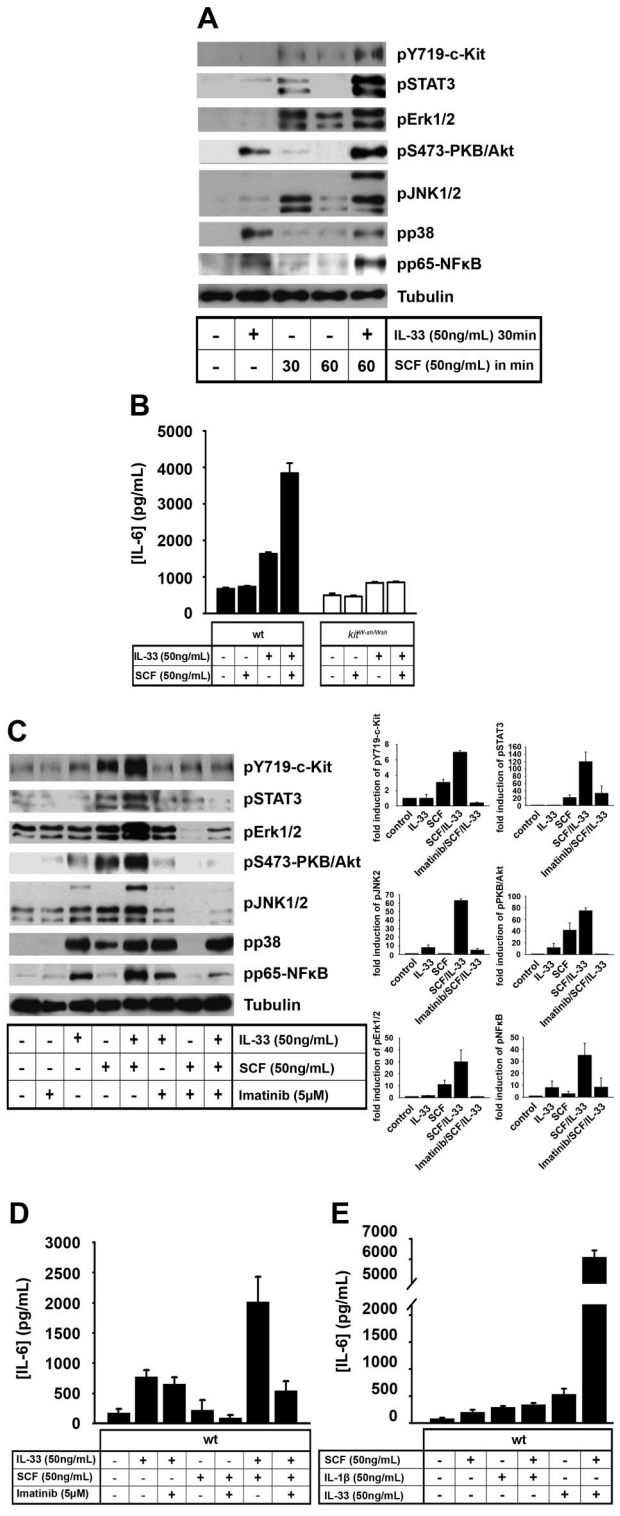

Figure 4. Prestimulation of wt BMMCs with SCF is crucial for the full biologic function of IL-33. (A) Wt BMMCs were stimulated with SCF or IL-33 as indicated. Costimulation was performed by prestimulation with SCF for 30 minutes followed by stimulation with IL-33 for additional 30 minutes. Lysates were analyzed by immunoblot for phosphorylated signaling molecules as indicated. (B) Wt or *kit*$^{W\text{-}sh/W\text{-}sh}$ BMMCs were stimulated with SCF and/or IL-33 for 24 hours. Supernatants were analyzed for IL-6. (C-D) Wt BMMCs were preincubated with imatinib for 30 minutes and subsequently prestimulated with SCF for 30 minutes followed by stimulation with IL-33 for additional 30 minutes (C) or 24 hours (D). Lysates were analyzed by immunoblot for phosphorylated signaling molecules as indicated. Phosphorylation was quantified in 3 independent experiments and results are shown as increase (mean ± SEM) relative to nonstimulated cells (C). Supernatants were analyzed for IL-6 (D). (E) Wt BMMCs were stimulated with SCF and/or IL-1β or IL-33 for 24 hours. Supernatants were analyzed for IL-6.

c-Kit interacts constitutively with IL-1RAcP and ligand dependently with IL-33R

We questioned whether activation of c-Kit establishes the structural basis for the functional interaction between c-Kit and IL-33R. Stimulation with SCF but not IL-33 induced the association of c-Kit and IL-33R time dependently (Figure 5A). Furthermore, this association could be blocked by imatinib (Figure 5B). Together, these findings strongly suggest that SCF-induced tyrosine kinase activity of c-Kit is pivotal for association of IL-33R and c-Kit. Because IL-33R, upon binding to its ligand, forms a complex with the IL-1RAcP, we also sought to detect IL-1RAcP in the precipitates. Even in unstimulated cells, IL-1RAcP accumulated significantly in the presence of c-Kit–specific antibody, and this coprecipitation remained unchanged after imatinib treatment or IL-33 stimulation (Figure 5B). In addition, we investigated whether IL-33 induces the interaction of c-Kit/IL-33R in cells that express constitutively active c-Kit mutants. In HMC-1.1 and *kit*^{D814V} tg cells, a constitutive association of IL-33R with c-Kit was detectable (data not shown).

Cholesterol-rich microdomains are necessary for the association of IL-33 with c-Kit

Interactions and reciprocal cross-activation of signaling molecules often depend on their enrichment in membrane microdomains.[35] SCF-induced recruitment of c-Kit into cholesterol-rich microdomains is necessary for c-Kit–mediated biologic effector functions.[36] To test whether the interaction between IL-33R and c-Kit depends on these microdomains, we analyzed the interaction of these receptors in wt BMMCs treated with the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD). Both, the SCF-induced activation of c-Kit and the SCF-induced association of IL-33R and c-Kit were abrogated in cholesterol-depleted cells (Figure 5C). In contrast, MβCD treatment did not influence the association of IL-1RAcP with c-Kit. Thus, association of IL-33R with c-Kit depends on cholesterol-rich membrane microdomains. Next, we asked whether the complex, once assembled in the intact microdomains, still required cholesterol for its stability. Therefore, we stimulated wt BMMCs with SCF, subsequently lysed the cells in MβCD-containing or conventional buffer, and performed coimmunoprecipitations. The quantity of IL-33R interacting with c-Kit was constant, irrespective of whether lysis conditions provided cholesterol depletion or not (supplemental Figure 7). The caveolae marker flotillin did not coprecipitate with c-Kit under conventional or under cholesterol-depleting conditions. This indicates that the lysis conditions were sufficient to extract c-Kit from cholesterol-rich microdomains (supplemental Figure 7). These data exclude coincidental coprecipitation of c-Kit and IL-33R resulting from their close proximity within these microdomains without physical interaction. Next we analyzed whether the integrity of microdomains is necessary for the IL-6 release induced by costimulation with SCF and IL-33. In contrast to the IL-6 release induced by IL-33 alone, the IL-6 response to costimulation was MβCD sensitive (Figure 5D). Taken together, cholesterol-enriched membrane microdomains are indispensable for the association of IL-33R with c-Kit and the resulting IL-6 release, but not for the stability of the IL33R/c-Kit complex, once it has been assembled.

These findings suggest that the assembly of a signalosome consisting of IL-33R, IL-1RAcP, and c-Kit is dependent on the tyrosine kinase activity of c-Kit and the integrity of cholesterol-rich microdomains and is the molecular basis for the c-Kit–dependent full biologic effector functions of IL-33.

Ligand-dependent formation of a signalosome containing IL-33R, MyD88, IL-1RAcP, and c-Kit

Our experiments revealed a constitutive association of c-Kit and IL-1RAcP and a SCF-induced association of c-Kit and IL-33R. These findings suggest a model in which SCF stimulation leads to recruitment of IL-33R into the constitutive c-Kit/IL-1RAcP complex. Alternatively, 3 different complexes, namely c-Kit/IL-1RAcP, c-Kit/IL-33R, and IL-1RAcP/IL-33R may be formed in mast cells. To distinguish between these possibilities, we analyzed lysates from differentially stimulated BMMCs with sucrose gradient separation, using a linear sucrose gradient. Fractions were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblot for the presence of c-Kit, IL-33R, and IL-1RAcP. The largest amount of all 3 receptors migrated in fractions 17 to 21 (15%-12% sucrose; supplemental Figure 8A). Detergent-resistant microdomains float in low-density sucrose fractions (5% sucrose) during gradient centrifugation because of their high lipid content.[37] Thus, the sedimentation characteristics of the 3 receptors indicate that solubilized protein complexes were detected. In lysates of unstimulated wt BMMCs, all 3 receptors, in particular IL-33R, were broadly distributed across fractions 16 to 21 (supplemental Figure 8A). In gradients of cell lysates from SCF-stimulated wt BMMCs, the migration of all 3 receptors was focused in fractions of higher density (fractions 17 and 18) (supplemental Figure 8B). This comigration of c-Kit, IL-33R, and IL-1RAcP was also observed for cell lysates of unstimulated kit^{D814V} tg BMMCs (data not shown).

Thus, in accordance with the coimmunoprecipitation experiments, the size fractionation approach substantiated the SCF-induced formation of a multimeric receptor complex. To analyze whether this complex contains additional signaling molecules, we separated aliquots of gradient fractions 18 and 20 on a native gel with subsequent immunoblot (supplemental Figure 8C). Thereby, we confirmed the presence of a complex of approximately 1000 kDa containing in addition to IL-33R, IL-1RAcP, and c-Kit the TIR adaptor protein MyD88.

Discussion

IL-33 induces effector functions in mast cells, basophils, eosinophils, and T lymphocytes.[4,5,38,39] Here, we report that in mast cells IL-33 induces the association of IL-33R with preactivated c-Kit and activates this receptor tyrosine kinase. IL-33–induced c-Kit cross-activation is critical for important effector functions mediated by IL-33R. Both, IL-33R–induced signaling and cytokine production are severely impaired in $kit^{W\text{-}sh/W\text{-}sh}$ BMMCs that do not express c-Kit.

Cross-activation of a receptor tyrosine kinase (RTK) by a TIR-family member has not been reported previously. Whereas c-Kit has been known to cross-activate cytokine and growth factor receptors including erythropoietin receptor, IL-3R, IL-7R, and granulocyte-macrophage colony-stimulating factor receptor,[40-43] we did not find any evidence for SCF-induced phosphorylation of

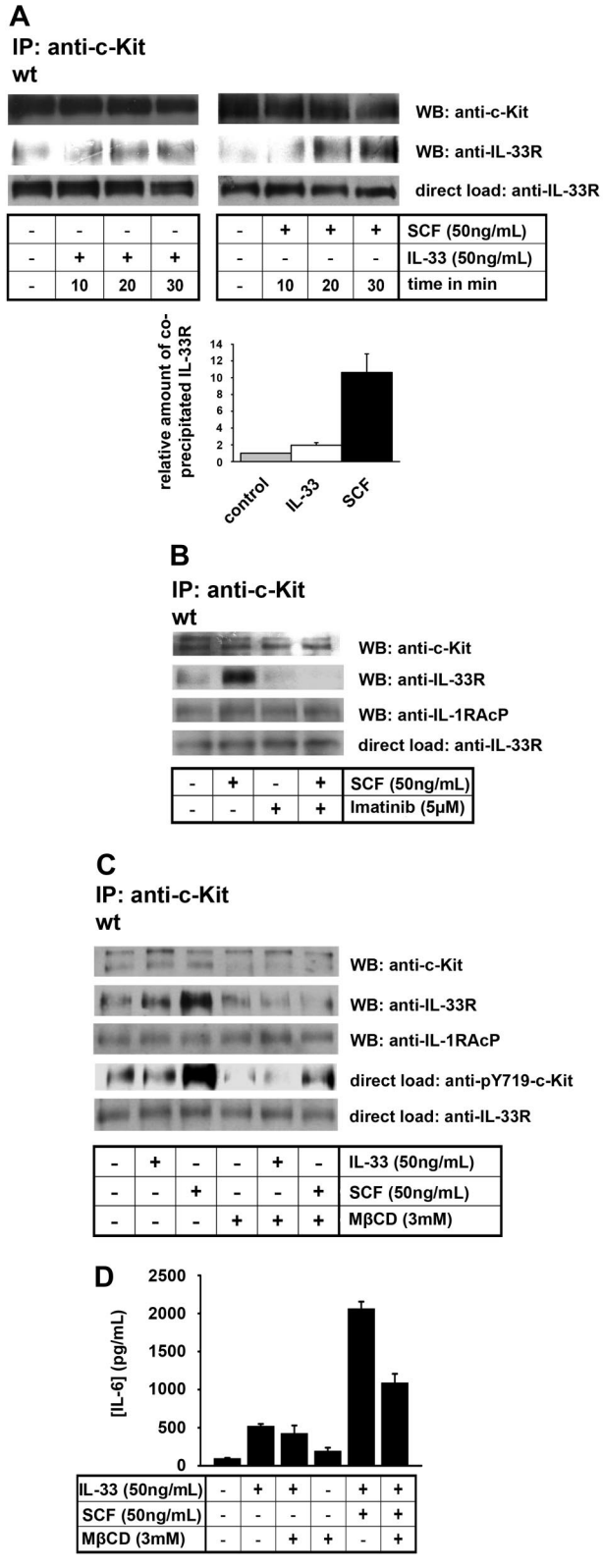

Figure 5. SCF mediates the association of IL-33R with c-Kit in dependency of its tyrosine kinase activity and the integrity of cholesterol-rich microdomains. (A) Wt BMMCs were stimulated with IL-33 or SCF as indicated, lysed, and subjected to c-Kit immunoprecipitation. Coprecipitated IL-33R was detected by immunoblot and quantified for 3 independent experiments. Results are shown as increase (mean ± SEM) relative to nonstimulated cells. Equal amounts of IL-33R in lysates were determined by immunoblotting (direct load). (B) Wt BMMCs were preincubated with imatinib and subsequently stimulated with SCF. Lysates were immunoprecipitated with an anti–c-Kit antibody and analyzed for coprecipitated IL-33R and IL-1RAcP. (C) Wt BMMCs were treated with the methyl-β-cyclodextrin (MβCD) for 2 hours and subsequently stimulated with either IL-33 or SCF. Lysates were immunoprecipitated with anti–c-Kit and analyzed for c-Kit, IL-33R, and IL1-RAcP. Phosphorylation of Y721–c-Kit and equal amounts of IL-33R were verified by immunoblot (direct load). (D) Wt BMMCs were left untreated or were treated with MβCD for 2 hours and subsequently stimulated with either IL-33, SCF, or both for 6 hours. Supernatants were analyzed for IL-6.

IL-33R. Neither matrix metalloproteinases nor reactive oxygen species nor Src kinases are involved in IL-33R–induced c-Kit activation. Instead, both IL-33R and IL-1RAcP physically associate with c-Kit, suggesting that the aggregation of these 3 receptors is the major mechanism for c-Kit cross-activation by IL-33R. Whereas the association of c-Kit and IL-1RAcP occurs constitutively, the association of IL-33R with IL-1RAcP or c-Kit are both ligand dependent.

Several lines of evidence show that the complex formation of IL-33R with c-Kit critically depends on c-Kit's tyrosine kinase activity. First and foremost, IL-33 induces this complex formation and c-Kit cross-activation only in cells with a constitutively active c-Kit mutant, such as HMC-1.1 cells and BMMCs from *kit*^{D814V} tg mice. In wild-type BMMCs, IL-33R and c-Kit associate only upon SCF stimulation, and this complex formation can be abrogated by treatment with the tyrosine kinase inhibitor imatinib. Furthermore, the SCF-induced formation of the c-Kit/IL-33R complex is strictly dependent on the integrity of cholesterol-rich plasma membrane microdomains that are also crucial for the effective SCF-induced activation of c-Kit.[36] Besides IL-33R and c-Kit, the receptor cluster also contains IL-1RAcP. In addition to its constitutive association with c-Kit, IL-1RAcP could be recruited into the IL-33R/c-Kit complex either by IL-33–dependent IL-33R/IL-1RAcP association or by SCF-enhanced IL-33R/c-Kit association. The exact biochemical mechanisms of c-Kit activation within this complex remain to be elucidated.

Size fractionation and native PAGE analysis of SCF-stimulated BMMCs revealed that IL-33R, IL-1RAcP, and c-Kit comigrate within a multimeric complex. Remarkably, we could also detect comigrated MyD88 in this complex. This surprising association of the TIR-interacting adaptor with the SCF-induced receptor complex could be mediated by 2 mutually nonexclusive types of recruitment: via IL-1RAcP[44] bound by c-Kit or via dimerized IL-33R.[23] Taken together, our findings are compatible with the concept that c-Kit, IL-33R, IL-1RAcP, and MyD88 form a signalosome that is required to unleash IL-33–induced effector function in mast cells. Although protein migration within native gels is not dependent solely on the molecular weight but also on charge, it is tempting to speculate how the complex with an estimated size of 1000 kDa is composed. Future experiments should identify both the stoichiometric ratio of integrated receptors and possibly additional receptor components or intracellular adaptors within this complex.

Functionally, the consequence of the complex formation of these receptors within microdomains is the integration of the signals emanating from the IL-33R and c-Kit into one network. IL-33 amplifies c-Kit signaling components such as STAT3 and Erk1/2, and c-Kit signaling synergizes with IL-33–induced pathways such as NF-κB and JNK2. Thus, costimulation with SCF plus IL-33 enhances the activation of signaling molecules that are only weakly activated upon IL-33 stimulation and not activated at all by SCF (NF-κB and JNK2). This cross-activation broadens the signaling network triggered by IL-33 and consequentially the biologic effector functions exerted by IL-33–responsive mast cells. Notably, the effective IL-33–induced IL-6 production in BMMCs depends on SCF costimulation.

Recent reports showed that IL-33 enhances the SCF-induced maturation of mast cell progenitors but cannot induce mast cell differentiation in the absence of SCF.[10,11] This provides an example of how the integration of signals from c-Kit and the IL-33R facilitates the exact adaptation of cellular responses to complex microenvironmental stimuli. The requirement for 2 different signals, IL-33 and SCF, for full activation of mast cells helps to prevent inappropriate activation by one receptor system and thus provides an additional level of regulation for IL-33–induced effector functions.

The dysregulation of such a cooperative signaling system, for example, via the expression of a constitutively active c-Kit mutant as in the *kit*^{D814V} tg mice or the inappropriate production of SCF or IL-33, could be important for disease development. One relevant example would be the inflamed airways of asthmatic subjects where both SCF and IL-33 are present at elevated concentrations.[38,45] In fact, a synergistic effect of SCF and IL-33 has recently been reported in allergic inflammation.[38] Tyrosine kinase inhibitors such as imatinib may provide a useful way to block pathogenic IL-33–induced mast cell effector functions in allergic subjects.

Does IL-33R interact with distinct RTKs in different cell lineages? IL-33R–expressing cells such as mast cells, eosinophils, basophils, and Th2 cells are critical mediators of inflammation at mucosal sites. Like mast cells, eosinophils and basophils express functional c-Kit.[46,47] It will be interesting to discern whether IL-33R cross-activates c-Kit in these cells and which effector functions depend on this cross-activation.

Murine and perhaps also human Th2 cells express IL-33R,[2,7,48] and signaling via IL-33R induces the expression of IL-4, IL-5, and IL-13 even in the absence of additional T-cell receptor triggering.[8,9] Because Th cells do not express c-Kit, we are currently investigating whether IL-33R cooperates with an RTK or signals autonomously in Th cells. It also remains to be elucidated whether signaling pathways emanating from other TIR-family members such as the IL-1R or Toll-like receptors also integrate signals from receptor tyrosine kinases. In summary, we report here on the physical association and cross-activation of c-Kit by IL-33R in human and murine mast cells. This novel interaction between a TIR-family member and an RTK may also apply to signaling activated by additional TIR-family members in other cell lineages and thus broaden the opportunities to modulate these important networks.

Acknowledgments

We are grateful to Drs E. Buchdunger (Novartis), J. H. Butterfield (Mayo Clinic), P. M. Kloetzel, E. Kruger, D. Ludwig, Marcus Maurer (Charité-Universitätsmedizin Berlin), A. N. McKenzie (Medical Research Council Laboratory of Molecular Biology), and F. Weih (Fritz Lippmann Institute) for cells, reagents, mice, or access to special equipment. We thank Drs O. Krämer and F. D. Böhmer (Friedrich Schiller Universität Jena) and all members of our laboratory for helpful discussion and C. Berns (Department of Dermatology, Universität Köln), C. Heinemann, and Dr I. Irmler (Department of Immunology, Universitätsklinikum Jena) for excellent experimental assistance.

This work was supported by grants from Deutsche Forschungsgemeinschaft (SFB 604, TPC5 [T.K. and J.N.]), and the Fritz Thyssen Foundation (grant nos. 10.05.2.171, 10.07.2.142 [K.H. and A.R.]), and Interdisziplinäres Zentrum für Klinische Forschung (IZKF J3 [S.D.]).

Authorship

Contribution: S.D. and S.H. designed the research, performed experiments, analyzed data, made the figures, and

wrote the paper; T.K. designed the research, analyzed data, and wrote the paper; S.W., T.L., L.B., J.S., and J.F. performed experiments and analyzed data; M.G., A.G., S.G., D.R., and O.R. performed experiments; and A.D., J.N., K.H., and A.R. analyzed data.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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