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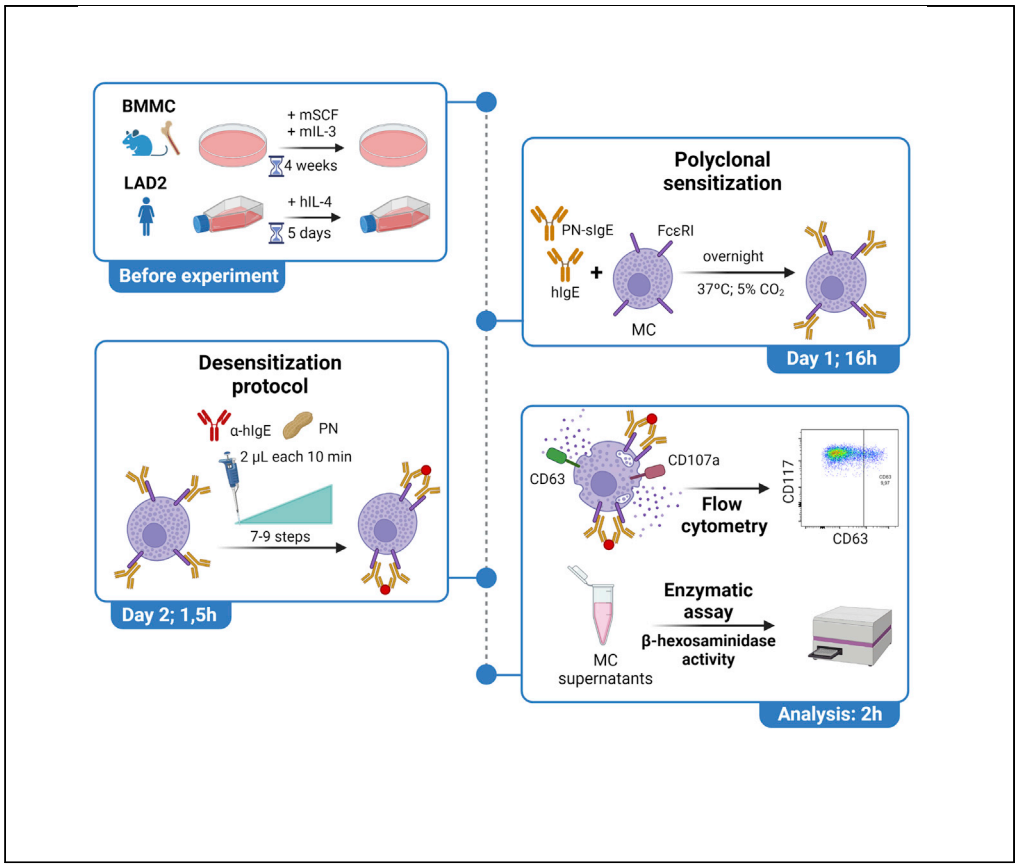
Protocol to desensitize human and murine mast cells after polyclonal IgE sensitization



In this protocol, we provide detailed instructions to desensitize human and murine mast cells (MCs) after polyclonal IgE sensitization. Moreover, we specify the steps for MC degranulation assessment after desensitization, measuring CD63 and CD107a expression by flow cytometry and β-hexosaminidase activity. Desensitized MCs can be used directly for co-culture with other cell types, immunofluorescence, live imaging, and omics approaches.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Human and murine mast cell (MC) culture and polyclonal IgE sensitization procedures

MC degranulation assessment via flow cytometry and β-hexosaminidase activity

Optimized polyclonal desensitization platform for MCs

Applicable for the study of MC biology in allergen immunotherapy

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Protocol to desensitize human and murine mast cells after polyclonal IgE sensitization

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SUMMARY

In this protocol, we provide detailed instructions to desensitize human and murine mast cells (MCs) after polyclonal IgE sensitization. Moreover, we specify the steps for MC degranulation assessment after desensitization, measuring CD63 and CD107a expression by flow cytometry and β-hexosaminidase activity. Desensitized MCs can be used directly for co-culture with other cell types, immunofluorescence, live imaging, and omics approaches.

For complete details on the use and execution of this protocol, please refer to López-Sanz et al. (2022).

BEFORE YOU BEGIN

The prevalence of allergic diseases has been on the rise for the last few decades. Allergen immunotherapy (AIT) is the only treatment with disease-transforming potential, and it is based on giving increasing allergen doses to allergic patients. The AIT early-phase response relies on mast cell (MC) desensitization to prevent allergic reactions upon allergen exposure. However, up to 70% of AIT patients suffer allergic reactions during desensitization, which are mainly driven by the IgE-MC axis. The biology under MC desensitization is incompletely understood, and the mechanisms reported are controversial. This is partly due to the lack of protocols describing MC desensitization and its readouts. Furthermore, current desensitization models largely use murine MCs and monoclonal IgE, while allergic patients present with a polyclonal IgE repertoire (López-Sanz et al., 2022).

The protocol below describes novel desensitization methods based on polyclonal strategies for human and murine MCs; this means that MCs are sensitized with a pool of IgEs that recognize more than one epitope. These novel methods will be useful to study cellular and molecular mechanisms of MC desensitization under conditions that more closely resemble human AIT.

Institutional permissions

Murine samples: bone marrow from femur and tibia of C57BL/6 mice were used to obtain bone-marrow-derived MCs (BMMCs) (Wang et al., 2021). Sera from peanut (PN)-allergic mice were generated following immunization with a classical model of food allergy and anaphylaxis (Jimenez-Saiz et al., 2017; Chu et al., 2014). All procedures were approved by the local Animal Experimentation Ethics Committee (CEEA) of the National Center for Biotechnology (CNB, Madrid, Spain) and by the Environmental Council of the Community of Madrid (Madrid, Spain) with PROEX reference 45.2/20.

Human samples: the human MC line LAD2 was kindly provided by Drs. Dean Metcalfe and Arnold Kirshenbaum (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA) (Kirshenbaum et al., 2003). Human sera were provided by Dr. Carlos Blanco (Allergy Service, Hospital Universitario de La Princesa, Madrid, Spain). The Research Ethics Committee of Hospital Universitario de La Princesa (Madrid, Spain) approved the study protocol (reference 4460). All the donors provided written informed consent with no conflict of interest.

For BMMC desensitization protocol

Reagent preparation

Timing: 60 min

1. Media (see detailed recipes in the materials and equipment section):
   a. Culture media.
   Complete IMDM supplemented with recombinant murine Stem Cell Factor (r-mSCF) and recombinant murine IL3 (r-mIL3).
   b. Desensitization media.
   Complete IMDM without cytokine supplementation.

   Note: Warm up fresh media at 37°C in water bath for at least 20 min before use.

   Note: To prevent any unspecific activation due to r-mSCF and r-mIL3 while performing the desensitization, the media can be prepared without cytokines. However, this is optional.

2. Serial stock dilutions for PN stock concentrations:

PN extract	10,000 ng/mL	868.30 ng/mL	543.90 ng/mL	181.30 ng/mL	90.65 ng/mL	38.70 ng/mL	21 ng/mL	9.10 ng/mL
PN volume (μL)	STOCK (see materials and equipment section)	4.3	37.6	20	20	19.2	21.8	10.8
Desensitization media (μL)		45.7	22.4	40	20	25.8	18.2	14.2
Total volume (μL)		50	60	60	40	45	40	25

Note: As the dilutions were not the same in all the step points, different total volumes were chosen, considering that we set a minimum volume of 20 μL after the next dilution is performed.

Cell culture preparation

Timing: 4 weeks

Erythrocyte-free bone marrow cells are cultured for 4 weeks in culture media to generate mature BMMCs (Wang et al., 2021). It is recommended to plate 0.2–0.3 × 10^6 cells per mL in a 100 mm × 20 mm cell culture dish at 37°C and 5% CO_2. The culture media is changed weekly and cellularity is kept below 0.5 × 10^6 cells/mL. After 4 weeks of culture, bone marrow precursors differentiate into mature BMMCs.

⚠ CRITICAL: BMMCs should not be used before complete differentiation/maturation is reached. This can be assessed by the co-expression of CD117 and FcεRI (IgE high affinity receptor) by flow cytometry. After 4 weeks in culture ≥90% of cells should be positive for CD117 and FcεRI, and the viability should be ≥85%.

⚠ CRITICAL: We recommend using BMMCs in the first and second week after cells being mature. In our hands, BMMC maturation is achieved in the 4th and the 5th week of culture. We have noticed that BMMCs in the 6th week of culture have a decreased viability and degranulation responses (*i.e.*, activation), which may distort the results.

For LAD2 desensitization protocol

Reagent preparation

Timing: 60–80 min

3. Media (see detailed recipes in the materials and equipment section):
   a. Culture media.
      Complete StemPro-34 supplemented with recombinant human SCF (r-hSCF).

Note: Warm up fresh media at 37°C in water bath for at least 20 min before use.

4. Serial stock dilutions for hIgE or PN-specific desensitization protocols:

anti-hIgE	10,000 µg/mL	50 µg/mL	25.50 µg/mL	12.75 µg/mL	6.38 µg/mL	3.20 µg/mL	1.60 µg/mL	1.14 µg/mL	0.50 µg/mL
Anti-hIgE volume (µL)	STOCK (see materials and equipment section)	2	20.4	20	20	20	20	21.4	8.8
Desensitization media (µL)		38	19.6	20	20	20	20	8.6	11.2
Total volume (µL)		40	40	40	40	40	40	30	20

PN extract	10,000 ng/mL	1,000 ng/mL	173.60 ng/mL	113.22 ng/mL	95.60 ng/mL	60 ng/mL	30 ng/mL	15 ng/mL	7.50 ng/mL	4.20 ng/mL	2.10 ng/mL
PN volume (µL)	STOCK (see materials and equipment section)	5	8.68	32.6	33.77	25.1	20	20	20	23.8	20
Desensitization media (µL)		45	41.32	17.4	12.46	14.9	20	20	20	16.2	20
Total volume (µL)		50	50	50	40	40	40	40	40	40	40

Note: As the dilutions were not the same in all the step points, different total volumes were chosen, considering that we set a minimum volume of 20 µL after the next dilution is performed.

Cell culture preparation

Timing: 5 days

LAD2 is a human SCF-dependent MC line derived from a patient with mastocytosis (Kirshenbaum et al., 2019). LAD2 are cultured as previously described (Radinger et al., 2010). LAD2 cells express the FcεRI, however culturing them with IL4 increases the steady-state expression of the FcεRI (Hano Toru et al., 1996).

5. Centrifuge the LAD2 cell suspension at 486 g modifying the default acceleration/deceleration settings to a low brake (e.g., level 3 of 9) for 5 min at 20°C–25°C.
6. Discard supernatant and suspend cells in 1–2 mL of fresh StemPro-34 culture media.
7. Perform cell counting.
8. Seed 2 × 10^6 cells in StemPro-34 culture media supplemented with r-hIL4 at 10 ng/mL. Cellularity should be 0.2–0.25 × 10^6 cells/mL.

Note: The number of total LAD2 cells cultured with r-hIL4 can vary depending on the total number of cells needed for the experimental conditions.

⚠ CRITICAL: LAD2 cells duplicate weekly, however cell growth rate is diminished when culturing cells in the presence of r-hIL4. We recommend seeding an excess of approximately 0.5 × 10^6 cells to prevent low cellularity for the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Armenian hamster anti-FcεRIα PE-Cy7; [clone MAR-1]; 1:200 dilution	BioLegend	Cat#134318
Rat anti-CD107a PerCP-Cy5.5; [clone 1D4B]; 1:200 dilution	BioLegend	Cat#121625
Rat anti-CD117 BV421; [clone ACK2]; 1:200 dilution	BioLegend	Cat#135124
Rat anti-CD63 APC; [clone NVG-2]; 1:200 dilution	BioLegend	Cat#143906
Rat anti-IgE PE; [clone RME-1]; 1:200 dilution	BioLegend	Cat#406908
Purified anti-mouse CD16/CD32; 1:50 dilution	BioLegend	Cat#101302
Mouse anti-FcεRIα FITC; [clone AER-37]; 1:100 dilution	BioLegend	Cat#334608
Mouse anti-CD107a APC; [clone H4A3]; 1:100 dilution	BioLegend	Cat#280.328620
Mouse anti-CD117 PE; [clone 104D2]; 1:600 dilution	BioLegend	Cat#313204
Mouse anti-CD63 BV421; [clone H5C6]; 1:200 dilution	BioLegend	Cat#353030
Mouse anti-IgE PE-Cy7; [clone MHE-18]; 1:100 dilution	BioLegend	Cat#325510
Human TruStain FcX (FcR blocking solution); 1:50 dilution	BioLegend	Cat#422302
Human IgE (hIgE), Myeloma	EMD Milipore	Cat#401152-100UG
Goat polyclonal anti-hIgE (ε-chain specific)	Sigma-Aldrich	Cat#I6284-1MG
Chemicals, peptides, and recombinant proteins		
IMDM, Hepes	Thermo Fisher Scientific	Cat#12440061
Mem Vitamin Solution (100×)	Thermo Fisher Scientific	Cat#11-120-052
Sodium pyruvate (100 mM)	Biowest	Cat#L0642-100
Non-essential amino acid (100×)	Biowest	Cat#X0557-100
Fetal bovine serum (FBS)	Cytiva	Cat# 16SV30160.03
Recombinant Murine SCF (r-mSCF)	PeproTech	Cat#250-03
Recombinant Murine IL3 (r-mIL3)	PeproTech	Cat#213-13
StemPro-34 SFM (1×)	Thermo Scientific	Cat#10639011
Glutamax™ Supplement	Thermo Fisher Scientific	Cat#35050061
Recombinant Human SCF Protein (r-hSCF)	R&D Systems	Cat#255-SC-200
Recombinant Human IL4 Protein (r-hIL4)	R&D Systems	Cat#204-IL-100
Penicillin/streptomycin 100×	Biowest	ID#MS00UN1006
Peanut (PN) lyophilized extract (allergen)	Stallergenes Greer	Cat#XPF171D3A25
Calcium Ionophore A23187	Merck Life Sciences	Cat#C7522
Ionomycin calcium salt	Sigma-Aldrich	Cat#I3909-1ML
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A2153-10G
Phosphate-buffered saline (PBS)	Gibco	Cat#10010056
Ethylenediaminetetraacetic acid (EDTA)	PanReac AppliChem	Cat#131026
Sodium hydroxide	Sigma-Aldrich	Cat#S5881
Sodium citrate dihydrate	Sigma-Aldrich	Cat#W302600
Citric acid	Sigma-Aldrich	Cat#251275-100G
p-nitrophenyl N-acetyl β-D-glucosamine	Sigma-Aldrich	Cat#N9376-100MG
Distilled water (H_2O)	Versylene Fresenius	Cat#255188
TrypanBlue	Gibco	Cat# 15250061
Efluor780 viability; 1:2,000 dilution	eBioscience	Cat#65-0865-14
Experimental models: Cell lines		
Mouse: C57BL/6 (6–12 weeks of age, male or female)	Charles River Laboratory	Strain No.: 027
Human: LAD2	Drs. Metcalfe & Kirshenbaum	(Kirshenbaum et al., 2003)

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Graph Pad Prism v 9.0	GraphPad	https://www.graphpad.com/demos/
FlowJo v10	BD	https://www.flowjo.com/solutions/flowjo
Biorender	BioRender	https://biorender.com/
Other		
Falcon conical tubes, 50 mL	DELTALAB	Cat#429927
Falcon conical tubes, 15 mL	DELTALAB	Cat#429946
Cell culture plate 6 well TC-treated	Falcon	Cat#353046
Cell culture plate 24 well TC-treated	Costar	Cat#3524
U/V-bottom 96 well TC-treated microplates	Falcon	Cat#353910
Flat-bottom 96 well high binding microplates	Costar	Cat#3590
Sterile Dish 100 mm × 20 mm CC-treated	Corning	Cat#430167
Corning 500 mL Filter System	Corning	Cat#431097
Neubauer chamber	BLAUBRAND	Cat#717810
Centrifuge	Hettich	Rotina 380
Flow cytometer	BD	FACSCantoII
Microplate Reader	Promega	GloMax Discover

MATERIALS AND EQUIPMENT

For BMMCs

r-mSCF stock (100 μg/mL): r-mSCF powder (50 μg) is reconstituted in 0.5 mL of sterile PBS (as indicated by the manufacturer) to a solution of 100 μg/mL. Store aliquots of 20 μL at −20°C (until use) or −80°C for long-term storage.

Note: We consider long-term storage a period ≥ 6 months.

r-mIL3 stock (100 μg/mL): r-mIL3 powder (50 μg) is reconstituted in 0.5 mL of sterile PBS (as indicated by the manufacturer) to a solution of 100 μg/mL. Store aliquots of 20 μL at −20°C or −80°C for long-term storage.

Complete IMDM: a bottle of 500 mL (keep covered from light) is supplemented with MEM (stored at −20°C), sodium pyruvate (stored at 4°C), non-essential amino acids (stored at 4°C), heat-inactivated FBS (stored at 4°C or −20°C for long term), penicillin/streptomycin (stored at −20°C) and the cytokines r-mSCF and r-mIL3 (stored at −20°C or −80°C for long term). To avoid media expiration, it is highly recommended to make fresh media of less volume as shown below. Once supplemented, this media is stored at 4°C for up to 1 month.

Reagent	Final concentration	Amount	Amount
IMDM (1×)	1×	85 mL	48 mL
MEM (100×)	1×	1 mL	500 μL
Sodium pyruvate (100 mM)	1 mM	1 mL	500 μL
Non-essential aminoacids (100×)	1×	1 mL	500 μL
FBS (10×)	1×	10 mL	5 mL
Penicillin/streptomycin (100×)	1×	1 mL	500 μL
r-mSCF (100 μg/mL)	0.01 μg/mL	10 μL	5 μL
r-mIL3 (100 μg/mL)	0.005 μg/mL	5 μL	2.5 μL
Total	**N/A**	**100 mL**	**50 mL**

Note: Freeze/thawing cycles of the cytokines (r-mSCF and r-mIL3) should not exceed 4 times. Keep on ice (4°C) until they are added to the culture media and, immediately after, store them at −20°C.

⚠ CRITICAL: For FBS inactivation, heat FBS at 56°C for 30 min. Filter inactivated FBS through a 0.22 μm filter system and store in aliquots of 50 mL at −20°C until use. Alternatively, directly purchase heat inactivated FBS.

For LAD2

r-hSCF stock (200 μg/mL): r-hSCF powder (200 μg) is reconstituted directly in the vial by adding 1 mL of sterile PBS containing 0.1% BSA (as indicated by the manufacturer). Final stock concentration is 200 μg/mL. Store working aliquots of 20 μL at −20°C or −80°C for long-term storage.

StemPro-34 medium: 500 mL bottle of StemPro-34 (keep protected from light) is supplemented with StemPro-34 Nutrient Supplement (stored at −20°C), penicillin/streptomycin (stored at −20°C), Glutamax™ (stored at 4°C) and r-hSCF (stored at −20°C). Once supplemented, this media is stored at 4°C up to 1 month.

Reagent	Final concentration	Amount	Amount
StemPro-34 medium (1×)	1×	477.25 mL	38.2 mL
StemPro-34 Nutrient Supplement (40×)	1×	12.5 mL	1 mL
Penicillin/streptomycin (100×)	1×	5 mL	400 μL
Glutamax™ (100×)	1×	5 mL	400 μL
r-hSCF (200 μg/mL)	100 ng/mL	250 μL	20 μL
Total	**N/A**	**500 mL**	**40 mL**

Note: The whole volume of the StemPro-34 media (500 mL) can be supplemented at once. However, to avoid media expiration, it is highly recommended to make fresh media of less volume (e.g., 40 mL) when needed.

r-hIL4 stock (200 μg/mL): r-hIL4 powder (200 μg) is reconstituted in 1 mL of sterile PBS (as indicated by the manufacturer) to a stock solution of 200 μg/mL. Store working aliquots of 10 μL at −20°C until use. Aliquots for long-term storage should be kept at −80°C.

hIgE stock (100 mg/mL): following initial thaw of the liquid solution of the vial, store working aliquots of 100 μL at −20°C until use to avoid freeze/thaw cycles. For long term storage, freeze aliquots at −80°C.

Polyclonal anti-hIgE stock (1 mg/mL): lyophilized powder (1 mg) is stored at 4°C and reconstituted in 1 mL of sterile PBS (as indicated by the manufacturer) to a solution of 1 mg/mL. Store working aliquots of 20 μL at −20°C or at −80°C for long-term storage.

Note: If needed, dilutions of this stock concentration can be done and kept at −20°C or −80°C for long-term storage.

Common reagents

NaOH solution (1 M): dissolve sodium hydroxide pellet in distilled H_2O. After mixing, store at 20°C–25°C until use.

Reagent	Final concentration	Amount
Sodium hydroxide	1 M	40 g
Distilled H_2O	N/A	1 L
Total	**N/A**	**1 L**

⚠ CRITICAL: Sodium hydroxide is a strong base and could cause chemical burns. Follow the safety datasheet recommendations when preparing the solution.

Citrate buffer (100 mM): dilute dehydrated sodium citrate and citric acid in distilled H_2O. After mixing both compounds, adjust to pH 4.5 if needed and add distilled H_2O until a final volume of 500 mL. Store at 4°C until use.

Reagent	Final concentration	Amount
Dehydrated sodium citrate (molecular weight = 294.1 g/mol)	45 mM	6.70 g
Citric acid (molecular weight = 192.1 g/mol)	55 mM	5.23 g
Distilled H_2O	N/A	400 mL
Total	**N/A**	**500 mL**

FACS buffer: dilute the BSA in a PBS solution and add EDTA. Store at 4°C until use.

Reagent	Final concentration	Amount
BSA	0.5%	2.5 g
0.5 M EDTA solution	2.5 mM	2.5 mL
PBS	1×	497.5 mL
Total	**N/A**	**500 mL**

β-hexosaminidase substrate solution: p-nitrophenyl N-acetyl β-D-glucosamine (2 mM): dilute p-nitrophenyl N-acetyl β-D-glucosamine (store at −20°C) in citrate buffer 100 mM. Once made, store at 4°C until use.

Reagent	Final concentration	Amount
p-nitrophenyl N-acetyl β-D-glucosamine (molecular weight = 342.3 g/mol)	2 mM	17 mg
Citrate buffer (100 mM)	N/A	25 mL
Total	**N/A**	**25 mL**

Note: It is recommended to weigh the p-nitrophenyl N-acetyl β-D-glucosamine directly in the Falcon tube.

Note: Once homogenized, store at 4°C until use, up to 1 month. To obtain a homogeneous mix, leave the Falcon tube rolling 16 h approximately.

⚠ CRITICAL: After 1-month storage at 4°C, the reactive adds background to the enzymatic assay and we do not recommend its use. Moreover, we recommend avoiding long-term storage at −20°C because freeze/thaw cycles add unspecific signal to the assay.

PN extract (10 mg/mL): PN extract is reconstituted in warm PBS to a final concentration of 10 mg/mL. Mix the solution gently for homogeneity and store aliquots of 1 mL at −20°C until use or −80°C for long-term storage.

STEP-BY-STEP METHOD DETAILS

The desensitization protocols consist in adding sequentially increasing doses of the allergen to eventually induce MC unresponsiveness to the given allergen. Clinically, a successful AIT protects allergic individuals from developing clinical symptoms upon allergen exposure. *In vitro*, it is observed a lack, or reduction, of MC activation/degranulation when challenging desensitized-MCs with a single-dose of allergen.

BMMC desensitization step-by-step

BMMC overnight sensitization with PN-allergic mice sera

Timing: 30 min + 16 h

This part of the protocol allows PN-sIgEs, from sera of PN-allergic mice, to bind to the FcεRI expressed on the cell surface of MCs.

1. BMMCs should be collected from a 100 mm × 20 mm cell culture dish into a Falcon tube.
2. Centrifuge the cell suspension at 486 g modifying the default acceleration/deceleration settings to a medium acceleration (e.g., level 7 of 9) and low brake (e.g., level 3 of 9) for 5 min at 20°C–25°C.

   ⚠ CRITICAL: Low brake to avoid unspecific cell activation.

3. Discard supernatant.
4. Suspend cell pellet in 1 mL of IMDM culture media.
5. Perform cell counting and assess cell viability.

   Note: When using trypan blue testing, cell viability should be > 85%.

6. Polyclonal sensitization is done at a cell density of 0.5–1 × 10^6 cells/mL, and 0.1 × 10^6 BMMCs per condition.
   a. In a Falcon tube, suspend BMMCs in a total volume of complete IMDM to reach the adequate sensitization cellularity.
   b. Add the murine polyclonal IgE sera at a concentration range of 1–10 ng/mL.
   c. Seed the cells in a 6- or 24-well cell culture plate, depending on the final volume obtained.

   Note: In this case, the desensitization protocol is optimized with a pool of sera from PN-allergic mice.

   ⚠ CRITICAL: To decrease variability between experiments, PN-sIgE levels (or at least total IgE levels) should be characterized before sensitizing BMMCs. In our settings, we measured PN-sIgE levels with an ELISA (Jimenez-Saiz et al., 2017), but other techniques can be employed. In this case, BMMCs were sensitized with a concentration range of 1–10 ng/mL PN-sIgEs (in our conditions, sera dilutions were made from 1:50 to 1:100) where BMMCs showed a robust activation upon a single-dose PN challenge.

7. Leave BMMCs approximately 16 h at 37°C and 5% CO_2.

BMMC desensitization

Timing: 80–100 min

The desensitization is a sensitive part of the protocol. It needs accuracy in making the correct allergen dilutions to avoid activation during the procedure.

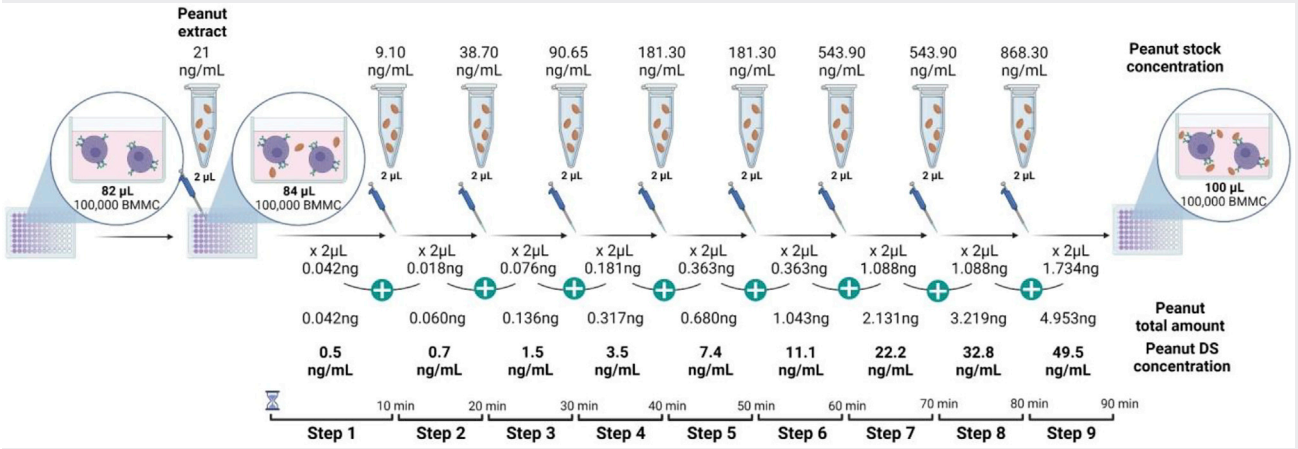
8. Collect sensitized BMMCs and spin cells at 486 g for 5 min at 20°C–25°C and discard supernatant.

   Note: It is important to perform this wash before starting the desensitization protocol to discard free IgE and other Igs contained in the sera that could interfere with the protocol.

9. Suspend pelleted BMMCs in supplemented IMDM desensitization media to get a cellularity of 1.22 × 10^6 cells/mL.

   Note: To prevent unspecific activation during desensitization, cytokines should not be added to the culture media.

Table 1. Peanut (PN) *in vitro* desensitization (DS) protocol for bone-marrow-derived mast cells (BMMCs)



⚠ CRITICAL: The BMMC desensitization protocol is optimized for 100,000 cells in a final volume of 100 µL. In every step of the desensitization protocol 2 µL are added; the protocol includes 9 steps, so by the end of the protocol, a total volume of 18 µL is added. To maintain the final volume at 100 µL, 0.1 × 10^6 BMMC should be seeded in 82 µL (100 µL–18 µL = 82 µL).

10. Seed 0.1 × 10^6 BMMCs in 82 µL in a U/V-bottom 96 well pate.
11. Carry out the desensitization protocol adding 2 µL of PN solution at the corresponding concentration every 10 min (Table 1).

Troubleshooting 1: if the concentration increase between steps is too high, MCs can be activated during the desensitization protocol. This situation can be solved attenuating the escalation between concentrations. In other words, adding more steps to the final desensitization protocol.

LAD2 desensitization step-by-step

LAD2 overnight sensitization with hIgE and sera from PN-allergic donors

Timing: 30 min + 16 h

12. After 5 days, spin cells at 486 g at maximal acceleration and low brake (e.g., level 3 of 9), for 5 min at 20°C–25°C.
13. Discard supernatant.
14. Suspend cell pellet in 1 mL of StemPro-34 culture media and r-hIL4 at 10 ng/mL.
15. Perform cell counting and assess cell viability.

Note: Viability should be > 85%.

16. Polyclonal sensitization is done at a cell density of 0.5 × 10^6 cells/mL, and 0.05 × 10^6 cells per condition:
    a. In a Falcon tube, suspend LAD2 cells in supplemented StemPro-34 + r-hIL4 at 10 ng/mL to reach the adequate sensitization cellularity.
    b. Add the specific volume of polyclonal sIgEs.
        i. For hIgE, sensitize LAD2 cells with 100 ng/mL of hIgE.
        ii. For PN-allergic human sera, sensitize cells with the chosen concentration of PN-allergic donor sera.

Note: The total number or LAD2 cells chosen for sensitizing will depend on the number of experimental conditions included in your experiment. For example, if you want to test LAD2 cells degranulation levels upon 10 increasing single-dose allergen challenges, you will need, at least 50,000 cells (the protocol is optimized for 50,000 cells) × 10 conditions = 0.5 × 10^6 cells will be required.

c. Seed the cells in a 6- or 24-well plate depending on the final volume obtained.

⚠ CRITICAL: PN-sIgE levels may differ between allergic donors. To obtain reproducible results among experiments, it is highly recommended to test degranulation of LAD2 cells (by flow cytometry or β-hexosaminidase activity) sensitized with different PN-sIgE sera dilutions and to evaluate total PN-sIgE levels (kUA/mL). For optimizing this protocol, we have used two different donor sera diluted to a concentration of approximately 10 kUA/mL, which induced a maximal LAD2 degranulating response of 40%.

17. Leave LAD2 cells 16 h approximately at 37°C and 5% CO_2.

LAD2 desensitization

Timing: 80–100 min

18. Collect sensitized LAD2 cells and spin at 486 g with maximal acceleration and low brake (e.g., level 3 of 9) for 5 min at 20°C–25°C. Discard supernatant.

Note: it is important to perform this wash before starting the desensitization protocol to eliminate free IgE and other Igs present in the sera from PN-allergic donors.

19. Suspend cells in supplemented StemPro-34 media.
    a. For hIgE protocol, LAD2 cellularity is approximately 0.58 × 10^6 cells/mL.
    b. For PN protocol, LAD2 cellularity is approximately 0.61 × 10^6 cells/mL.
20. Seed 0.05 × 10^6 LAD2 cells in each well of a U/V-bottom 96 well pate in 86 µL or 82 µL, depending on the desensitization protocol performed.

Table 2. LAD2 *in vitro* cell desensitization (DS) protocol with anti-IgE (α-hIgE)

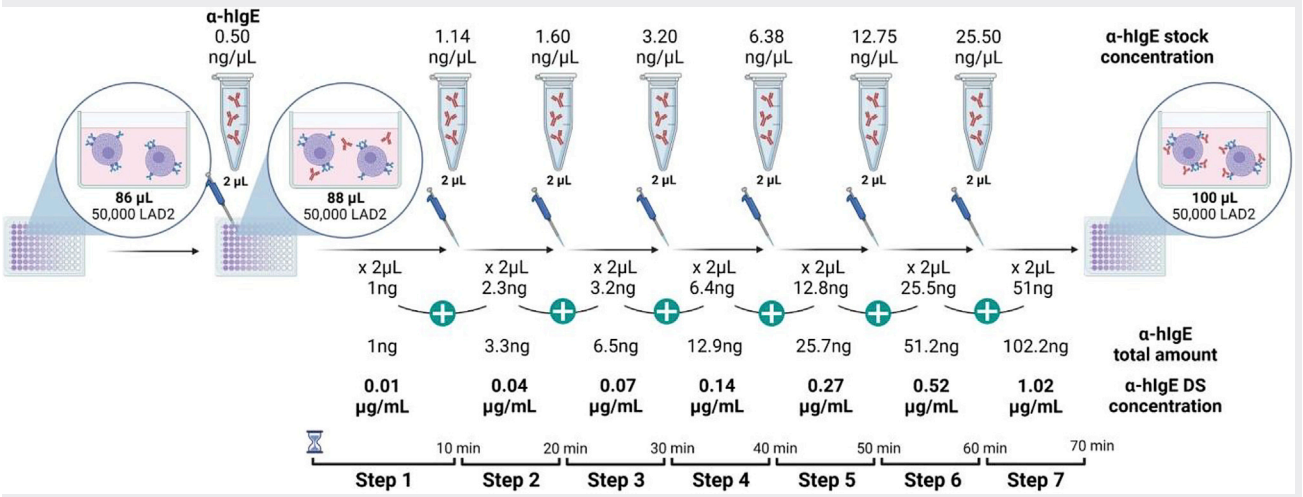
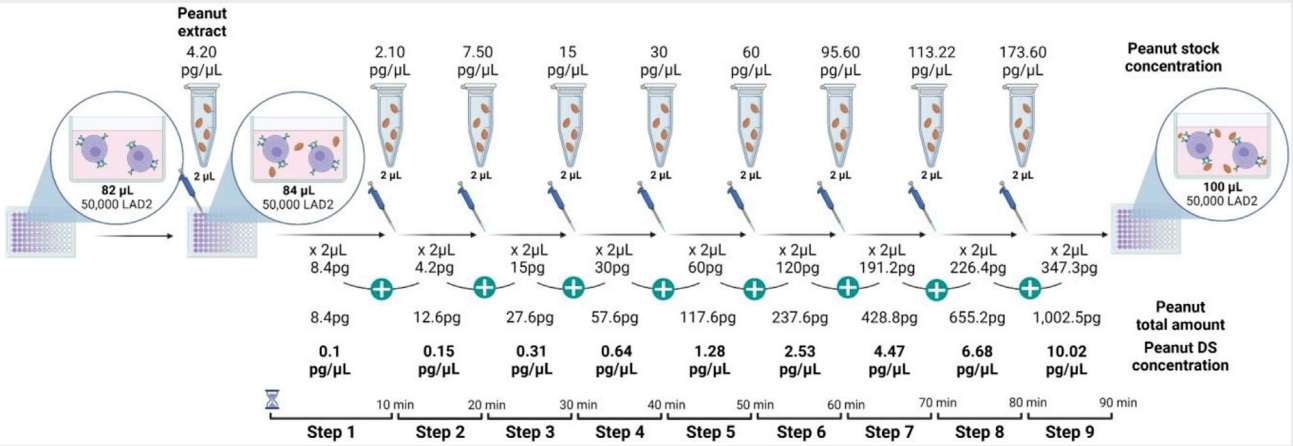


Table 3. LAD2 *in vitro* cell desensitization (DS) protocol with peanut (PN)



⚠ CRITICAL: The LAD2 desensitization protocol is optimized for 50,000 cells in a final volume of 100 µL. The protocol has a different number of steps whether we are performing hIgE or PN protocol.

⚠ CRITICAL: The hIgE desensitization protocol entails 7 steps. In every step of the desensitization protocol 2 µL are added, so by the end of the protocol a total volume of 14 µL is added. To keep the final volume of 100 µL, 50,000 LAD2 should be seeded in 86 µL (100 µL–14 µL = 86 µL).

⚠ CRITICAL: The PN desensitization protocol has 9 steps. In every step of the desensitization protocol 2 µL are added, so by the end of the protocol a total volume of 18 µL is added. To keep the final volume at 100 µL, 50,000 LAD2 should be seeded in 82 µL (100 µL–18 µL = 82 µL).

21. Carry out the desensitization protocol adding 2 µL of the anti-hIgE or PN at the corresponding concentration every 10 min (Tables 2 and 3).

EXPECTED OUTCOMES

The desensitization protocol should prevent, or substantially reduce, BMMC or LAD2 activation following a single-dose allergen challenge in comparison to non-desensitized MCs (Figure 1). To evaluate the success of the desensitization protocol, the degranulation of MCs has been measured by two methods.

β-hexosaminidase assay

Desensitized and non-desensitized MCs were challenged with PN or anti-IgE at 10 µg/mL and 20 µg/mL, respectively. After 10 min at 37°C, the reaction is stopped on ice and cells were centrifuged at 486 g with maximal acceleration and low brake (e.g., level 3 of 9) for 5 min at 4°C–8°C to recover cell supernatant. A volume of 45 µL is added in duplicates to 45 µL of the β-hexosaminidase substrate solution in a flat-bottom 96 well plate. After a 45 min incubation at 37°C in the dark, 90 µL of NaOH 1 M were added to stop the reaction. OD was measured at 405 nm with a microplate reader. Media, buffers alone and MC lysates were used as a control, absorbance background (B) and total β-hexosaminidase release (A), respectively. The % of β-hexosaminidase activity was calculated according to the following formula:

$$degranulation\ (\%) = \frac{OD\ supernatant - B}{OD\ total\ lysis\ (A) - B} \times 100$$

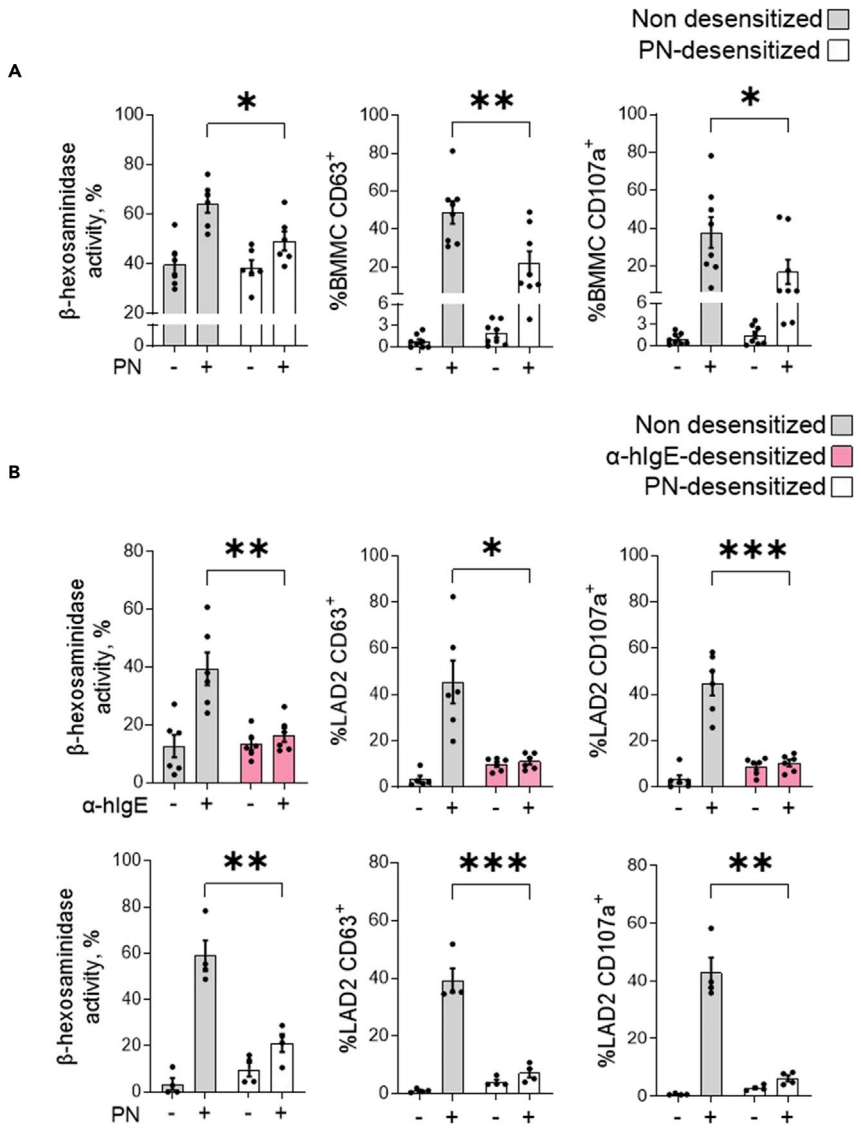


Figure 1. Polyclonal desensitization impairs mast cell (MC) degranulation after a single-dose allergen challenge

(A) % of β-hexosaminidase activity (left), and CD63 and CD107a expression (middle and left) by flow cytometry of desensitized bone-marrow-derived MCs (BMMCs) after a single-dose peanut (PN) challenge.

(B) % of β-hexosaminidase activity (left), CD63 and CD107a expression (middle and left) of desensitized LAD2 after a single-dose anti-hIgE or PN challenge. Comparisons between 2 groups were made with a two-tailed t-student or Mann-Whitney tests. The latter was used when data were non-parametric. Welch correction was used when equal SD between data could not be assumed. Data are shown as mean ± SEM. N = 4–8 independent experiments. $p<0.0332$ (*); $p<0.0021$ (**); $p<0.0002$ (***); $p<0.00001$ (****).

Flow cytometry

Once MCs were challenged with a PN or anti-hIgE single-dose, cell pellets were suspended in ice-cold FACS buffer and blocked with 1:50 of purified anti-mouse CD16/CD32 for BMMCs and Human TruStain FcX for LAD2 cells for 15 min. Then, cells were incubated with a combination of fluorophore-labelled antibodies (see key resources table) on ice for 30 min. Cell viability was assessed with efluor780 dye. After washing with FACS buffer, MC were analyzed on a BD FACSCanto II flow cytometer. On average, 10,000 events of live and single cells were recorded. Data were analyzed using FlowJo v10 software.

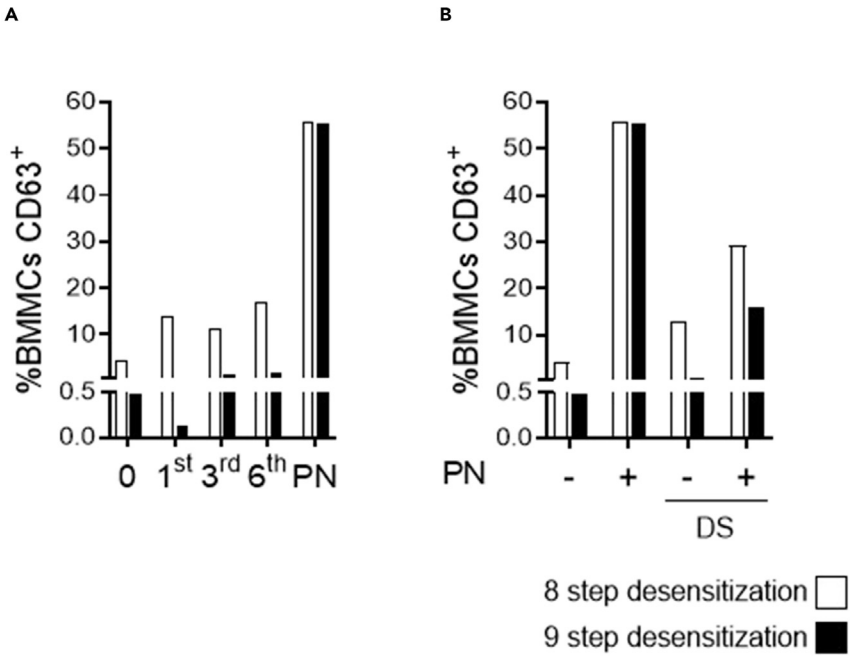


Figure 2. CD63 expression results for two different desensitization approaches in bone-marrow-derived mast cells (BMMCs)

(A) CD63 expression in different in-between step points of the desensitization protocol is reduced when the concentration increase between steps is lessen.

(B) CD63 expression of sensitized and desensitized (DS) BMMCs after a single dose challenge is lower when the concentration increase between steps is reduced. N = 1 experiment.

LIMITATIONS

The protocol describes how to desensitize BMMCs and LAD2 MCs that have been sensitized with polyclonal IgE. It does not describe the desensitization of other MC types, such as peritoneal MCs, or other human MC lines such as LUVA, HMC-1 or LADR. In addition, this protocol does not describe desensitization against allergens other than PN. Notwithstanding, it lays the foundation to develop desensitization methods to other common allergens such as chicken egg white or house dust mite using LAD2 cells sensitized with sera from allergic patients.

TROUBLESHOOTING

Problem 1

A clear reduction in MC activation (assessed by β-hexosaminidase enzymatic assay or phenotypically by flow cytometry) is not seen following a single-dose challenge in desensitized MCs 10 min after the protocol is ended (BMMC desensitization step 11 and LAD2 desensitization step 21).

Potential solution

Previous work of Oka et al. suggested in their monoclonal desensitization protocol that allergen concentration should not be increased by 10-fold at each desensitization step (Oka et al., 2013), however in different systems, the concentration increase can differ. Thus, when setting up the protocol, we recommend analyzing several in-between steps of the desensitization procedure to ensure that MCs do not respond during it. Or if they do so, to better characterize and optimize the protocol in your system. As shown in Figure 2, a desensitization attempt with 8 steps induced a higher in-between MC response when compared with the 9 steps protocol.

Problem 2

There is no reduction in the level of MC degranulation upon a single-dose allergen challenge few hours after the desensitization protocol is ended.

Potential solution

In our conditions, we confirmed that MC degranulation is impaired 10 min and even 1 h after finishing the protocol. However, we did not study MC responses at longer times. Thus, we recommend evaluating the time frame your specific desensitization protocol lasts before planning long-time experiments.

Problem 3

There is no surface expression of CD63 or CD107a in MCs 24 h after a single-dose allergen challenge.

Potential solution

The surface expression of these two molecules is affected by receptor recycling kinetics. For instance, CD107a expression is lost 24 h after challenge. In consequence, when the experiment requires long-term incubation (e.g., measuring late-phase inflammatory cytokines), we recommend assessing MC degranulation by methods other than flow cytometry, such as β-hexosaminidase activity in cell supernatants.

Problem 4

The β-hexosaminidase enzymatic activity is high in the basal condition in BMMCs (expected outcomes).

Potential solution

In our settings, we noticed that heated-inactivated FBS added background signaling to the β-hexosaminidase enzymatic assay. We recommend keeping FBS out of the experimental desensitization media culture if this technique is going to be used as a MC degranulation readout. On the other hand, if the experimental conditions required long-time incubations (e.g., 4 h), we suggest adding FBS alone in the enzymatic assay and remove this signal from every other experimental condition.

Problem 5

Cell viability is lower than 80%–85%.

Potential solution

We do not recommend using MCs with low viability for desensitization and later degranulation assay because these responses may be affected. Thus, we strongly recommend starting new cell cultures or extending the culture until cell viability is adequate. In our experience, a common cause of low MC viability is cell overgrowth; so, checking cellularity and replacing media on demand is helpful.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Rodrigo Jiménez-Saiz (rodrigo.jimenez@ufv.es).

Materials availability

This study did not generate new unique reagents. All materials are noted in the key resources table including the catalog numbers.

Data and code availability

This study did not generate or analyze datasets/code.

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AUTHOR CONTRIBUTIONS

C.L.-S. and E.S.-M. participated in the design of the study and performed experiments. C.L.-S. wrote the manuscript and prepared the tables and figures. C.L.-S. and E.S.-M. designed and prepared the graphical abstract. R.J.-S. designed the study, raised funding, performed experiments, edited the manuscript, and oversaw the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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