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Original Article

IL-33 fine-tunes mast cell degranulation and chemokine production at the single cell level

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ABSTRACT**Background**

Mast cells are versatile key components of allergy and inflammation, known to respond to both innate and adaptive immunological stimuli. However the response of individual mast cells to cumulative stimuli remains poorly understood.

Objectives

To dissect mast cell responses at the single-cell level and their potentiation by IL-33.

Methods

We monitored mast cell degranulation in real time by exploiting the capacity of fluorochrome-labeled avidin to stain degranulating cells. During the degranulation process, granule matrix is externalized and immediately bound by fluorochrome-labeled avidin present in the culture medium. The degranulation process is monitored either by time-lapse microscopy or FACS analysis.

Results

Single cell analysis revealed a strong heterogeneity of individual mast cell degranulation responses. We observed that the number of degranulating mast cells was graded according to the FcεRI stimulation strength whereas the magnitude of individual mast cell degranulation remained unchanged, suggesting an all-or-none response of mast cell following FcεRI triggering. IL-33 pretreatment increased not only the number of degranulating and chemokine producing mast cells but also the magnitude of individual mast cell degranulation and chemokine production.

Conclusion

We illustrate the impact of IL-33 on mast cell biology at the single cell level by showing that IL-33 potentiates IgE-mediated mast cell responses by both increasing the number of responding cells and by enhancing the responses of individual mast cells.

50

51 **Key Messages**

- 52 • Fluorescent avidin allows to monitor mast cell degranulation at the single-cell and
53 population levels by time-lapse microscopy and flow cytometry.
- 54 • Single-cell analysis reveals that increasing the stimulation via FcεRI results in
55 degranulation of an increasing number of mast cells but does not change the
56 degranulation extent of individual cells
- 57 • IL-33 favors mast cell degranulation and chemokine production by increasing the
58 magnitude of individual mast cell response.

59

60 **Capsule summary**

61 We show that the alarmin IL-33 induces high responder mast cells upon IgE-mediated
62 stimulation. Our results indicate that IL-33 production by microenvironment might impact
63 allergic reactions by enhancing the inflammatory potential of individual mast cells.

64

65 **Key words**

66 Mast cells; degranulation; IL-33; time-lapse flow cytometry; avidin

67

68 **Abbreviations used**

69 Ab, antibody; DNP, Dinitrophenol, FI, fluorescence intensity, hMC, human mast cell; HSA, human
70 serum albumin; IFI, Integrated fluorescence intensity; SCF, Stem cell factor; gMFI, geometric
71 mean fluorescence intensity; TSLP, thymic stromal lymphopoietin.

INTRODUCTION

Mast cells are innate immune cells hosted in all tissues and more particularly in tissues facing the environment such as skin and mucosa. Mast cells are not only sentinel cells capable of sensing danger signals but also effector cells in virtually all inflammatory processes¹. They are characterized by a cytoplasm filled with storage granules (secretory lysosomes) where several bioactive molecules are stocked². This property endows mast cells with the ability to respond swiftly and efficiently by releasing their granule content in the extracellular milieu, a process called degranulation. Moreover, mast cells are able to synthesize *de novo* a vast array of cytokines and chemokines³. Mast cells respond to a large panel of stimuli and notably to adaptive immune stimuli via Fc receptors (FcRs) and danger signals present in the microenvironment⁴ and are involved in several inflammatory and allergic processes⁵⁻⁷.

Upon allergen contact, epithelial cells produce IL-33, IL-25 and TSLP. These cytokines contribute to the pathogenesis of allergic diseases by boosting function of ILC2s, basophils and mast cells⁸⁻¹⁰. The genes encoding for IL-33 and its receptor have been identified as susceptibility loci for asthma¹¹ and IL-33 is implicated in several allergic processes¹². Moreover, mast cells seem to react mainly to IL-33¹³⁻¹⁵. IL-33 functions as an alarmin secreted by dying or damaged cells^{16, 17}. Its production is exacerbated in asthma¹⁸, in chronic obstructive lung disease¹⁹ or in inflammatory bowel diseases²⁰. On the one hand, IL-33 promotes mast cell maturation (notably by increasing the amount of pre-stored mediators^{21, 22}), induces cytokine production and increases degranulation induced by IgE/antigen stimulation²³⁻²⁶. On the other hand, prolonged treatment with IL-33 has been found to down-regulate mast cell signaling²⁷ suggesting that IL-33 exerts different effects on mast cells in chronic conditions when compared to acute allergic phase. Moreover, stimulation of mast cells with IL-33 in the absence of IgE crosslinking can even play an anti-inflammatory role by inducing Treg expansion, as shown in a papain-induced innate-type airway inflammation model²⁸. These results show that IL-33 is a multifaceted cytokine that can play different roles in the immune response, not all of them are presently fully characterized. In the present work, to better understand the role played by IL-33 in the early steps of mast cell activation, we analyzed in detail several mast cell responses triggered by FcεRI aggregation following pretreatment with IL-33 at the single cell level.

Pioneering studies that investigated the impact of IL-33 on mast cell degranulation and cytokine production were based on bulk assays²³⁻²⁶. Indeed, mast cell degranulation is usually assessed by measuring the amount of released granule molecules in the cell supernatant such as β -hexosaminidase, tryptase or histamine. These assays do not take into account the heterogeneity of the single-cell responses. Accordingly, several lines of evidences indicate that population data do not faithfully reflect how single cells respond to stimuli²⁹. Indeed, bulk assays can provide similar results

for stimulated populations exhibiting either a high frequency of low-responder cells or low frequency of high-responder cells. Nevertheless, the presence of low- or high-responder cells might have utterly different outcomes in pathophysiological settings. Single cell analysis of degranulation was addressed more than 30 years ago in mast cells and basophils by measuring cellular parameters associated with the degranulation process using flow cytometry³⁰⁻³⁵. These studies pointed out the importance of single cell analysis by showing a heterogeneity in the cellular responses and by outlining that, depending on the stimulus or the cell type, different cell behaviors could be observed. Nevertheless the methods employed so far measured basophil or mast cell responses across the degranulation process such as F-actin polymerization, CD11b expression, CD63 exposure or forward scatter changes but did not measure directly the terminal step of the process (i.e. granule exteriorization). Besides, these measurements did not always reflect the amount of mediators released upon degranulation³⁴. To go further, we set up a method that allows us to measure the final step of degranulation in individual mast cells. This method allows to monitor degranulation by microscopy or flow cytometry at the single-cell level and relies on the mast cell granule matrix properties. Mast cell granules contain a serglycin proteoglycan matrix where several mast cell mediators are embedded³⁶. Serglycin is made of a protein core to which sulfated and thereby negatively charged glycosaminoglycan (GAG) such as heparin/heparin sulfate or chondroitin sulfate are linked. Avidin was shown to selectively bind to heparin GAG³⁷ and to bind highly preferentially to mast cell granules rather than to other cellular structure. In human and rodent, avidin conjugated to fluorochrome was shown to selectively stain mast cells in fixed preparation/tissues^{38, 39} suggesting that this non cell-permeant dye might be used to monitor degranulation in live cells. Upon degranulation, the granule and plasma membrane fuses together and the granule matrix enters in contact with the extracellular milieu and is immediately bound by the avidin probe. In a previous study, this method allowed us to show that mast cell form degranulatory synapses with antibody-targeted cells⁴⁰.

In the present study, we used this avidin-based method to analyze human mast cell degranulation simultaneously with either intracellular $[Ca^{2+}]_i$ increase or chemokine production at the single-cell level. This analysis showed a high heterogeneity of individual mast cell responses. While only a fraction of the mast cell population degranulated in response to FcεRI triggering, responding cells exhibited different levels of degranulation. Interestingly, increasing the strength of stimulation augmented the fraction of responding cells but did not influence the mean level of individual cell responses, suggesting that FcεRI triggering induces an all-or-none degranulation response. We also monitored responses of individual mast cells to cumulative stimuli by investigating how individual mast cell responses to FcεRI aggregation were influenced by IL-33. Our results reveal that IL-33 increases the frequency of mast cells responding to FcεRI aggregation and induces cells with a high-

responder phenotype by augmenting, at the single cell level, the magnitude of degranulation and chemokine production.

Methods

Confocal microscopy analysis of hMC degranulation. 5×10^4 hMCs previously sensitized with human IgE were plated on Poly-D-Lysine (Sigma)-coated Lab-Tek™ chambered coverglass (Nunc) in Tyrode's buffer supplemented with 8 $\mu\text{g}/\text{mL}$ avidin-sulforhodamine 101 (Av.SRho, highly cationic glycoprotein that selectively stains mast cell granules) or Annexin-V-Alexa647 (final 3% v/v) or FM1-43 (5 $\mu\text{g}/\text{mL}$) as indicated and warmed at 37°C for 20 min. Cells were stimulated with 2.5 $\mu\text{g}/\text{mL}$ goat IgG anti-human IgE at time=0. Fluorescence was acquired every 2.3 seconds using Zeiss LSM 710 confocal microscope and ZEN software, environmental chamber (37°C and 5% CO₂), 63x Plan-Apochromat objective (1.4 oil). The same protocol was applied to 1×10^4 mast cells embedded in 200 μL BD Matrigel™ (2.5 mg/mL in Tyrode's buffer). z-stacks images were acquired with an interval of 0.4 μm , 30 min post stimulation. In other experiments, hMCs were stimulated as above onto Poly-D-Lysine-coated diagnostic slides (Thermo Scientific) for 30 min at 37°C then fixed and stained for chymase and tryptase. The samples were mounted and examined using a Zeiss LSM 710 confocal microscope and ZEN software with a 63x Plan-Apochromat objective (1.4 oil), electronic zoom 3.

Image quantification. Movies were analyzed using the Region Measurements function of Metamorph software (Universal Imaging, Downingtown, PA). 3D projection of degranulated mast cell was obtained using the interactive 3D surface plot (Image J) and 3D reconstruction of degranulated mast cell in Matrigel was obtained with Imaris software (Bitplane, Oxford Instruments). Colocalization analysis was performed using the Linescan function of Metamorph software (Universal Imaging, Downingtown, PA).

Mast cell degranulation assays. hMCs were sensitized with 1 $\mu\text{g}/\text{mL}$ human IgE overnight. For time-lapse flow cytometry analysis, 1×10^6 hMCs were washed and distributed in Tyrode's buffer containing 5 $\mu\text{g}/\text{mL}$ Av.A488. Acquisition was performed in a final volume of 1 mL at 37°C. Baseline (F_0) was set during 3.8 minutes then cells were stimulated with indicated concentrations of anti-IgE Abs for ~ 20 minutes (F) using the time function of the LSRII cytometer (BD Biosciences). For degranulation assays, 5×10^4 human IgE-sensitized hMCs were distributed in 96-well flat-bottom plates at a density of 1×10^5 cells in 50 μL Tyrode's buffer and adapted to 37°C for 20 minutes. hMCs were then stimulated with anti-IgE Abs in 50 μL prewarmed Tyrode's buffer for 30 min at 37°C, 5% CO₂. Supernatants were harvested and β -hexosaminidase was assayed by measuring release of p-nitrophenol from the substrate p-nitrophenyl N-acetyl- β -D-glucosaminide. The cells were next stained with 5 $\mu\text{g}/\text{mL}$ of Av.A488 or anti-CD63-PE at 4°C for 30 min then proceeded to flow cytometry analysis (MACSQuant®

Analyzer 10, Miltenyi Biotec). FlowJo software (Tree Star, Inc, Ashland, Ore) was used to analyze the data. In some parallel experiments, hMCs were sensitized overnight with 1 µg/mL anti-DNP IgE and stimulated with DNP-HSA.

Ca²⁺ measurements. IgE-sensitized hMCs were preloaded for 45 minutes with 5 µmol/L Indo-1 AM calcium probe then washed with PBS. Cells were prewarmed at 37°C in Tyrode's buffer containing 5µg/mL Av.A488 and the baseline ratio was acquired for 3.8 minutes before the addition of anti-IgE Ab with LSR II flow cytometer (Becton Dickinson). Relative calcium response (F/F_0) means are from five independent experiments.

Fluorescent cell barcoding. Cells were stained for 20 minutes with either low (0.25nM), medium (1nM), or high (5µM) concentration of CellTrace™ violet, or low (0.056nM), medium (0.25nM), or high (1µM) concentration of CellTrace™ far red. Cells were next washed, pooled in the same tube and immediately used for $[Ca^{2+}]_i$ or degranulation measurement by flow cytometry.

Data analysis. Correlations between β-hexosaminidase released and flow cytometry measurements were analyzed (10 independent experiments) using linear or nonlinear (Hill equation, $y = \frac{x^a}{k^a + x^a}$) regressions.

Statistical analysis. Unpaired Student's *t*-tests or Mann Whitney tests were used for comparing two groups (P value range is indicated: ns> 0.05, *P <0.05, **P <0.01, and ***P <0.001, ****P<0.0001. Av.A488 fluorescence intensities were extracted from FlowJo software (n~10 000 for each condition) and plotted using Box and Whisker plots and Mann Whitney test was used to compare fluorescence intensities and Wilcoxon matched-paired test was used to compare fluorescence intensity medians from at least 5 different experiments. Flow cytometry profiles and microscopy images are representative of repeated (three times or more) experiments.

A complete description of the methods is available in this article's Online Repository.

RESULTS

Avidin binds to mast cell surface upon degranulation

IgE-sensitized primary human mast cells (hMCs) were stimulated with anti-IgE Abs in the presence of avidin-sulforhodamine (Av.SRho) in the incubation medium to monitor degranulation dynamics by high-resolution time-lapse confocal microscopy. As shown in Video E1 and Fig. 1A, the budding granules were detected on the hMC surface after about 5 minutes of stimulation. During the degranulation process some exocytosed granules were released in the extracellular milieu whereas a substantial part of exocytosed granules was retained on the hMC surface by forming a corona. We used the Region Measurement function of the MetaMorph software to measure the integrated fluorescence intensity (IFI) of the Av.SRho staining on the hMC surface (Fig.1B). As shown in the figure the cells analyzed exhibited different response curves plateauing at different fluorescence intensities.

To ascertain whether avidin staining revealed exteriorized granules bound on the mast cell surface, we stained for tryptase and chymase 30 min after stimulation. Confocal images (Fig. 1C) and fluorescence intensity analysis (Fig. 1D-E) showed a superimposition of the Av.SRho staining with tryptase and chymase on the cell surface. To rule out the possibility that granules remnants might stay attached on the cell surface due to culture conditions in the absence of surrounding extracellular matrix, we performed similar experiments in a surrogate extracellular matrix. Three-dimensional reconstitution of degranulated cells in Matrigel showed that, in parallel with granule release, mast cells retained on their surface exteriorized granules (Fig. 1F).

We next compared the avidin-based method with two other methods previously employed to monitor cell secretion: annexin-V binding and FM1-43 staining. Since phosphatidylserine exposure occurs at sites of secretory granule exteriorization following FcεRI triggering⁴¹, annexin-V has been previously used to analyze mast cell degranulation⁴². FM1-43 has been widely employed to monitor exocytosis in secretory cells^{43, 44}. We induced mast cell degranulation in the presence of Av.SRho, annexin V-Alexa647 and FM1-43 (added to the incubation medium) and analyzed the fluorescence of these three probes by confocal time-lapse microscopy (Fig. E1 and video E2). Recorded IFI (Fig. E1 B) showed a simultaneous increase of the Av.SRho and FM1-43 Integrated Fluorescence Intensity (IFI) while annexin V-Alexa 647 IFI increase was slightly delayed. The three probes co-localized on the mast cell surface at the end of the recording time (Fig. E1 C-D).

Together, the above results show that fluorochrome-labeled avidin allows to monitor degranulation kinetics and to directly visualize the degranulated material at the single-cell level using confocal laser scanning microscopy.

Heterogeneity of hMC degranulation at the population level notwithstanding homogeneous calcium responses

We next employed flow cytometry to measure the degranulation extend of individual cells at the population level using fluorescent avidin. IgE-sensitized hMCs were incubated in Tyrode's buffer containing Avidin-A488 at 37°C and acquired for a few minutes using the Time function of the cytometer to set the fluorescence baseline (F_0 , Fig. 2A-B and video E3). At $t=3.8$ min, hMCs were stimulated by adding 2.5 $\mu\text{g}/\text{mL}$ anti-IgE Abs. Acquisition of cells up to a total time of 25 min showed that hMCs progressively stained with avidin-A488 and that fluorescence increased in some cells while others remained negative (Fig. 2 A-B and video E3). Relative Avidin-A488 fluorescence F/F_0 of the whole population showed a rapid increase that reached a plateau within ~ 5 min after stimulation (Fig. 2C) indicating that mast cell surface covering with granule remnants (i.e. corona formation) reached its maximum within 5 min. Noteworthy, also the maximum of β -hexosaminidase release in the medium (a gold standard method to detect degranulation) was reached 5 min after stimulation with 2.5 $\mu\text{g}/\text{mL}$ anti-IgE-Abs (Fig. E2). Nevertheless, β -hexosaminidase release is a bulk assay that does not illustrate the behavior of individual cells. Conversely, avidin binding assay allowed single cell analysis and revealed the behavior of individual cell responses. If all the hMCs would degranulate to the same extent, the avidin-A488 fluorescence intensities would distribute in a Gaussian manner at the end of the degranulation process. In opposition, our analysis showed that after plateauing, the hMC avidin fluorescence intensity profiles of avidin⁺ hMC population (analyzed 8, 12, 16, 20 minutes post stimulation, see gates in Fig. 2B) did not exhibit a Gaussian distribution and remained stable during the time (Fig. 2D). To further evaluate the hMC response heterogeneity, we performed similar experiments using another stimulation system: anti-DNP IgE/DNP-HSA. We observed a non-Gaussian distribution of the degranulated hMCs (Fig. E3 A-B). This non-Gaussian distribution was also observed with suboptimal or supra-optimal stimuli. To rule out the possibility that the heterogeneity we observed might be due to the *in vitro* expansion of hMCs, we also investigated the degranulation distribution profile of *ex vivo* mouse peritoneal mast cells using the mouse anti-DNP IgE/DNP-HSA as a stimulation system. We made conspicuous a strong heterogeneity of the degranulation magnitudes (Fig. E4 A-B). Taken together, these results show heterogeneity of hMC degranulation responses at the single cell level, with cells exhibiting a responder profile ranging from very low to high.

Since $[\text{Ca}^{2+}]_i$ increase is a key event in granule exocytosis, we analyzed by time-lapse flow cytometry both calcium responses and mast cell degranulation following Fc ϵ RI triggering. Mast cells were pre-loaded with the ratiometric calcium probe indo-1 and incubated in Tyrode's medium containing

avidin-A488 at 37°C. Resting cells were monitored by flow cytometry during 3.8 min (for baseline setting), then cells were stimulated with anti-IgE abs and immediately analyzed by flow cytometry for 10 min. $[Ca^{2+}]_i$ increase of the hMC population was proportional to the stimulus strength and all the cells underwent comparable $[Ca^{2+}]_i$ increase while only a fraction of cells underwent degranulation (Fig. 2E-F and video E4). Interestingly, comparison of indo-1 ratios measured 2 min before and 2 min after stimulation (see grey and red boxes in Fig. 2F) showed that the whole mast cells population underwent $[Ca^{2+}]_i$ increase at any anti-IgE abs concentration tested (Fig. 2G). In a parallel approach, we measured phosphotyrosine (PTyr) staining by FACS analysis following stimulation with anti-IgE abs, a parameter associated to cell protein kinase function^{45, 46}. In line with $[Ca^{2+}]_i$ measurements, this approach showed that the whole mast cell population underwent a dose-dependent tyrosine kinase signaling following stimulation.

These results indicate that the whole hMC population is triggered via FcεRI aggregation. Nevertheless, activation of main signaling pathways in individual cells is not always followed by degranulation.

Mast cell degranulation induced by FcεRI triggering is an all-or-none response

We next investigated whether the stimulation of mast cells with increasing concentrations of anti-IgE Abs would result in an increased degranulation at the single-cell level. Cells were analyzed 30 minutes after stimulation (when the degranulatory process is completed, Fig 2 C) by flow cytometry and by measuring β-hexosaminidase release. The percentage of avidin-A488⁺ cells and avidin-A488 geometric MFI (gMFI) of the entire hMC population increased with the strength of the stimulation, similarly to the percentage of β-hexosaminidase release estimated using the classical β-hexosaminidase assay (Fig 3 A-C). We next analyzed whether the avidin-based single cell measurements reflected the extent of the degranulation, as measured by a standard bulk assay (β-hexosaminidase release). We first determined the relationship between the percentage of degranulated cells as measured by flow cytometry and the percentage of β-hexosaminidase release. The data were fit either to a linear function or to a Hill equation similarly to a previous study on basophil degranulation³⁴ (Fig 3 D). Linear regression showed a good fitting ($0.95 < r^2 < 0.98$) for all experiments analyzed and non-linear regression showed a good fitting with a Hill coefficient a very close to 1 and a very high k constant value indicating a linear relationship. This analysis showed that while the two assays do not measure the same parameter of the degranulation process, the percentage of avidin⁺ cell is linearly correlated with the percentage of degranulation as measured by a standard assay. A similar analysis indicated also that the amount of β-hexosaminidase released and

the Av.A488 gMFI of the hMC population were linearly correlated (Fig 3E). Thus these results show that avidin-A488 gMFI is a good measurement of the degranulation extent at the population level and that the fraction of mast cells that degranulate increases with the strength of the FcεRI stimulation.

The Avidin-A488-based assay therefore allowed us to study the degranulation magnitude of individual cells within the mast cell population by analyzing the avidin-A488 fluorescence intensity of cells undergoing degranulation (avidin-A488⁺ cells). Surprisingly, the magnitude of degranulation of avidin⁺ mast cells did not increase according to the stimulus strength (Fig 3 F). Box and Whisker plots made conspicuous a similar repartition of avidin-A488 fluorescence intensity among hMCs with no statistical differences between hMCs stimulated with low or high concentration of anti-IgE Abs. Moreover, analysis of the median avidin-A488 fluorescence intensity using hMCs from different donors showed that the median response did not change with the strength of the applied stimulus (Fig 3 G). Additional sets of experiments were conducted using different stimulation systems: anti-DNP IgE/DNP-HSA (to avoid a possible involving of FcγRIIB via anti-IgE Abs stimulation) and cell bound antigens (anti-CD20 IgE/B cells⁴⁰). We observed that in conditions in which sensitized hMCs were stimulated with a multivalent antigen or with cell bound antigens, degranulation occurred in an all-or-none fashion (Fig E3 C-F). Similar results were obtained using ex vivo mouse peritoneal mast cells sensitized with anti-DNP IgE and stimulated with increasing concentrations of DNP-HSA (Fig E4 C and D), showing that this all-or-nothing response is not limited to primary cell lines.

Taken together, the above results show that, independently of the method used to aggregate FcεRI and of the degree of FcεRI aggregation, an increased stimulation strength recruits an increased number of hMCs to degranulate without increasing the magnitude of degranulation at the single-cell level. They imply that FcεRI-mediated degranulation is an all-or-none response in individual mast cells.

IL-33 augments the extent of hMC degranulation without affecting calcium signaling

IL-33 is involved in allergy and has been shown to potentiate mast cell responses⁴⁷, nevertheless its impact on human mast cell degranulation has not been thoroughly studied. We first investigated the impact of IL-33 on hMC degranulation kinetics. We performed a set of experiments similar to that described in Fig 2, A with cells pretreated with 1-5 ng/mL IL-33 for one hour. To compare the degranulation kinetics of hMCs pretreated or not with IL-33, it was important to stimulate all the cells under identical experimental conditions and at the same time. To this end, we employed a

barcoding method by staining each IL-33 pretreated cells with a known concentration of a fluorescent cell tracer (Fig 4, A). Degranulation analysis at the population level showed that IL-33 potentiated in a dose-dependent manner the degranulation induced by a suboptimal concentration of anti-IgE Abs (Fig 4, B-C, video E5) without changing FcεRI expression level (Fig E5). IL-33 did not modify the time course of degranulation that started at the same time and reached a plateau after about 5 minutes in all tested conditions. Noticeably, pretreatment with 5 ng/mL IL-33 induced a degranulation magnitude comparable to that obtained with a high concentration of anti-IgE Abs (compare Fig 4, C to Fig 2, C). In parallel experiments, we investigated whether IL-33 pretreatment might affect $[Ca^{2+}]_i$ increase following FcεRI aggregation. We observed that $[Ca^{2+}]_i$ increase exhibited similar time kinetics and amplitude of responses both in untreated and in IL-33 pretreated hMCs (Fig 4, D-F). Taken together, these results indicate that IL-33 potentiates hMC degranulation via a mechanism that does not affect calcium signaling.

IL-33 drives individual mast cells toward a high responder phenotype

The degranulation kinetics analysis by flow cytometry showed the heterogeneity of the hMC response and suggested that IL-33 induced the emergence of cells exhibiting high degranulation responses (Fig 4, B and video E5). We thus analyzed whether IL-33 could enhance the magnitude of degranulation by individual hMC using the same approach used in Fig 3. As shown in Fig 5, A and B, the frequency of degranulated cells following anti-IgE Abs stimulation augmented with the concentration of IL-33 pretreatment, in particular at suboptimal concentrations of anti-IgE Abs. IL-33 *per se* did not induce degranulation. Statistical analysis of the avidin staining (avidin-A488 fluorescence intensity) of responder cells (avidin-A488⁺) following stimulation with a suboptimal concentration of anti-IgE Abs showed that not only the frequency but also the magnitude of the degranulation did increase according to the IL-33 concentration. Box and Whisker plots indeed showed that avidin-A488 median fluorescence intensity of individual hMC, in the responder population, increased according to the concentration of IL-33 (Fig 5, C). Moreover, analysis of the avidin-A488 median fluorescence intensity as measured in different donors showed that the response increased with the strength of IL-33 stimulation (Fig 5, D). Similar results were obtained using anti-DNP IgE sensitized mast cells stimulated with DNP-HSA (Fig E6). Moreover, to test whether IL-33 potentiated degranulation also in *ex vivo* mast cells, we employed mouse peritoneal cells sensitized with anti-DNP IgE. We observed that following stimulation with DNP-HSA, IL-33 pretreatment enhanced both the frequency of responder cells and their degranulation extent (Fig E4 E-

F). Taken together, our results indicate that IL-33 augments both the frequency of degranulating hMCs and the magnitude of individual hMC degranulation following FcεRI triggering.

To test whether IL-33 modulates individual mast cell function beyond degranulation, we analyzed the production of the pro-inflammatory chemokines CCL4 and CXCL8 in IL-33-pretreated hMCs. hMCs pretreated or not with IL-33 for one hour, were stimulated with anti-IgE Abs for 3 hours to induce chemokine production. Cells were stained with avidin-A488 then fixed, permeabilized and stained for CCL4 or CXCL8 and analyzed by flow cytometry. Flow cytometry dotplots showed a heterogeneity of the response with cells exhibiting a large range of response intensities (Fig 6, A). In the absence of IL-33 pretreatment, CCL4⁺ frequency augmented according to the concentration of the anti-IgE Abs stimulus. IL-33 pretreatment by itself induced a low percentage of CCL4⁺ or CXCL8⁺ hMCs. Interestingly, IL-33 augmented both the frequency of CCL4⁺ and CXCL8⁺ cells and the extent of both chemokine production at the single cell level mostly in conditions in which mast cells were stimulated using a suboptimal concentration of anti-IgE Abs (Fig 6, B-E).

These results indicate that IL-33 induces the emergence of hMCs with an “high responder” phenotype, both for degranulation and chemokine production, within an heterogeneous mast cell population.

DISCUSSION

We have previously shown that fluorescent avidin staining allows to monitor spatiotemporal degranulation events in mast cells and that mast cells can polarize their secretion toward antibody-targeted cells⁴⁰. Here, we analyzed in depth mast cell degranulation by investigating the individual cell behavior within the whole population. Our study relies on fluorochrome-labelled avidin added in solution during mast cell stimulation. This probe immediately binds to the heparin rich matrix exposed on the cell surface during the exocytosis process. Using this approach, we found a strong heterogeneity of mast cell biological responses following FcεRI aggregation. Individual mast cells provided all-or-none responses when triggered by anti-IgE Abs, while they were activated in a graded fashion after pre-treatment with increasing concentrations of IL-33.

It is established that IgE-mediated degranulation involves a phenomenon of compound exocytosis by which membranes of surrounding individual granules fuse with each other and with the plasma membrane forming intracellular degranulation chambers^{48, 49}. Taken together with previous electron microscopy studies⁵⁰ our confocal microscopy results suggest that granules can stay in those chambers or are extruded as pellets that can spread out in the extracellular space or remain attached to the cell surface. The advantage of our method over previous observations is that it allows to measure the extent of degranulation in live cells and at the single cell level by quantifying the exteriorized granule fraction that remains on the cell surface. It should be noted that this method can be used to monitor secretory lysosomes exteriorization in other cell systems such melanoma cells.⁵¹

Non-genetic cell-to-cell functional variability is an emerging concept that helps to better understand how individual cells behave following cell stimulation or drug treatment^{52, 53}. Degranulation analysis by flow cytometry makes conspicuous a strong heterogeneity of mast cell response. This is in agreement with the confocal microscopy results showing that the fluorescent corona formation during the degranulation process is a progressive phenomenon plateauing at variable fluorescence intensity in individual cells. Interestingly, our approach also allowed us to measure simultaneously, in live cells, degranulation and $[Ca^{2+}]_i$ increase revealing that while mast cell degranulation magnitude is heterogeneous at the single-cell level, $[Ca^{2+}]_i$ increase showed a noticeable homogeneity in virtually all cells. These results suggest that heterogeneity of degranulation responses in mast cell population is downstream of early FcεRI-mediated signaling.

Another degree of heterogeneity of mast cell responses was observed when chemokine production was measured in parallel with degranulation. This analysis showed a large array of responses ranging from non-responding cells to cells that provided a strong degranulation response without chemokine production and to cells that exhibited both degranulation and chemokine production. These results

indicated that these mast cell functional outcomes are not necessarily linked in individual cells and that multifunctional subpopulation can emerge following a given simulation. Such a functional heterogeneity has also been reported in human macrophage cell lines stimulated by LPS^{54, 55}. It is tempting to speculate that a large array of individual mast cell behaviors might be instrumental for robust *in vivo* responses to different challenges. On the one hand, heterogeneous behaviors might allow to avoid large synchronous responses of all tissue mast cells following antigen exposure, thus preventing possible deleterious reactions. On the other hand, they might be helpful to keep some mast cells in a non-degranulated status, ready to respond to repeated antigen stimulation or to other challenges by different antigens.

It is worthy to note that the hMC primary cell lines we generated from several donors were homogeneous in terms of CD117, FcεRI, tryptase and chymase expression (Fig E7). These results, together with the observation that the whole hMC population increased $[Ca^{2+}]_i$ and augmented PTyr levels in an homogeneous fashion in response to FcεRI triggering, rule out the possibility that the heterogeneity observed in functional responses might be due to heterogeneity in FcεRI triggering or in granule content. Accordingly, it is well known that very low FcεRI expression allows efficient mast cell activation⁵⁶ indicating that the level of FcεRI expression is not linked to the magnitude of functional responses. In other words, although all the cells were activated via FcεRI (as detected by $[Ca^{2+}]_i$ and PTyr measurements), a vast range of functional outcomes was observed.

We can hypothesize that important housekeeping cellular mechanisms or biochemical processes might interfere with cellular effector functions such as degranulation or chemokine production. In this line, heterogeneity in mast cell degranulatory response was reported by Liu *et al.*⁵⁷ using an integrated microfluidic platform. The authors suggested that heterogeneity might stem from cell-to-cell variability in the copy number of the tyrosine kinase Lyn.

The concept of functional response heterogeneity is emerging in several cell models including tumor cells, macrophages and CD8⁺ T lymphocytes^{29, 54, 58-60}. In mast cells, heterogeneity of the response was previously proposed by D. Mac Glashan⁶¹. In our study we reinforce this notion by showing that homogeneous mast cell populations derived from blood progenitors still display different functional responses at the single cell level. Because primary cell lines heterogeneity might not always reflect an *in vivo* heterogeneity, we analyzed mouse peritoneal mast cell responses. We observed also in this *ex-vivo* mast cell model, an important functional heterogeneity.

Taken together, the above discussed findings strongly suggest that the definition of functional signature in single mast cell can provide some information that the study of their responses at the

whole population level might fail to reveal. Indeed, in bulk assay the responses are averaged out and specific information from each individual cell can be lost.

Our approach also revealed that, when the mast cell population was triggered by increasing concentrations of anti-IgE Abs, the global Av.A488 gMFI increased according to the stimulus strength and paralleled β -hexosaminidase release in the culture medium. Interestingly, the global increase of Av.A488 fluorescence was the result of the recruitment of an increased number of mast cells to degranulate rather than the result of an increased extent of individual cell degranulation. This finding was made possible by a detailed analysis of the degranulated cells in which the distribution of Av.A488 FI was presented using box-and-whisker plots. This analysis showed that, on the one hand, increasing the stimulation strength through Fc ϵ RI augmented the probability to degranulate for individual cells. On the other hand, the magnitude of degranulation (as detected by Av.A488 FI among Av.A488⁺ cells) remained similar for all anti-IgE Abs concentrations tested, suggesting an all-or-none response. In this line, studies based on different cellular models and stimulation systems reported that degranulation is an all-or-nothing response³⁰⁻³³. Conversely, other studies reported that degranulation appears to be a graded process according to stimulation strength^{34, 35}. The discrepancies between the above mentioned studies might stem from the use of different cellular models and stimuli or from the readouts used.

The methods employed so far to investigate degranulation were based on the measurement of cellular parameters indirectly related to granule exteriorization such as alcian blue staining loss^{30, 31}, change in forward scatter, loss of acridine orange labeling or CD63 exposure^{34, 62}. Those pioneering studies revealed the interest of single cell analysis of degranulation. However some employed methods (ex. CD63 exposure in basophils stimulated with anti-IgE) provided measurements that were not always correlated to the amount of mediators released as discussed by D. MacGlashan³⁴. Those results prompted us to analyze mast cell degranulation by using the CD63 exposure assay and the β -hexosaminidase release assay. Our results show that under our experimental conditions, CD63 exposure did not always follow β -hexosaminidase release notably for supraoptimal stimulations (Fig. E8A). Indeed, the percentage of CD63⁺ hMCs did not linearly correlate with the percentage of β -hexosaminidase release, in agreement with previous reports (Fig E8B)³⁴. In mast cells, on the contrary to the avidin-based assay, the CD63 exposure assay did not always reflect the process of mediator release and seems to not measure strictly the same phenomenon as compared to the avidin-binding assay.

Mast cells are able to sense danger signals and notably express the ST2 receptor for the alarmin IL-33. Accordingly, the primary hMCs derived from peripheral blood progenitors used in this study

expressed ST2 (Fig E7) in agreement with Bandara *et al.*⁶³. Since IL-33 was shown to potentiate β -hexosaminidase release by RBL cells stimulated with IgE/antigen²³ we analyzed the impact of IL-33 on human mast cells at the single cell level.

To accurately compare activation kinetics of cells pretreated with different concentrations of IL-33 we barcoded hMCs with a cell tracer⁶⁴. To our knowledge, this is the first time that live cells are barcoded to analyze cellular responses by time-lapse flow cytometry. As typically described for alarmins, IL-33 is expected to act swiftly on target cells. We thus investigated the impact of IL-33 on mast cell biology after one hour incubation. Time-lapse flow cytometry analysis revealed that IL-33 did not modify the kinetics of degranulation responses but dramatically increased the extent of those responses. Statistical analysis of Av.A488 FI of the degranulated cell population showed that pretreatment with IL-33 augmented not only the fraction of degranulated cells in response to Fc ϵ RI triggering, but also the magnitude of individual cell degranulation. Those observations are in agreement with recent results by Sjöberg *et al.* showing that IL-33 causes increased mast cell β -hexosaminidase and serotonin secretion following antigen-induced degranulation *in vivo*, without increasing the number of tissue mast cells²¹. Mast cell response enhancement by IL-33 is expected to be critical in allergic processes since this cytokine is produced by stressed epithelia upon allergen contact^{8, 65}. Since other cytokines such as TSLP and IL-25 are also produced together with IL-33 in allergic context⁸, we analyzed the effects of TSLP and IL-25 on mast cell degranulation. Our results showed no significant effect of TSLP or IL-25 on mast cell degranulation in our cellular model (Fig. E9).

We next investigated whether a delayed mast cell response distinct from degranulation might be also impacted by IL-33. Our avidin-based method allowed us to simultaneously measure chemokine production and degranulation. CCL4 and CXCL8 production analysis by flow cytometry showed that IL-33 both potentiated the frequency of CCL4⁺ and CXCL8⁺ hMC at the population level and augmented the amount of intracellular CCL4 and CXCL8 in individual cells. The signaling events underlying this phenomenon are poorly understood. Degranulation is mainly driven by rapid signaling events such as intracellular calcium fluxes. In agreement with Andrade *et al.*²³, we did not find any effect of IL-33 on calcium fluxes elicited by Fc ϵ RI triggering, indicating that IL-33/ST2 signaling pathways, that remain to be elucidated, might prime mast cells to be more prone to degranulate and to exteriorize more granules.

All in all, these results suggest that mast cell degranulation is controlled by two mechanisms: a first mechanism that functions similarly to a switch that induces mast cells to degranulate and a second mechanism that sets the magnitude of the degranulation. The IL-33/ST2 axis potentiates hMC

responses induced by FcεRI engagement by increasing both mast cell degranulation frequency and degranulation magnitude. It is tempting to speculate that this potentiation mechanism exerted by IL-33 on mast cell degranulation and chemokine secretion might have a deep impact in conditions in which IgE sensitized mast cells are primed with IL-33 such as in asthma¹⁸ or in atopic dermatitis⁶⁶. In such conditions allergen/IgE might stimulate mast cells and license degranulation while IL-33 secretion might boost degranulation magnitude and exacerbate the clinical symptoms.

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Author contributions

R.J. and E.E. performed experiments; R.J. and E.E analyzed results and made the figures; F.L designed advanced flow cytometry experiments; E.E. and S.V. designed the research and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interest.

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Figure Legends

FIG 1. Avidin-based analysis of mast cell degranulation by confocal microscopy. **A**, time lapse analysis of one hMC stimulated with anti-IgE Abs. **B**, Single cell analyses of Av.SRho IFI. **C**, Avidin, chymase and tryptase extracellular staining following degranulation. **D** and **E**, colocalization analysis. **F**, hMC embedded in matrigel, 30min after stimulation. Data are from one representative experiment out of three.

FIG 2. Fc ϵ RI aggregation induces different degranulation profiles despite homogeneous early signaling events. **A-D**, Av.A488 staining kinetics following stimulation. Data are from one representative video out of five. **E**, Analysis of calcium fluxes and degranulation, representative dotplots. **F**, relative calcium response. **G**, Indo-1 Ratio distribution before (black lines) and after stimulation, numbers indicate indo-1 ratio mean. **H**, Phosphotyrosine staining.

FIG 3. Mast cell degranulation induced by Fc ϵ RI triggering follows an all-or-none response. **A**, Representative FACS profiles. **B**, frequency of Av. A488⁺ hMCs. **C**, Av.A488 gMFI and β -hexosaminidase release (Mean $\pm$ SD). **D-E**, Correlation analysis. **F-G**, Av.A488 FI of the degranulated hMCs (see gate panel A) from one representative experiment (F) or plotted as median FI (G). *P* values were determined using Mann-Whitney test. ns *P*>0.05; ****P*<0.001; *****P*<0.0001.

FIG 4. IL-33 augments the hMC degranulation magnitude. Time lapse degranulation analysis of barcoded hMCs pretreated with IL-33. **A**, Barcoding. **B**, Av.A488 and CellTraceTM violet fluorescence. **C**, Av.A488 relative gMFI over control. **D-F**, Calcium fluxes of barcoded hMCs. Barcoding (D), representative FACS profiles depicting Indo-1 ratio over time (E), relative calcium response represented as means of three independent experiments (F).

FIG 5. IL-33 elicits high responder mast cells. A-B, frequency (mean $\pm$ SEM) of Av.A488⁺ hMCs. *P* values were determined using paired *t* test. **C and D**, Av.A488 FI of the degranulated hMCs from one representative experiment (C) or plotted as median FI (mean $\pm$ SEM) (D). *P* values were determined using Mann-Whitney test (C) or Wilcoxon test (D). ns *P*>0.05; **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

FIG 6. IL-33 potentiates chemokine production and induces high CCL4 or CXCL8 producers following FcεRI triggering. Simultaneous FACS analysis of degranulation and chemokine production. Representative FACS profiles (A). Frequency (mean $\pm$ SEM) of CCL4⁺ (B) or CXCL8⁺ (D) hMCs. CCL4⁺ (C) or CXCL8⁺ (E) hMC FI median. *P* values were determined using paired *t* test. ns *P*>0.05 **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 1

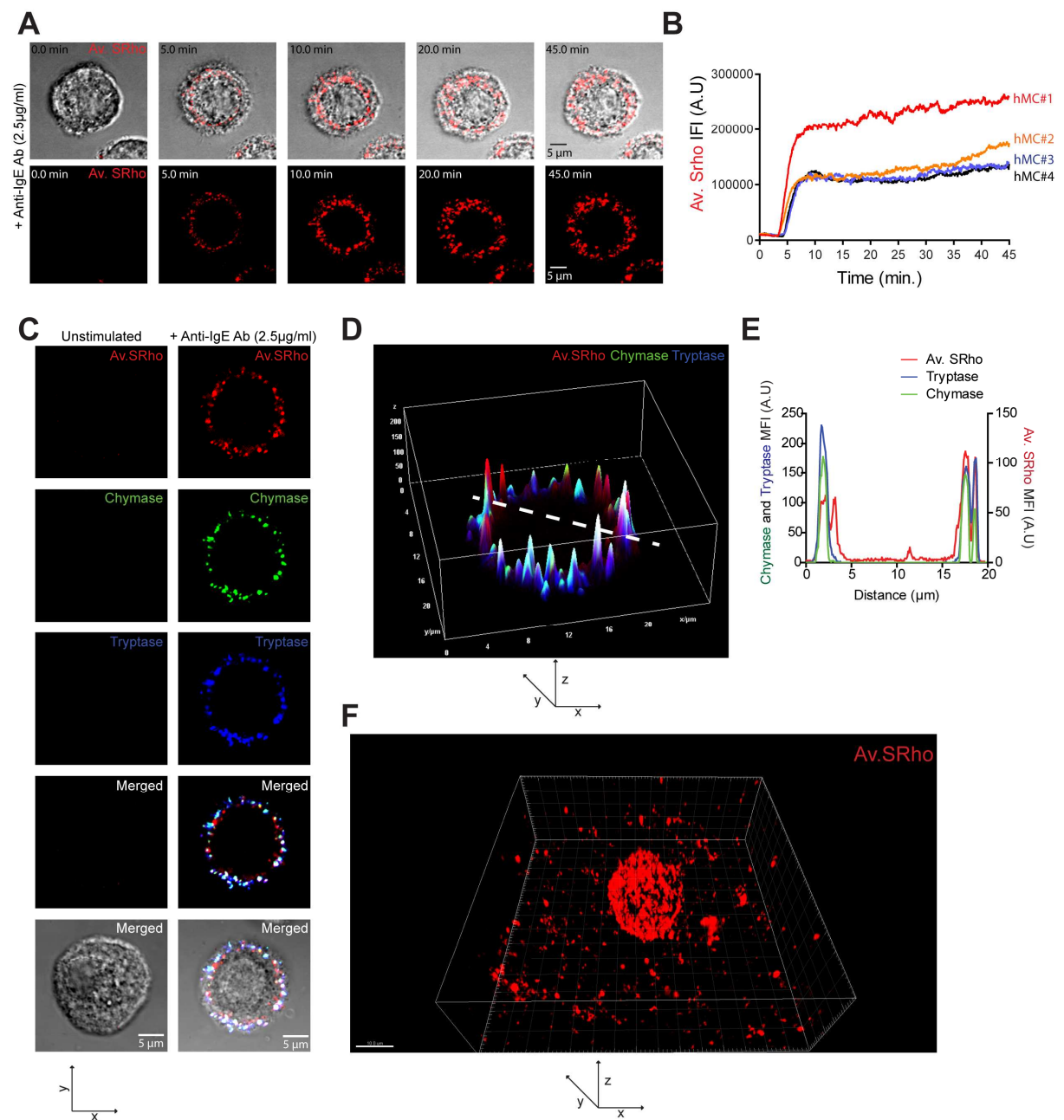


Figure 2

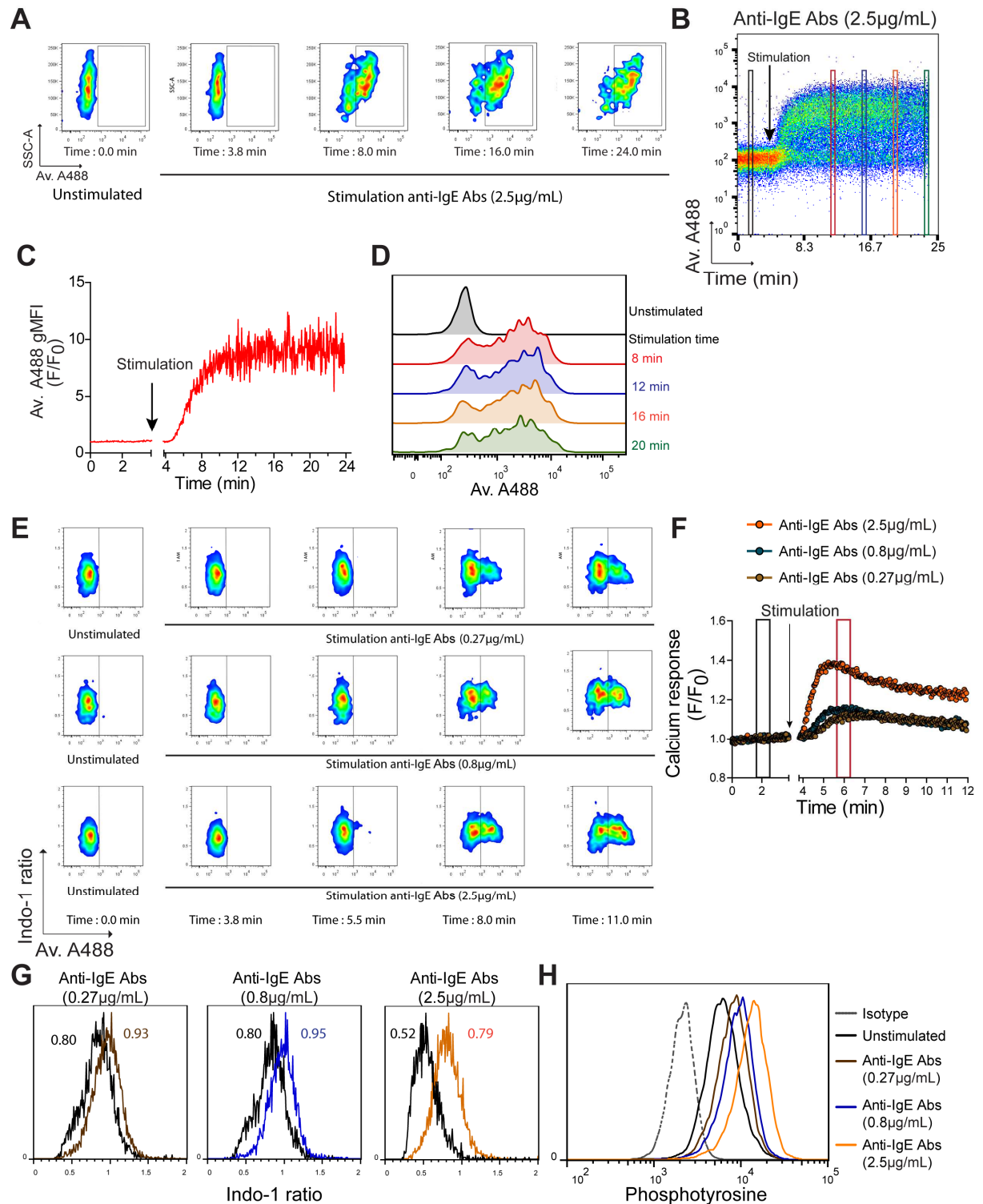


Figure 3

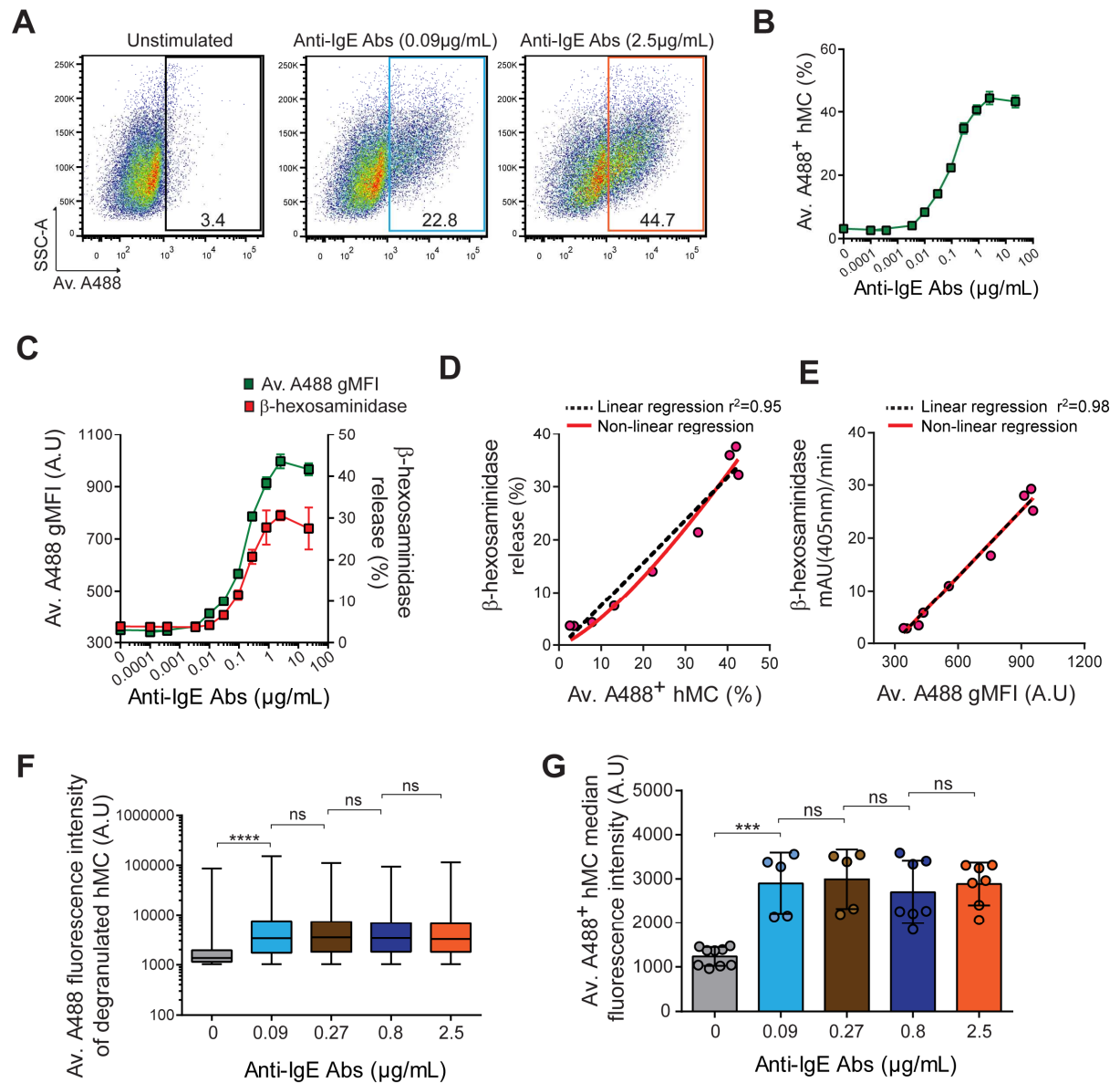


Figure 4

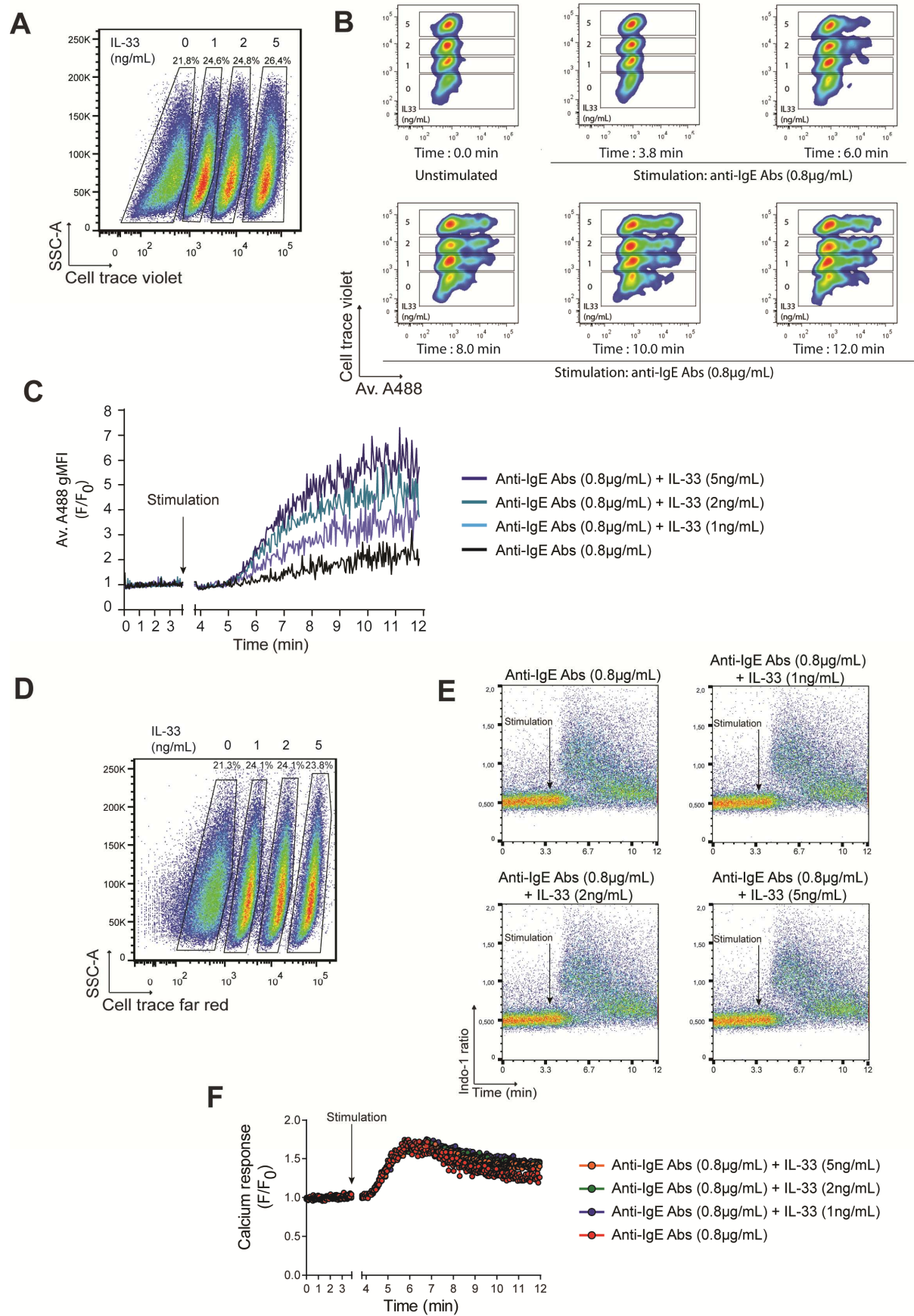


Figure 5

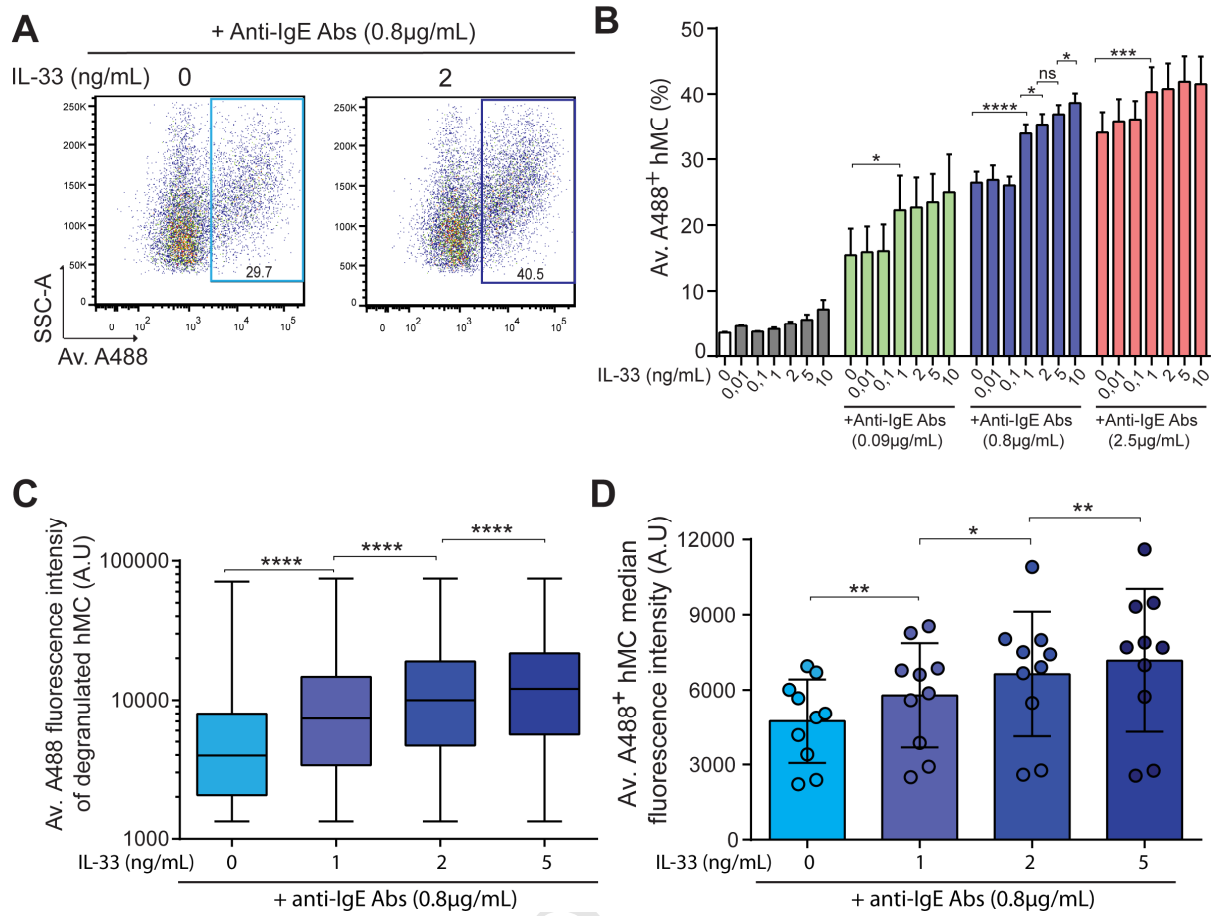
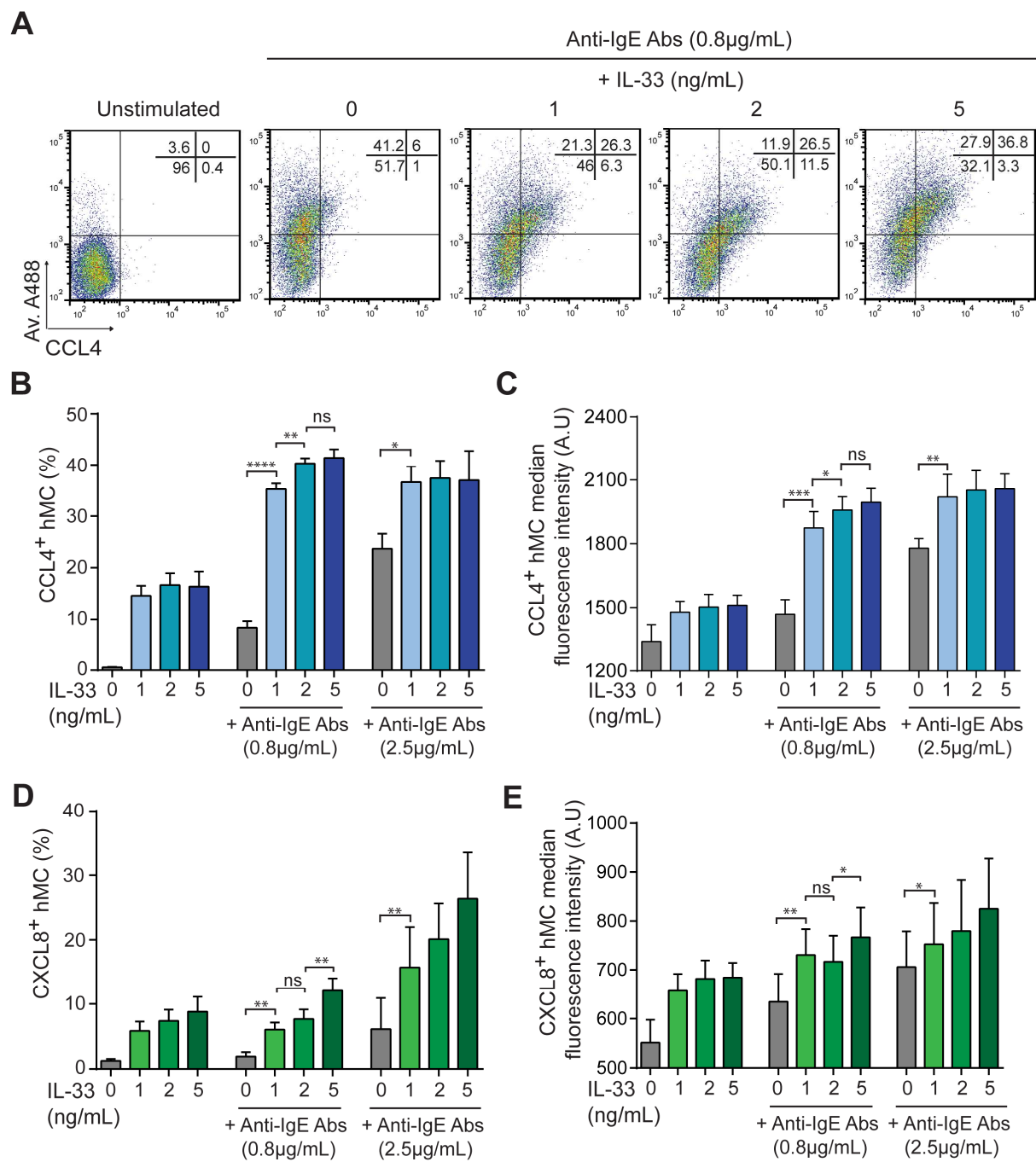


Figure 6



Appendix

Supplementary Methods

Reagents. Anti-mast cell tryptase (clone G-12), anti-phosphotyrosine (clone PY99) all from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-CD117-APC (clone 104D2), anti-CCL4-PE Cy7 (clone D21-1351), anti-CXCL8-PE (clone AS14), anti-CD63-PE (clone H5C6), all from BD Biosciences, San Jose, CA. Anti-mast cell chymase (clone B7, Millipore, Billerica, MA), Anti-FcεRI-FITC (clone AER-37, eBioscience, San Diego, CA). Goat anti-human ST2/IL-1R4 PE from R&D Systems, Minneapolis, MN. Human IgE (1μg/mL, Abcam) was used to sensitize hMCs. Goat anti-human IgE (Life technologies) was used to stimulate hMCs. All the secondary antibodies used (Alexa Fluor-conjugated) were from Molecular Probes, Inc., Eugene, OR. Anti-DNP Mouse IgE (clone SPE-7) and DNP-HSA, Sigma-Aldrich. In some experiments, cells were labelled with CellTrace™ violet, CellTrace™ far red and Indo-1 AM (Molecular probes) according to manufacturer's instructions. Mast cell granule matrix was stained using avidin-sulforhodamine 101 (Av. SRho, Sigma-Aldrich) or Alexa488-labelled avidin (Av. A488, Life Technologies, USA). Annexin-V-Alexa647 and FM1.43 (Invitrogen) were used to monitor degranulation. For some experiments, cells were pretreated for 1 hour with human recombinant IL-33, TSLP or IL-25 (Peprotech, USA).

Human mast cells (hMCs). Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats (Etablissement Français du Sang). CD34⁺ precursors cells were isolated from PBMCs (EasySep™ Human CD34 Positive Selection Kit, STEMCELL Technologies) and grown under serum-free conditions using StemSpan™ medium (STEMCELL Technologies) supplemented with recombinant human IL-6 (50 ng/mL; Peprotech), human IL-3 (10 ng/mL; Peprotech) and 3% supernatant of CHO transfectants secreting murine SCF (a gift from Dr. P. Dubreuil, Marseille, France, 3% correspond to ~50 ng/mL SCF) for one week. Cells were next grown in IMDM Glutamax I, sodium pyruvate, 2-mercaptoethanol, 0.5% BSA, Insulin-transferrin selenium (all from Invitrogen), ciprofloxacin (10 μg/mL; Sigma Aldrich), IL-6 (50 ng/mL) and 3% supernatant of CHO transfectants secreting murine SCF for 8 weeks then tested phenotypically (Tryptase⁺, CD117⁺, FcεRI⁺) and functionally (β-hexosaminidase release in response to FcεRI crosslinking) before use for experiments. Only primary cell lines showing more than 95% CD117⁺/FcεRI⁺ cells were used for experiments.

Phosphotyrosine analysis. 5x10⁴ human IgE-sensitized hMCs were distributed in 96-well flat-bottom plates at a density of 1x10⁵ cells in 50 μL Tyrode's buffer and adapted to 37°C for 20

minutes. hMCs were then stimulated with anti-IgE Abs in 50 μ L prewarmed Tyrode's buffer for 2 min at 37°C, 5% CO₂. Cells were next fixed (2% PFA), washed, permeabilized (0.1% saponin in PBS containing 1% BSA, Sigma-Aldrich) and stained with anti-phosphotyrosine mAbs for 45 min at 4°C. Cells were next stained with goat anti-mouse IgG2b A647 for 30 min at 4°C. Flow cytometric data were acquired on a MACSQuant® Analyzer 10 (Miltenyi Biotec) and were analyzed by using FlowJo software (Tree Star, Inc, Ashland, Ore).

Intracellular chemokine staining. 5×10^4 hMCs were pretreated with IL-33 for 1 hour in Tyrode's buffer at 37°C. Cells were next stimulated with increasing concentration of anti-IgE Abs for 3 hours. Cells were next stained with 5 μ g/mL of Av.A488 at 4°C for 30 min, washed, fixed (2% PFA), permeabilized (0.1% saponin in PBS containing 1% BSA, Sigma-Aldrich), and stained with anti-CCL4-PE-Cy7 or anti-CXCL8-PE mAbs for 45 min. Flow cytometric data were acquired on a MACSQuant® Analyzer 10 (Miltenyi Biotec) and were analyzed by using FlowJo software (Tree Star, Inc, Ashland, Ore).

Mouse peritoneal cells. Peritoneal cells from 5 C57BL/6 mice were collected by peritoneal washing. Non adherent cells were washed in Tyrode's buffer, sensitized with 1 μ g/mL anti-DNP IgE for two hours, washed in Tyrode's buffer, pretreated or not with IL-33 for one hour and next stimulated with DNP-HSA for 30 min. Cell were then stained with av.A488, anti-mouse CD117-APC (clone 2B8, BD Biosciences, San Jose, CA) and Fixable Viability Dye eFluor® 506 (eBioscience, San Diego, CA). for 30 min at 4°C and analyzed by flow cytometry.

hMCs stimulation by cell-bound antigens. hMCs were sensitized or not with human IgE anti-CD20 (1 μ g/mL, Invivogen, San Diego, CA) for 16 hours. The cells were then washed and distributed in 96-well flat-bottom plates at a density of 1×10^5 cells in 50 μ L Tyrode's buffer and adapted to 37°C for 20 minutes. hMCs were then treated with different ratios of JY cells (Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line) in 50 μ L prewarmed Tyrode's buffer for 60 min at 37°C, 5% CO₂. The cells were next stained with 5 μ g/mL of avidin-FITC at 4°C for 30 min then proceeded to flow cytometry analysis.

Supplementary Figure Legends

FIG E1. Avidin staining correlates with Annexin-V and FM1-43 binding assay. A ,Sequence of snapshot from time lapse analysis of one hMC stimulated with anti-IgE Ab in Tyrode's buffer supplemented with Av.SRho

(red), Annexin-V A647 (blue) and FM1-43 (green), scale bars 5 μ m. **B**, measurement of Av.SRho (red), Annexin-V A647 (blue) and FM1-43 (green) integrated fluorescence intensities (data are from Video E2). **C**, 3D projection of Av.SRho (red), Annexin-V A647 (blue) and FM1-43 (green) of a degranulated mast cell 30 minutes after stimulation. **D**, colocalization analysis of Av.SRho, FM1-43 and Annexin-V A647 fluorescences. Data are from one representative experiment out of three.

FIG E2. Kinetics of β -hexosaminidase release. IgE-sensitized hMCs were stimulated with anti-IgE Abs, supernatants were harvested and β -hexosaminidase release was analyzed. Data are shown as mean $\pm$ SEM of one independent experiment out of three.

FIG E3. Mast cell degranulation induced by Fc ϵ RI triggering with multivalent antigens exhibits an all-or-none response. **A-D**, Anti-DNP IgE-sensitized hMCs were stimulated with DNP-HSA for 30 min then stained with Av.A488. **A**, Representative dotplots and associated Av.A488⁺ histogram profiles **B**, frequency of Av.A488⁺ hMCs. **C, D** Av.A488 fluorescence intensity of the degranulated hMCs represented as box-and-whisker plots from one representative experiment (C) or plotted as median fluorescence intensity from 6 independent experiments, histograms represent mean $\pm$ SEM (D). **E-F**, Anti-CD20 IgE sensitized hMCs were stimulated with increasing number of CD20⁺ JY cells (B-EBV cell line) for one hour at 37°C then stained with Av.A488 and analyzed by flow cytometry. **E**, frequency of Av. A488⁺ hMCs. **F**, Median value of Av.A488 fluorescence intensity of Av.A488⁺ mast cells from 5 independent experiments was plotted against B cell : hMC ratio. Each symbol represents an experiment. *P* values were determined using Mann-Whitney test (C) or Wilcoxon test (D,F). ns *P*>0.05; * *P*<0.05; ** *P*<0.01.

FIG E4. Mast cell degranulation induced by Fc ϵ RI triggering follows an all-or-none response and IL-33 pretreatment elicits high responder mast cells in *ex-vivo* mouse peritoneal cells. Anti-DNP IgE-sensitized peritoneal cells pretreated or not with IL-33 for 1 hour were stimulated with DNP-HSA for 30 min then stained with Av.A488, Anti-CD117 and viability dye and analyzed by flow cytometry. **A**, Gating strategy to isolate peritoneal mast cells (pMCs). **B**, Representative dotplots and associated Av.A488⁺ histogram profiles. **C, D** Av.A488 fluorescence intensity of the degranulated pMCs represented as box-and-whisker plots from one representative experiment (C) or plotted as median fluorescence intensity from 5 independent experiments, histograms represent mean $\pm$ SEM (D). **E**, frequency of Av. A488⁺ pMCs. Data are shown as mean $\pm$ SEM from four independent experiments. **F**, Av.A488 fluorescence intensity of the degranulated hMCs represented as median fluorescence intensity from 6 independent experiments, histograms represent mean $\pm$

SEM (F). *P* values were determined using Mann-Whitney test (C) or paired *t* test (D-F). ns $P>0.05$; * $P<0.05$; ** $P<0.01$.

FIG E5. IL-33 stimulation does not increase FcεRI expression. hMCs were stimulated one hour with 1, 2, or 5 ng/mL of IL-33, then cells were stained with anti-FcεRI-FITC and analyzed by flow cytometry. Shown are data from two different donors' cell lines.

FIG E6. IL-33 pretreatment elicits high responder mast cells following FcεRI aggregation by multivalent antigen. Anti-DNP IgE-sensitized hMCs pretreated or not with IL-33 for 1 hour were stimulated with DNP-HSA for 30 min then stained with Av.A488. **A**, Representative FACS profiles. **B**, frequency of Av. A488⁺ hMCs. Data are shown as mean ± SEM from 6 independent experiments. *P* values were determined using paired *t* test. **C** and **D**, Av.A488 fluorescence intensity of the degranulated hMCs represented as box-and-whisker plots from one representative experiment (C) or plotted as median fluorescence intensity from 6 independent experiments, histograms represent mean ± SEM (D). *P* values were determined using Mann-Whitney test (C) or Wilcoxon test (D). ns *P*>0.05; * *P*<0.05; ** *P*<0.01; *** *P*<0.001; **** *P*<0.0001.

FIG E7. Phenotypical characterization of hMC. **A**, Flow cytometry analysis of CD117, FcεRI and ST2 expression of hMCs from two donors. **B**, Intracellular flow cytometry analysis of tryptase and chymase expression.

Fig E8. Single cell analysis of hMC degranulation using CD63 exposure assay. IgE sensitized mast cell were stimulated with anti-IgE Abs then stained with anti-CD63-PE mAbs. **A**, Representative CD63 histogram profiles from one experiment out of three. **B**, correlation analysis between the percentage of CD63⁺ hMCs and β-hexosaminidase release. Shown are a representative scatter plot out of 3 independent experiments and curve fitting using a linear regression (black dotted line) or nonlinear regression (Hill equation, red curve).

Fig E9. TSLP or IL-25 pretreatment do not potentiate mast cell degranulation. IgE sensitized hMCs were pretreated with increasing concentration of IL-33 or TSLP or IL-25 for one hour then stimulated with 0.8 μg/mL anti IgE Abs for 30 min. Cells were stained with Av.A488 and analyzed by flow cytometry. **A**, Shown are the frequency of Av.A488⁺ hMCs. **B**, Shown are the median fluorescence intensity of degranulated hMCs. *P* value are determined using paired student *t* test.

Histograms represent mean $\pm$ SEM from at least 4 independent experiments. ns $P>0.05$; * $P<0.05$;
**** $P<0.0001$

Video legends

Video E1. Granule corona formation following stimulation with anti-IgE Abs. (Video E1 corresponds to FIG 1). IgE-sensitized hMCs stimulated with 2.5 $\mu\text{g/mL}$ anti-IgE Abs (added at $t=0$) in the presence of 8 $\mu\text{g/mL}$ Av.SRho were inspected using time-lapse confocal microscopy. Image sequences of the time-lapse recording were processed using Zen software. This video is from one representative experiment out of five. Bars, 5 μm .

Video E2. Granule exocytosis monitored by Annexin-V and FM1-43 binding assay. (Video E2 corresponds to FIG E1). IgE-sensitized hMCs stimulated with 2.5 $\mu\text{g/mL}$ anti-IgE Abs (added at $t=0$) in the presence of Av.SRho, annexin-V-Alexa647 and FM1-43 were inspected using time-lapse confocal microscopy. Image sequences of the time-lapse recording were processed using Zen software. This video is from one representative experiment out of three. Bars, 5 μm .

Video E3. Time lapse flow cytometry analysis of hMC degranulation. (Video E3 corresponds to FIG 2). IgE-sensitized hMCs stimulated with 2.5 $\mu\text{g/mL}$ anti-IgE Abs (added at $t=3.8$ min) in the presence of 5 $\mu\text{g/mL}$ Av. A488 were inspected using time-lapse function of the LSRII cytometer. Image sequences of the time-lapse recording were processed using FlowJo software. This video is from one representative experiment out of five.

Video E4. Time lapse flow cytometry analysis of hMC calcium fluxes and degranulation. (Video E4 corresponds to FIG 2). IgE-sensitized hMCs loaded with Indo-1 were stimulated with 0.27, 0.8 or 2.5 $\mu\text{g/mL}$ of anti-IgE Abs (added at $t=3.8$ min.) in the presence of 5 $\mu\text{g/mL}$ Av. A488. Cells were inspected using time-lapse function of the LSRII cytometer. Image sequences of the time-lapse recording were processed using FlowJo software. This video is from one representative experiment out of five.

Video E5. Time lapse flow cytometry analysis of IL-33 potentiation of hMC degranulation. (Video E5 corresponds to FIG 4). IgE-sensitized hMCs were pretreated for 1 hour with 1, 2, or 5 ng/mL of IL-33 and loaded with different concentrations of Cell trace violet. hMCs were stimulated with 0.8 $\mu\text{g/mL}$ of anti-IgE Abs (added at $t=3.8$ min.) in the presence of 5 $\mu\text{g/mL}$ Av. A488. Cells were inspected using time-lapse function of the LSRII cytometer. Image sequences of the time-lapse

recording were processed using FlowJo software. This video is from one representative experiment out of four.

Figure E1

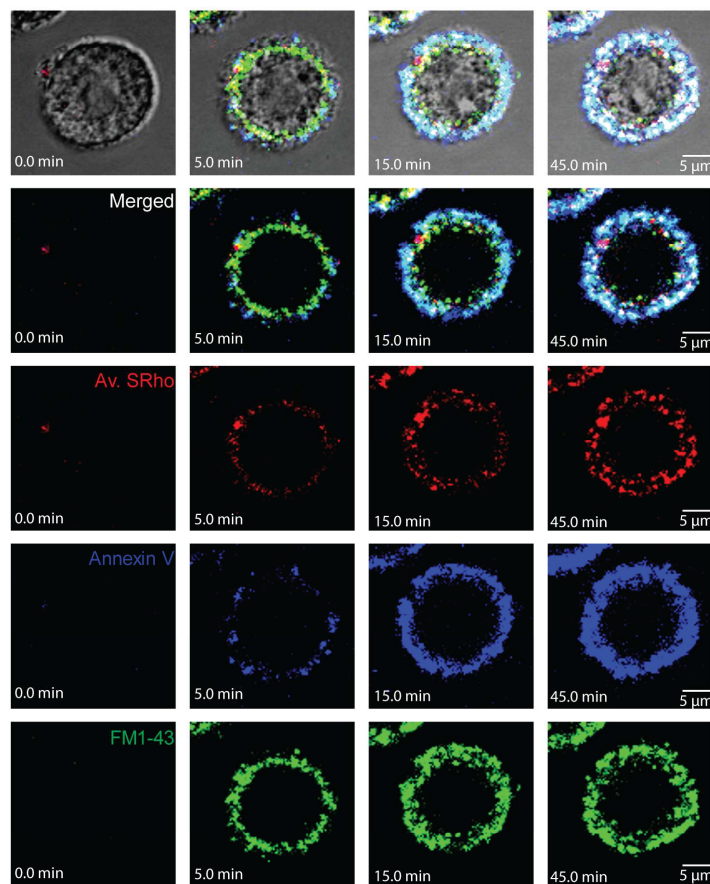
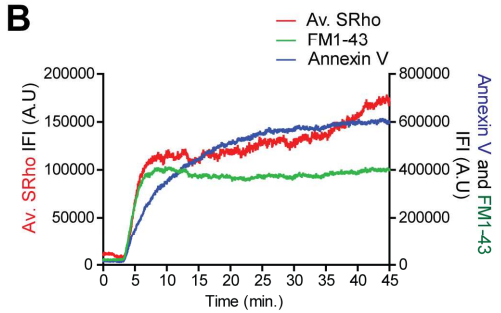
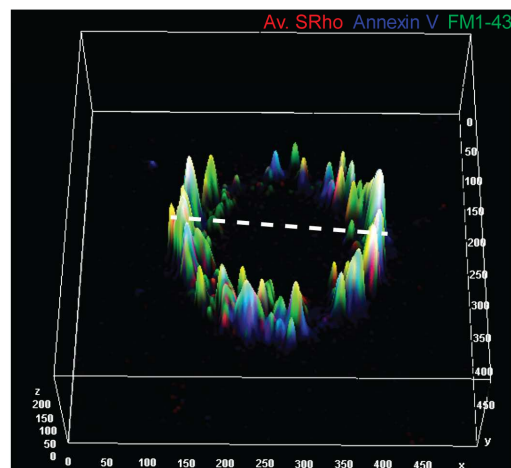
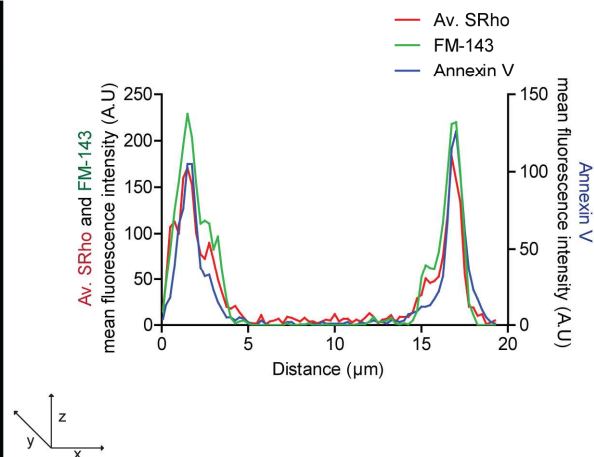
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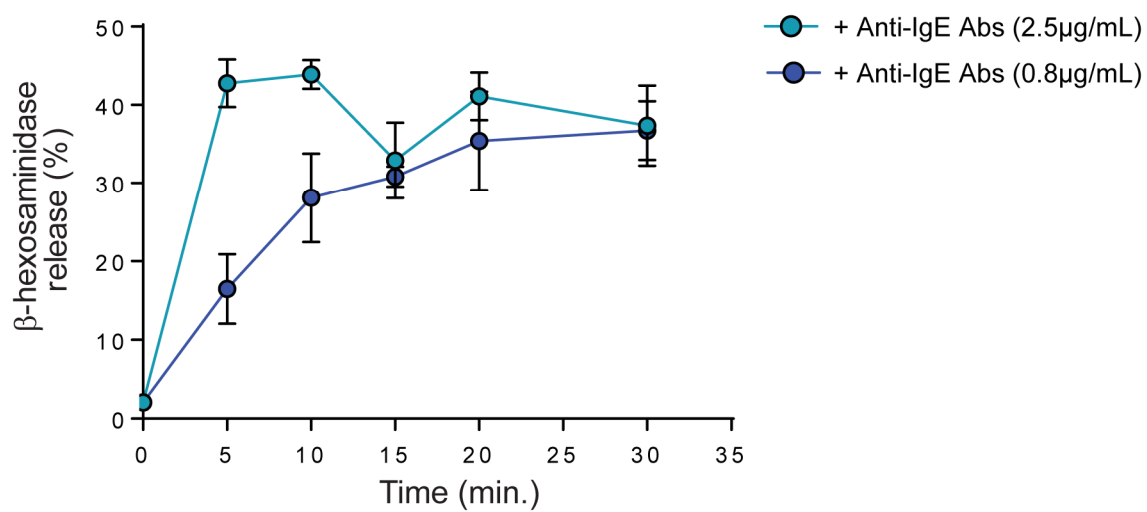


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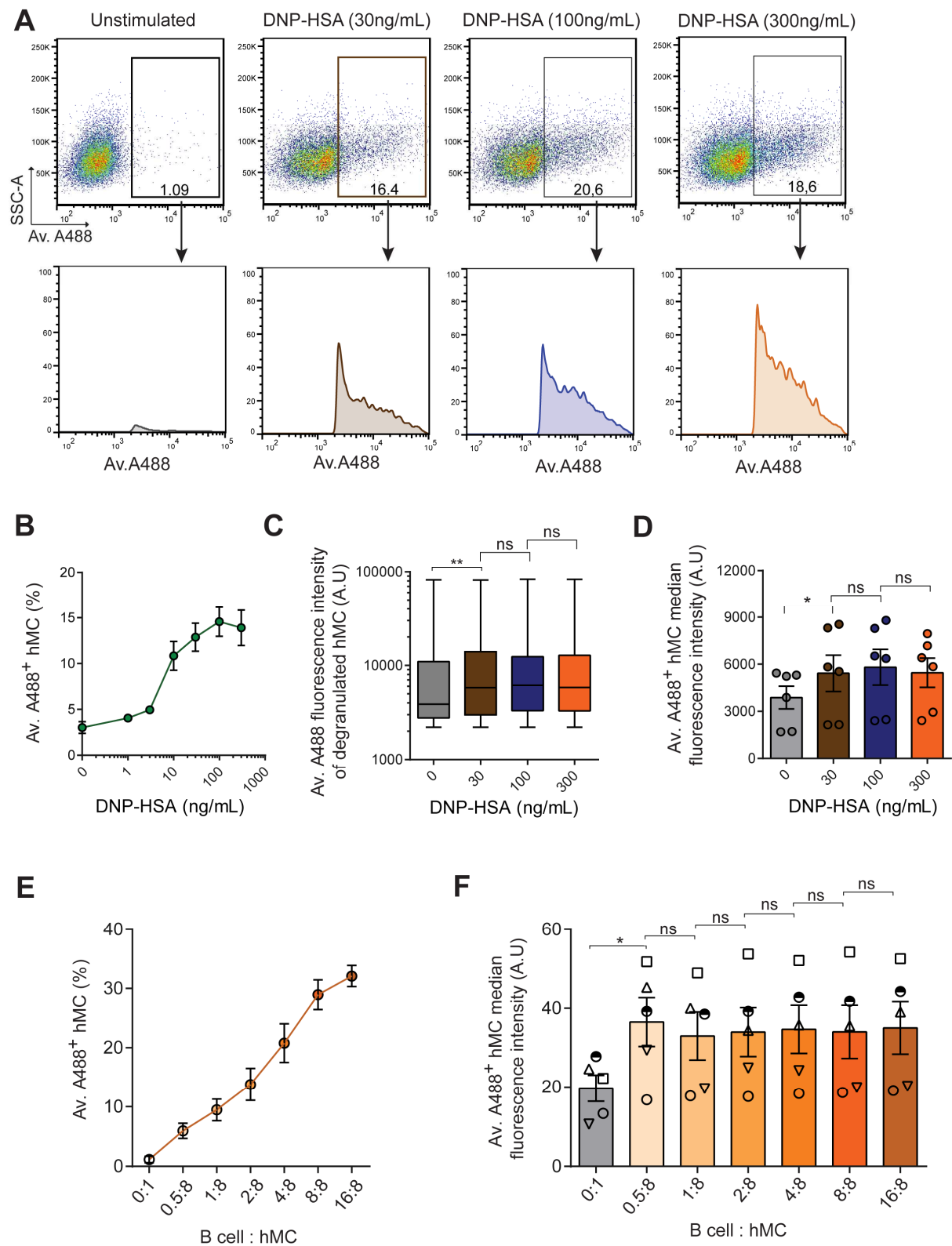


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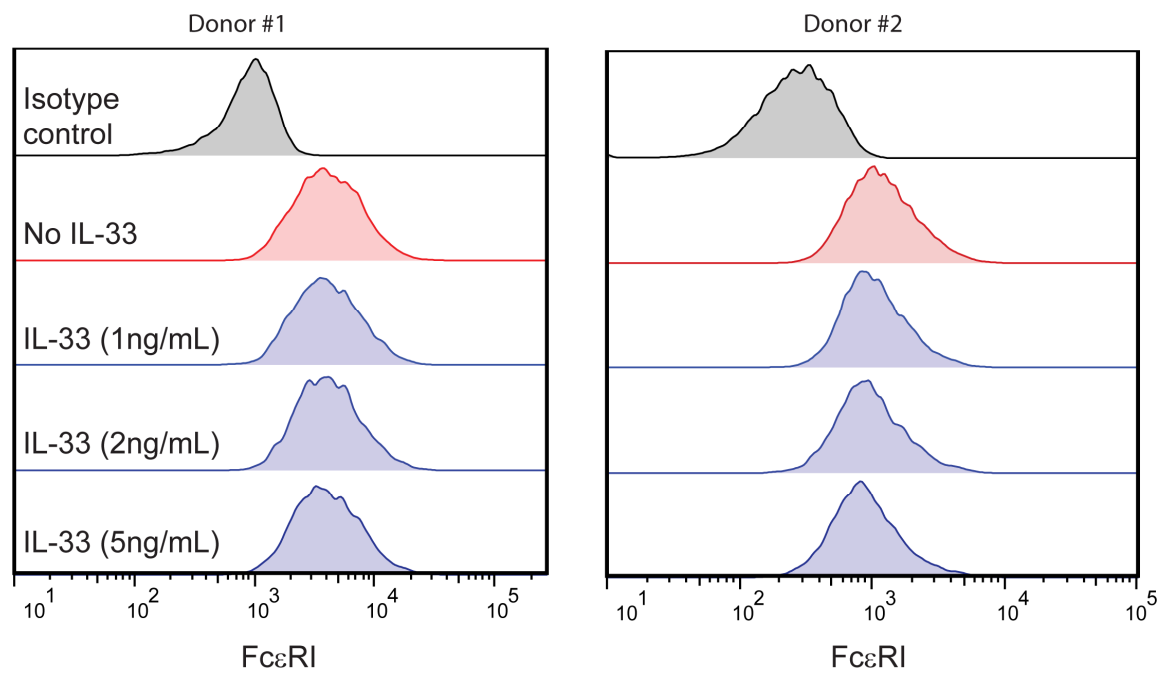


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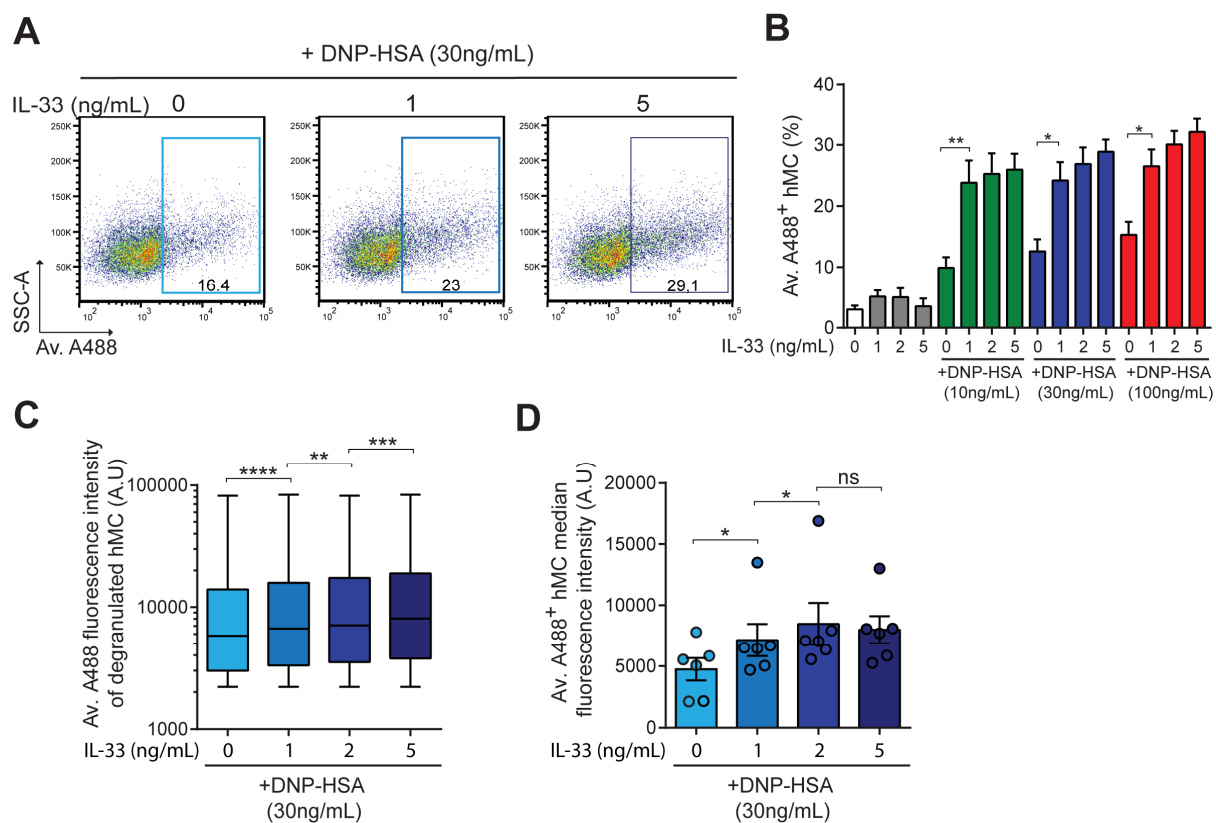


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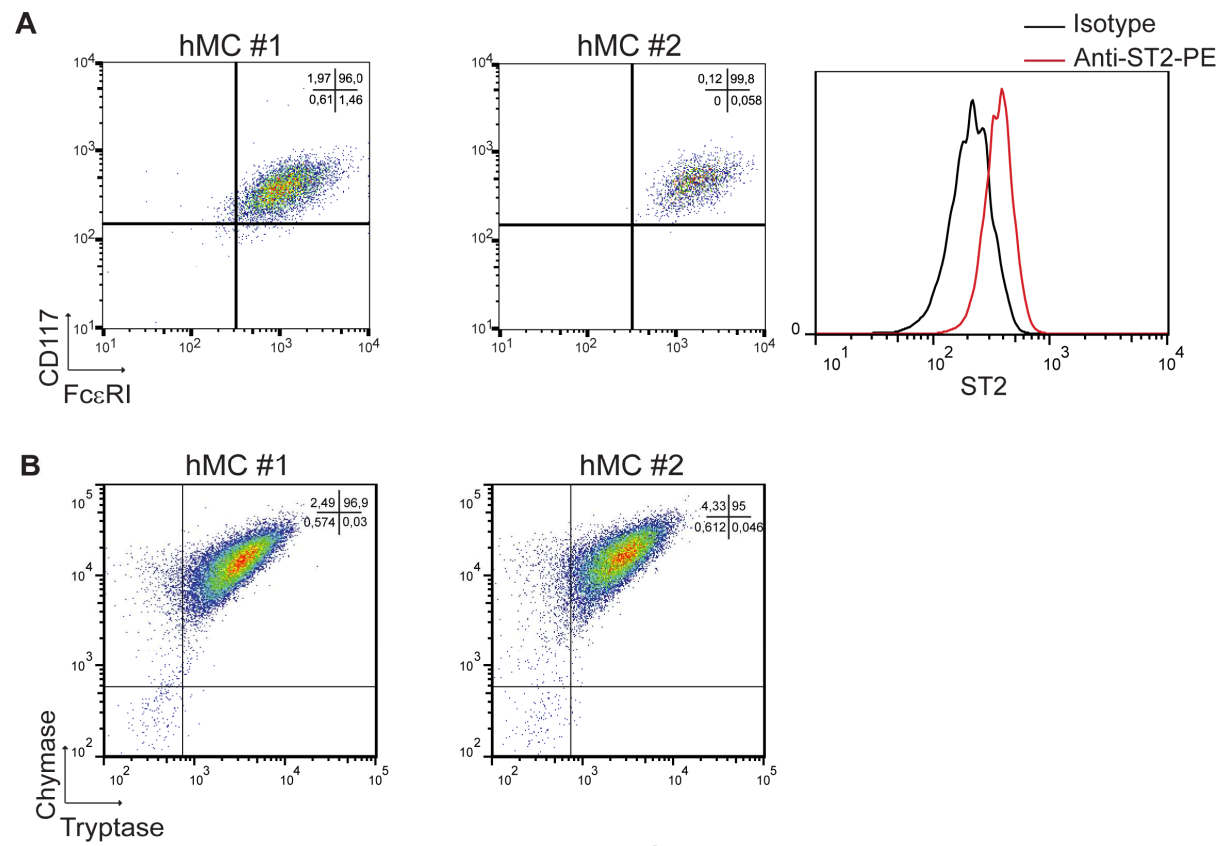


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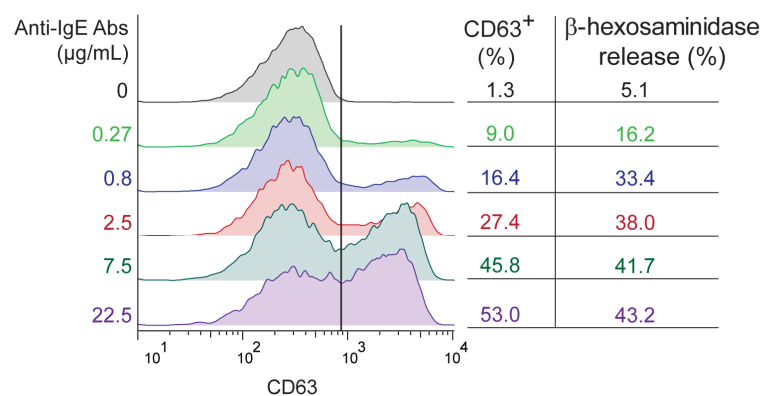
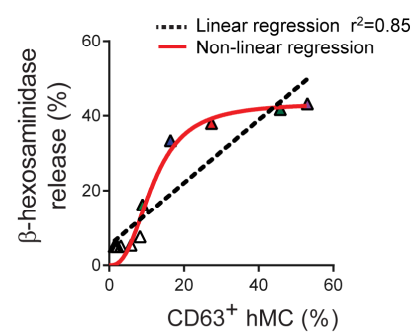
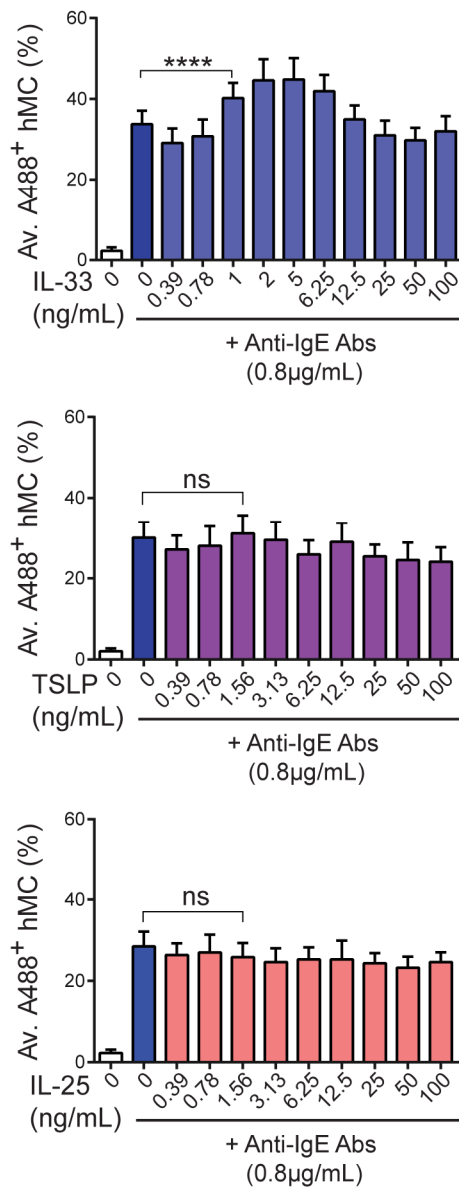
A**B**

Figure E9

A**B**