A novel functional mast cell assay for the detection of allergies

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Background: Clinical management of allergic diseases has been hampered by the lack of safe and convenient tests to reliably identify culprit allergens and to closely follow changes in disease activity over time. Because allergy diagnosis is a complex and laborious multistep procedure, there is an urgent need for simpler but still functionally accurate *ex vivo* assays allowing objective diagnosis, substantiating treatment choices, and quantifying therapeutic responses.

Objective: In this study, we sought to develop a novel functional cell-based assay that relies on passive sensitization of allergic effector cells with patient serum, circumventing current limitations in allergy diagnosis.

Methods: We genetically engineered a conditional homeobox B8 (Hoxb8)-immortalized progenitor line from the bone marrow of mice that are transgenic for the human high-affinity IgE receptor (FcεRIα). These cells can be reproducibly differentiated into mature Hoxb8 mast cells within 5 days of culture in virtually unlimited numbers.

Results: We demonstrate that the established Hoxb8 mast cell assay can be used to accurately measure total IgE levels, identify culprit allergens, longitudinally monitor allergen-specific immunotherapy, and potentially determine the time point of tolerance induction upon allergen-specific immunotherapy in patients with allergy. To facilitate the analysis of large testing volumes, we demonstrate a proof-of-concept for a high-throughput screening application based on fluorescent cell barcoding using the engineered Hoxb8 mast cells.

Conclusions: Our results indicate that this novel mast cell assay could represent a valuable tool to support clinicians in the identification of IgE-mediated allergies and in the quantification of treatment efficacy as well as duration of therapeutic response. (J Allergy Clin Immunol 2022;149:1018-30.)

Key words: *Allergies, IgE, diagnostic testing, functional assay, human high-affinity IgE receptor, mast cells, immunotherapy monitoring, homeobox B8*

Roughly one-third of the global population is suffering from allergic hypersensitivity disorders, according to recent estimations.[1] For many patients, allergies are associated with a marked reduction in physical and mental well-being and lead to a significant loss in quality of life due to disease activity.[2-4] To provide efficient and personalized treatment options, physicians are dependent on solid and reliable diagnostic tools. Generally, allergy diagnosis is a complex and laborious multistep procedure.[5,6] It involves the examination of the patient's medical history, serological determination of total and allergen-specific IgE antibody levels,[7] and various *in vivo* allergen skin prick tests and other *in vivo* allergen challenge protocols.[8] Even though the determined levels of total and allergen-specific IgE antibodies provide information about the atopic status of an individual, these values often poorly correlate with disease activity and clinical symptoms.[9] Furthermore, important parameters contributing to aggravation or suppression of allergic responses such as diversity and affinity of the allergen-specific IgE antibodies and the presence of allergen-specific IgG antibodies are generally neglected in the interpretation of diagnostic laboratory results. Occasionally, a functional basophil activation test with whole blood samples of the patient is performed to determine reactivity against certain allergens.[10] While such assays are useful because they provide important quantitative and functional information about the allergic status of an individual,[10] they are hampered by the use of fresh whole blood that has to be processed within hours in specialized diagnostic laboratories.[11,12] Immediate analysis of whole blood is associated with major logistical challenges because its storage is not possible due to instability of the biological material. More recently, diagnostic testing of allergies based on primary human blood–

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Abbreviations used
AIT: Allergen-specific immunotherapy
c-KIT: Stem cell factor receptor
Hoxb8: Homeobox B8
Hoxb8 MC: Hoxb8 mast cell
huFcεRIα: Human high-affinity IgE receptor alpha chain subunit
LAD2: Laboratory of Allergic Diseases 2 mast cell line
NIP-BSA: 4-hydroxy-3-iodo-5-nitrophenylacetyl hapten conjugated to BSA
PSCMC: Human peripheral $CD34^+$ stem cell–derived mast cell
RBL: Rat basophilic leukemia
rpc: Receptors per cell
$RPMI^c$: Complete RPMI medium
SCIT: Subcutaneous immunotherapy
4-OHT: 4-hydroxytamoxifen

derived mast cells or immortal human mast cell lines (ie, mast cell activation test) has been suggested by independent groups.[13-15] While the overall goal of this approach is appealing, the generation of the cells is laborious and requires extended culturing periods of more than 2 months. Despite these interesting recent developments, a convenient, safe, standardized, and reliable diagnostic assay predicting functional reactivity against culprit allergens is still not available.

Patients with allergy are often advised to undergo allergen-specific immunotherapy (AIT), which has been reported to be the only disease-modifying intervention currently available for allergy treatment.[16,17] In AIT, the identified culprit allergen is directly applied to the patient with the aim of inducing tolerance. In subcutaneous AIT (SCIT) protocols, patients receive monthly allergen injections over a duration of 3 to 5 years after an initial updosing phase. Various molecular and cellular mechanisms such as the induction of allergen-specific protective IgG have been described for AIT[18]; however, it remains still unclear why some patients respond better to the treatment than others. Studies in food-allergic individuals report that only 13% of patients show sustained unresponsiveness to an allergen after 1 year of AIT completion as assessed in a recent peanut desensitization study.[19] While basophil activation test has been performed in proof-of-concept studies,[20] there is currently no standardized read-out system available to assess whether and when a patient is responding to the treatment. Therefore, physicians must rely on the clinical course and symptom scores when assessing the effectiveness of immunotherapy. In selected cases, they perform *in vivo* allergen challenge tests to determine the degree of unresponsiveness after AIT, which is unpleasant for the patient and associated with the risk of inducing an allergic reaction in case the patient has not responded to the treatment.[21]

In this study, we sought to overcome current limitations of allergy diagnosis through the establishment of a functional allergy screening assay. To do so, we aimed at developing a novel and time-efficient method to generate highly functional mast cells from conditionally immortalized progenitors that can be passively sensitized with serum from patients with allergy and used in allergen-mediated activation tests. We further envisioned that such a test might be useful for the assessment of treatment responses including immunotherapy or anti-IgE antibody administration.

METHODS

See the Methods section in this article's Online Repository at www.jacionline.org for detailed information about reagents, cell culture of different allergic effector cell lines and bone marrow–derived cells, receptor quantification and IgE-binding capacity, activation with recombinant proteins and patient sera as well as an exact protocol for fluorescent cell barcoding.

Generation and differentiation of conditional homeobox B8–immortalized progenitors

Subcloning of the mouse homeobox B8 (Hoxb8) coding sequence into the pF-5xUAS-SV40-puro-Gev16 lentiviral vector system and generation of viral particles have been described elsewhere.[22,23] In short, lentiviral particles carrying a 4-hydroxytamoxifen (4-OHT)-inducible Hoxb8 expression system were produced in HEK 293T cells by transient transfection using X-tremeGENE HP transfection reagent (Roche Diagnostics, Rotkreuz, Switzerland). A total of 15 μg of DNA was transfected per 10-cm tissue culture dish, using a 2:5:3 ratio of pMD2.VSV-G (envelope proteins):pCMVδR8.2 (packaging elements):pF-5xUAS-Hoxb8(mm)-Sv40puroGev16 with 30 μL of transfection reagent. Medium was replaced the next day, and virus-containing supernatant was collected 24 and 48 hours later. Supernatants were pooled and passed through a 0.2-μm filter and used fresh for infection. Hematopoietic progenitor cells were enriched from bone marrow of B6.Cg-*Fcer1a*^{tm1Knt} Tg(FCER1A)1Bhk/J mice by magnetic cell separation using a lineage depletion cocktail (BD IMag, BD Biosciences Europe, Allschwil, Switzerland) following the manufacturer's instructions. A total of 5×10^5 lineage-marker depleted cells were incubated for 48 hours in complete RPMI medium ($RPMI^c$ = RPMI-1640 AQmedia, 10% FCS, 100 U/mL penicillin, 100 ug/mL streptomycin, 50 μM 2-mercaptoethanol) in the presence of 300 to 400 pg/mL IL-3 (added as WEHI-3B cell conditioned medium as a source for mouse IL-3). Cells were spin infected with ^{cond}Hoxb8 lentiviral particles (1 mL virus-containing supernatant, supplemented with 8 μg/mL polybrene) at 30°C for 90 minutes. Cells were transferred to $RPMI^c$/IL-3 medium, and Hoxb8 expression was induced by addition of 0.1 μM 4-OHT. Puromycin selection (1 μg/mL) was started 4 days after infection and maintained until outgrowth of surviving cells. Obtained cell lines were cultured in $RPMI^c$/IL-3/4-OHT and selected on the basis of combination of best growth, viability, and functional performance in the functional assay. The best-performing line was furthermore used for testing of subclones by limited single-cell dilution and clonal expansion in 96-well plates using 50% preconditioned growth medium. To differentiate progenitors into mature allergic effector cells, cells were washed twice in PBS and reseeded at 7.5×10^4 cells/mL in $RPMI^c$/IL-3 medium for 5 to 6 days.

Flow cytometric and morphological characterization of cells

To characterize the Hoxb8 mast cell (Hoxb8 MC) progenitors at various stages of their differentiation, the cells were stained at day 0, 2, 4, or 6 after differentiation start with anti-mouse stem cell factor receptor (c-KIT) CD117 and anti-human FcεRIα antibodies. To visualize the expression of the surface markers and IgE binding to human high-affinity IgE receptor alpha chain subunit (huFcεRIα) in a spatial context by multispectral imaging flow cytometry, 1×10^5 differentiated Hoxb8 MCs were incubated with anti-mouse c-KIT CD117 and anti-human FcεRIα antibodies, as well as the corresponding isotypes. Hoxb8 MCs after overnight incubation with 0, 0.5, and 5 μg/mL JW8-IgE were stained with anti-human IgE and anti-human FcεRIα antibodies. To assess the expression of the inhibitory mouse CD32 (FcgR2) receptor on Hoxb8 MCs on day 6 of differentiation, 5×10^4 cells were washed twice with 200 μL of PBS, pH 7.4, at 600*g* for 5 minutes at 4°C. Subsequently, the cells were stained with the anti-mouse CD32 antibody or its corresponding isotype control antibody for 15 minutes, light protected at room temperature. Expression and distribution of the surface marker anti-mouse c-KIT and anti-human FcεRIα and the binding of IgE-JW8 to the receptor were assessed using an Amnis ImageStream X MKII and the corresponding IDEAS software (Luminex Corporation, Austin, Tex).

To visualize the progressive increase in cellular granularity of the Hoxb8 MC progenitor line throughout their differentiation, 5×10^4 cells were immobilized by use of a Cellspin I centrifuge (Tharmac, Wiesbaden, Germany) on microscopy slides (Thermo Fisher Scientific, Waltham, Mass) at day 0, 2, 4, or 6 after differentiation start. Cells were stained with 1% Toluidine blue in methanol solution, dehydrated, cleared, and mounted with Cyto Seal XYL (Thermo Fisher Scientific). Images were acquired in brightfield mode using the HCX PL APO objective for 63× magnification at the LEICA DMI4000 microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the corresponding LAS V4.2 software (Leica Microsystems GmbH).

Human samples and animals

Human serum samples of allergic donors were received from the Center of Laboratory Medicine at the University Hospital Bern with approval from the local ethics committee (KEK 2017-01590). Human serum samples of donors with allergy who underwent SCIT were received from a study approved by the Regional Committee on Biomedical Research Ethics (M2009-0121) at Aarhus University Hospital.[24-26] Informed consent was obtained in accordance with the Declaration of Helsinki. Information on total and allergen-specific IgE levels, the ratio thereof as well as allergen-specific IgG levels can be found in the Online Repository (see Tables E1-E4 in this article's Online Repository at www.jacionline.org). Mice transgenic for human FcεRIα and with the mouse FcεRIα knocked out were obtained from Prof. Jean-Pierre Kinet. Double-transgenic mice expressing human IgE and human FcεRIα were licensed from GenOway S.A. All animal experimentation was approved by the local animal committee (BE66/18).

Statistics

Statistical analysis and calculation of linear as well as nonlinear fitting models as indicated in the figure legends were carried out in Prism 8.0 software (GraphPad Software, La Jolla, Calif). Wherever suitable, individual data points are shown. For all other graphs, data are displayed as mean ± SEM.

RESULTS

Differentiation of Hoxb8 immortalized progenitor cells results in mature mast cells

To generate allergic effector cells for use in a functional diagnostic allergy test, we conditionally immortalized mast cell progenitors from mice that are transgenic for the human high-affinity IgE receptor alpha chain (FcεRIα) with a previously described 4-OHT–inducible Hoxb8 expression system including a puromycin resistance gene[23] (Fig 1, *A*).

It has previously been demonstrated that shutdown of exogenous Hoxb8 expression on withdrawal of 4-OHT in such stably transduced progenitor cultures readily induces cell differentiation along the myeloid lineage. Depending on the cytokines present, generation of neutrophils, macrophages, and basophils has been described.[23,27] Thus, we first assessed whether our progenitor cells might differentiate into mature allergic effector cells (ie, c-KIT–negative basophils or c-KIT–positive mast cells) in the presence of IL-3. Importantly, the generation of mast cells using conditional Hoxb8 has not yet been reported. Indeed, we observed differentiation into allergic effector cells on 4-OHT removal from the culture medium (Fig 1, *B*). While all selected progenitor lines expressed high levels of huFcεRIα, flow cytometric analysis revealed that there was considerable heterogeneity regarding c-KIT expression after 5 days of differentiation, indicating that our immortalization and selection strategy enriched for both basophil (ie, huFcεRIα$^+$/c-KIT$^-$) and mast cell progenitors (huFcεRIα$^+$/c-KIT$^+$), respectively (Fig E1).[23,28,29] We decided to further proceed with the cells showing the most uniform expression of c-KIT. With the goal to ultimately obtain a robust mast cell progenitor line, we characterized these cells at various stages of differentiation (ie, day 0-6). Flow cytometric analysis demonstrated that c-KIT expression gradually increased over time and that by day 6 more than 90% of the cells were double-positive for c-KIT and huFcεRIα (Fig 1, *B*). To further visualize these cell surface markers in a spatial context, we additionally performed image stream flow cytometry. These measurements revealed an even distribution of both huFcεRIα and c-KIT on the cell surface (Fig 1, *C*) and confirmed upregulation of c-KIT on differentiation. Toluidine blue staining of the cells at various differentiation stages additionally indicated a progressive increase in cellular granularity and a clear mast cell phenotype with metachromatic elements (Fig 1, *D*). In addition, we performed limited dilution of the selected polyclonal progenitor cells and subsequent clonal expansion, which resulted in the generation of a monoclonal Hoxb8 MC line (ie, NT-1). NT-1 showed remarkably similar characteristics as the parental polyclonal line in terms of c-KIT/huFcεRIα expression and IgE binding (Fig E2, *A* and *B*). We ultimately decided to continue using the polyclonal line for subsequent experiments until the monoclonal line has been tested more thoroughly. Together, these data provide strong evidence that our selection strategy resulted in the identification of an immortalized huFcεRIα transgenic mast cell progenitor line, from which differentiated Hoxb8 MCs can be derived in as little as 5 days on removal of 4-OHT from the cell culture in the presence of mouse IL-3–containing differentiation medium.

Differentiated Hoxb8 MCs are functionally active

To further characterize differentiated Hoxb8 MCs, we quantified the absolute amount of huFcεRIα receptors per cell (rpc) and compared it with levels measured on either bone marrow–derived mast cells from mice transgenic for the human high-affinity IgE receptor, bone marrow–derived mast cells from mice double-transgenic for human IgE and the human high-affinity IgE receptor, rat basophilic leukemia (RBL) cells that were stably transfected with huFcεRIα and huFcεRIγ (ie, RBL-SX38 cell),[30] human peripheral CD34$^+$ stem cell–derived mast cells (ie, PSCMCs),[31] and the immortal human mast cell lines Laboratory of Allergic Diseases 2 mast cell line (LAD2),[32] human mast cell line 1,[33] and Laboratory of University of Virginia mast cell line[34] (Fig 2, *A*, and see Table I). In the absence of IgE sensitization, Hoxb8 MCs displayed an average of 18,415 rpc, which is similar to the 16,190 rpc measured on PSCMCs, whereas bone marrow–derived mast cells from mice transgenic for the human high-affinity IgE receptor expressed almost twice as many receptors with 35,391 rpc. Bone marrow–derived mast cells from mice double-transgenic for human IgE and the human high-affinity IgE receptor and LAD2 cells showed an intermediate level with 25,020 rpc and 29,656 rpc, respectively. RBL-SX38 cell displayed the highest receptor numbers with 51,862 rpc. Surface expression of huFcεRIα was undetectable on both human mast cell line 1 and Laboratory of University of Virginia mast cell line. In the presence of 5 μg/mL recombinant human IgE, the amount of huFcεRIα increased about 5-fold on all cells that have been analyzed, except for LAD2 cells, which showed only a 2-fold increase (Fig 2, *A*, and see Table I). This finding is in line with previous reports demonstrating that IgE binding stabilizes its receptor on the mast cell surface.[35] Image stream flow cytometry analysis further revealed a homogeneous distribution and

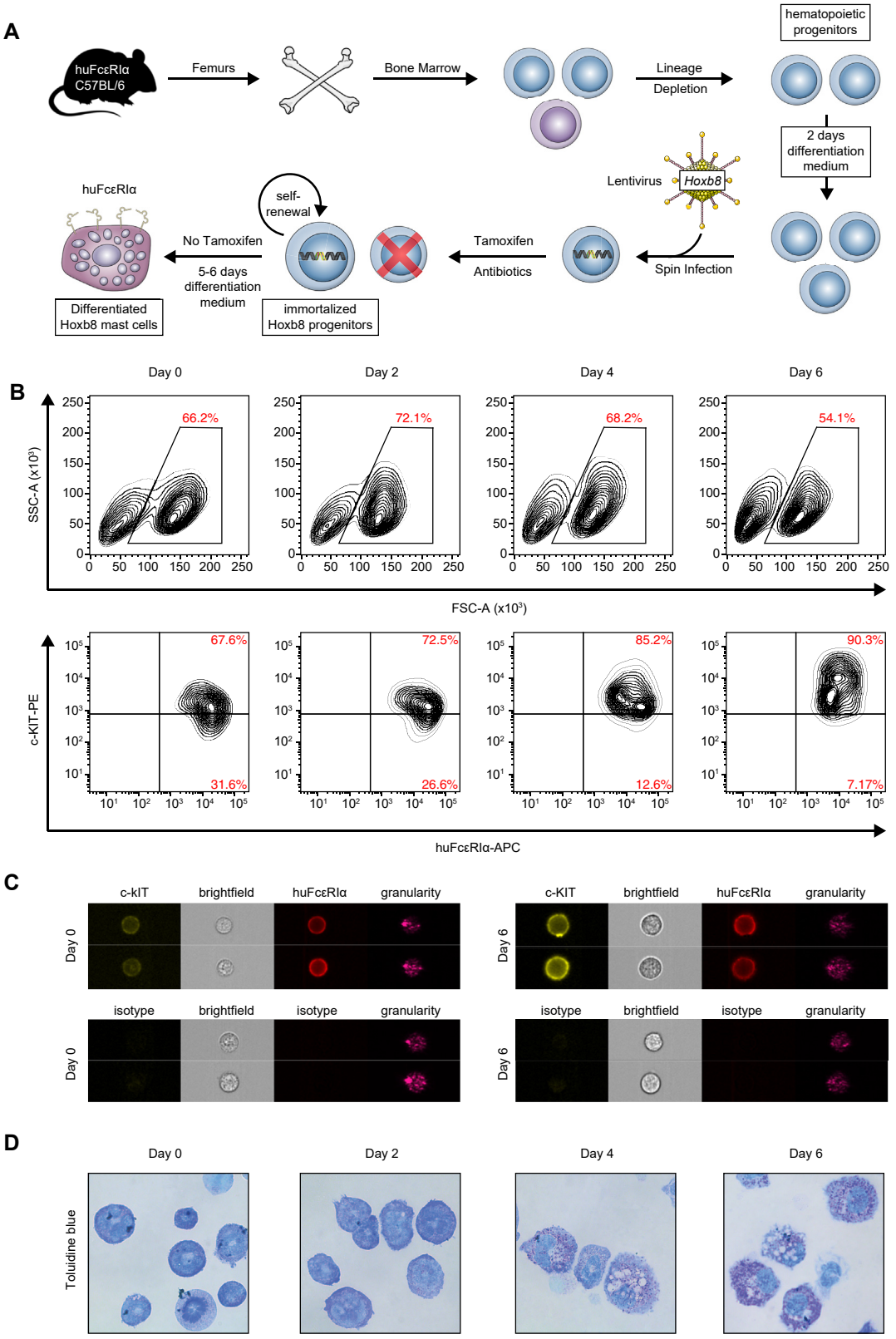

FIG 1. Generation and differentiation of conditional immortalized Hoxb8 MC progenitors. **A,** Schematic overview of progenitor line generation and differentiation. **B,** Flow cytometric assessment of the selected Hoxb8 MC progenitor line on removal of 4-OHT. Representative contour plots for day 0, 2, 4, and 6 of differentiation. Cells are gated on side- and forward-scatter (*top row*) and subsequently analyzed for c-KIT/huFcεRIα expression (*bottom row*). **C,** Imaging flow cytometric assessment of the selected Hoxb8 MC progenitor line at day 0 (to the left) and 6 (to the right) of differentiation. Representative brightfield pictures as well as fluorescent images of granularity, c-KIT, and huFcεRIα expression for stained cells (*top row*) and isotype control stained cells (*bottom row*). **D,** Morphological analysis of the selected Hoxb8 MC progenitor line at day 0, 2, 4, and 6 of differentiation by Toluidine blue staining. *FSC-A*, Forward scatter-area; *SSC-A*, side scatter-area.

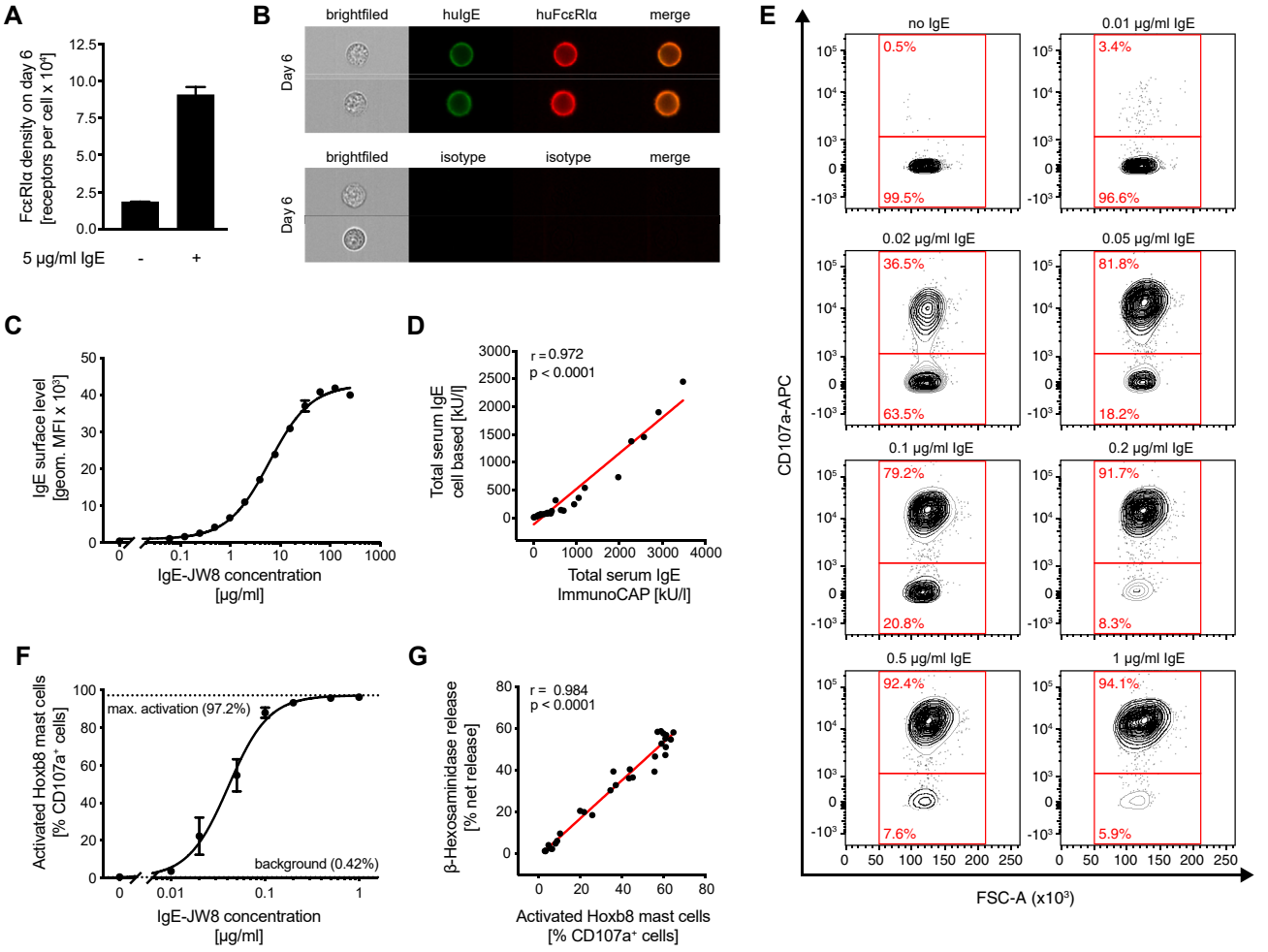

FIG 2. Functional characterization of Hoxb8 MCs after 6 days of differentiation. **A,** The absolute number of huFcεRIα rpc in the absence or presence of human recombinant IgE. **B,** Imaging flow cytometric assessment of IgE binding to the cell surface. Representative brightfield pictures as well as fluorescent images of huIgE binding and huFcεRIα expression as well as a merged panel for huIgE and huFcεRIα are shown for stained cells (*top row*) and isotype control stained cells (*bottom row*). **C,** Dose-dependent binding of human recombinant IgE to differentiated Hoxb8 MCs by flow cytometry. **D,** The correlation between total serum IgE as determined on Hoxb8 MCs or by singleplex immunoassay for 25 allergic patient sera. **E,** Representative contour plots for antigen- mediated activation of Hoxb8 MCs in an IgE dose-dependent manner as measured by flow cytometry. **F,** Quantification of antigen-mediated activation of Hoxb8 MCs in an IgE dose-dependent manner. **G,** Correlation between released β-hexosaminidase and the cell surface activation marker CD107a for antigen-activated Hoxb8 MCs. Statistical analysis in *D* and *G* has been performed using a standard linear regression model. Data in *A*, *C*, and *F* are shown as mean ± SEM. *FSC-A*, Forward scatter-area; *huIgE*, human IgE.

colocalization of huFcεRIα with human IgE (Fig 2, *B*). No clustering or aggregation on the Hoxb8 MC surface has been observed. Titration experiments with recombinant human IgE-JW8 on Hoxb8 MCs demonstrated that IgE can be detected in a linear range at concentrations from more than 1 µg/mL to less than 100 µg/mL by flow cytometry. Thus, this assay was able to detect IgE binding with a dynamic range of more than 2 logs (Fig 2, *C*). Next, we assessed whether it is possible to use the Hoxb8 MC system to determine total IgE concentrations in human serum samples. Using standard curves, we quantified IgE levels in sera from 25 individuals on Hoxb8 MCs and compared those results to total serum IgE values measured by the current gold standard singleplex immunoassay (ie, ImmunoCAP, Phadia, Uppsala, Sweden). Remarkably, the results demonstrated that the Hoxb8 MC system was accurate and showed a high degree of correlation ($r = 0.972$; $P < .0001$) with the measurements by singleplex immunoassay (Fig 2, *D*).

It is well established that antigen- or allergen-mediated aggregation of FcεRIα-bound IgE on mast cells leads to their activation and immediate degranulation.[36,37] However, to be functionally active, huFcεRIα has to pair with mouse γ-chains containing an intracellular signaling domain[38] (ie, formation of the trimeric $\alpha\gamma_2$ receptor) and ideally associate with the mouse β-chain also containing an intracellular signaling domain and serving as a signal amplifier[39] (ie, formation of the tetrameric $\alpha\beta\gamma_2$ receptor). To assess the expression of α-, β-, and γ-chains in Hoxb8 MCs, we performed western blot analysis. All 3 chains were detectable, strongly suggesting that the heterotetrameric $\alpha\beta\gamma_2$ receptor form

TABLE I. Comparison of different allergic effector cell models

Name	Hoxb8 MC	BMMCα-sTG	BMMCα-dTG	RBL-SX38 cell	LAD2	PSCMC
Growth rate (doubling time in hours)	28.8	NA*	NA*	24.8	194.8	NA*
Human FcεRIα per cell (absence of IgE)	18,415	35,391	25,020	51,862	29,656	16,190
Human FcεRIα per cell (presence of IgE)	90,831	200,605	188,487	227,021	66,217	61,499
Maximal activation no wash (CD107a)	97.2%	60.8%	67.5%	NA†	91.6%	93.9%
Maximal activation with wash (CD107a)	63.5%	41.5%	41.5%	NA†	82.1%	67.2%

No expression of human FcεRIα could be detected on human mast cell line 1 and Laboratory of University of Virginia mast cell line cells.
BMMCα-dTG, Bone marrow–derived mast cells from mice double-transgenic for human IgE and the human high-affinity IgE receptor; *BMMCα-sTG*, bone marrow–derived mast cells from mice transgenic for the human high-affinity IgE receptor; *LAD2*; laboratory of allergic diseases mast cell line; *PSCMC*, human peripheral $CD34^+$ stem cell-derived mast cells; *NA*, not available.
*NA because cells are not immortalized and do not proliferate in differentiated state.
†NA because cells grow adherent.

was expressed on the surface of Hoxb8 MCs (Fig E3, *A*). Activation and degranulation of allergic effector cells can be assessed by various means. On the one hand, researchers quantify specific enzymes or mediators (eg, β-hexosaminidase) that get released on degranulation in the cell culture supernatants.[40] Alternatively, cell surface markers that get exposed on degranulation, such as the lysosome-associated membrane protein-1/CD107a, may be quantified by flow cytometry.[41] To assess whether huFcεRIα in differentiated Hoxb8 MCs is functionally active, we sensitized cells with increasing concentrations of antigen-specific IgE-JW8 and challenged them with a fixed concentration (ie, 100 ng/mL) of the cognate antigen (NIP_{24}-BSA). Using cell surface CD107a positivity as a read-out (Fig 2, *E*), our results indicated dose-dependent activation of Hoxb8 MCs, which was highly reproducible in its maximal activation and which overall showed a remarkable signal-to-noise ratio (Fig 2, *F*). While maximal activation was reached at approximately 95%—a value that has previously neither been observed with mast cell lines nor with bone marrow–derived mast cells (Table I)—background activation remained low at less than 1%. We also tested Hoxb8 MCs derived from a monoclonal progenitor line (ie, NT-1), which behaved almost identically to cells derived from its parental polyclonal line (Fig E2, *C* and *D*). In addition to the exceptionally high maximal activation values, Hoxb8 MCs showed other favorable characteristics. The total yield of differentiated cells after 5 days is 5.8 times the number of seeded progenitor cells (Fig E3, *B*), which is in a similar range as previously described for human blood–derived mast cell cultures after 8 weeks.[42] However, with a calculated doubling time of 28.8 hours, the Hoxb8 progenitor cultures grow at a similar rate as RBL-SX38 cell and clearly faster than other previously described mast cell lines including LAD2 (Fig E3, *C*, and Table I). Importantly, Hoxb8 MCs were still functional after 4 months of continuous progenitor cell culture, showing no sign of losing activity (Fig E3, *D*). Because previous studies involving *in vitro* models of mast cell activation preferentially used quantification of β-hexosaminidase in the supernatants rather than flow cytometric analysis of activation markers, we also assessed how these 2 parameters relate to each other for Hoxb8 MCs. By performing both measurements, we found that there was a close correlation of released β-hexosaminidase and exposed CD107a surface marker (Fig 2, *G*).

Differentiated Hoxb8 MCs are suitable to assess and quantify IgE-mediated reactions

In a next step, we wanted to assess whether passive sensitization of Hoxb8 MCs with predefined sera from patients with allergy could be used to test allergen-specific activation. To get a representative picture for different allergen sources, we incubated Hoxb8 MCs with sera from individuals allergic to peanut, cat, wasp, honeybee, house dust mite, birch, or timothy grass and additionally determined total and allergen-specific IgE in these samples by singleplex immunoassay (Table E1). Moreover, we ensured that sera containing different amounts of allergen-specific IgE were represented in the tested samples. For all allergens used, we observed dose-dependent activation of Hoxb8 MCs (Fig 3, *A-G*), suggesting that this experimental setup was suitable to screen patients for unknown allergies. Interestingly, activation did not correlate with the amount of either allergen-specific or total IgE in the serum, nor with the ratio thereof, indicating that additional parameters,[43] including the presence of protective allergen-specific IgG, influence the outcome in this functional assay. These data strongly suggest that the Hoxb8 MC assay can be used to identify IgE-mediated allergies to virtually any allergen as well as to determine the severity of the allergic response based on maximal activation at a given allergen concentration.

Differentiated Hoxb8 MCs may be used for monitoring of AIT

It is well understood that the induction of protective IgG represents one of the mechanisms underlying the establishment of tolerance upon AIT.[18] Nevertheless, there is no standardized functional assay available to test the implications of allergen-specific IgG induction and to monitor treatment success of AIT. Here, we addressed the question of whether passive sensitization of Hoxb8 MCs with patient sera could be useful for the monitoring of patients with allergy undergoing AIT and whether this approach might help to predict treatment outcome. As a proof-of-concept, we sensitized Hoxb8 MCs with a fixed concentration of recombinant NIP-specific human IgE-JW8 in the presence or absence of a 200-fold excess of human IgG-JW183. On activation of the cells with different concentrations of NIP_7-BSA antigen, we observed a shift of the activation curve toward higher antigen concentrations for the NIP-specific IgG-containing sample. In addition, a decrease in maximal activation became apparent if NIP-specific IgG was present (Fig 4, *A*). Next, we tested serum samples of patients with allergy to timothy grass who had undergone AIT over the course of at least 36 months (Table E2). This longitudinal observation revealed that patient sera showing reactivity on Hoxb8 MCs to timothy grass at baseline (ie, before treatment initiation) became unresponsive after 12 months of treatment or later time points (Fig 4, *B*). However, serum from

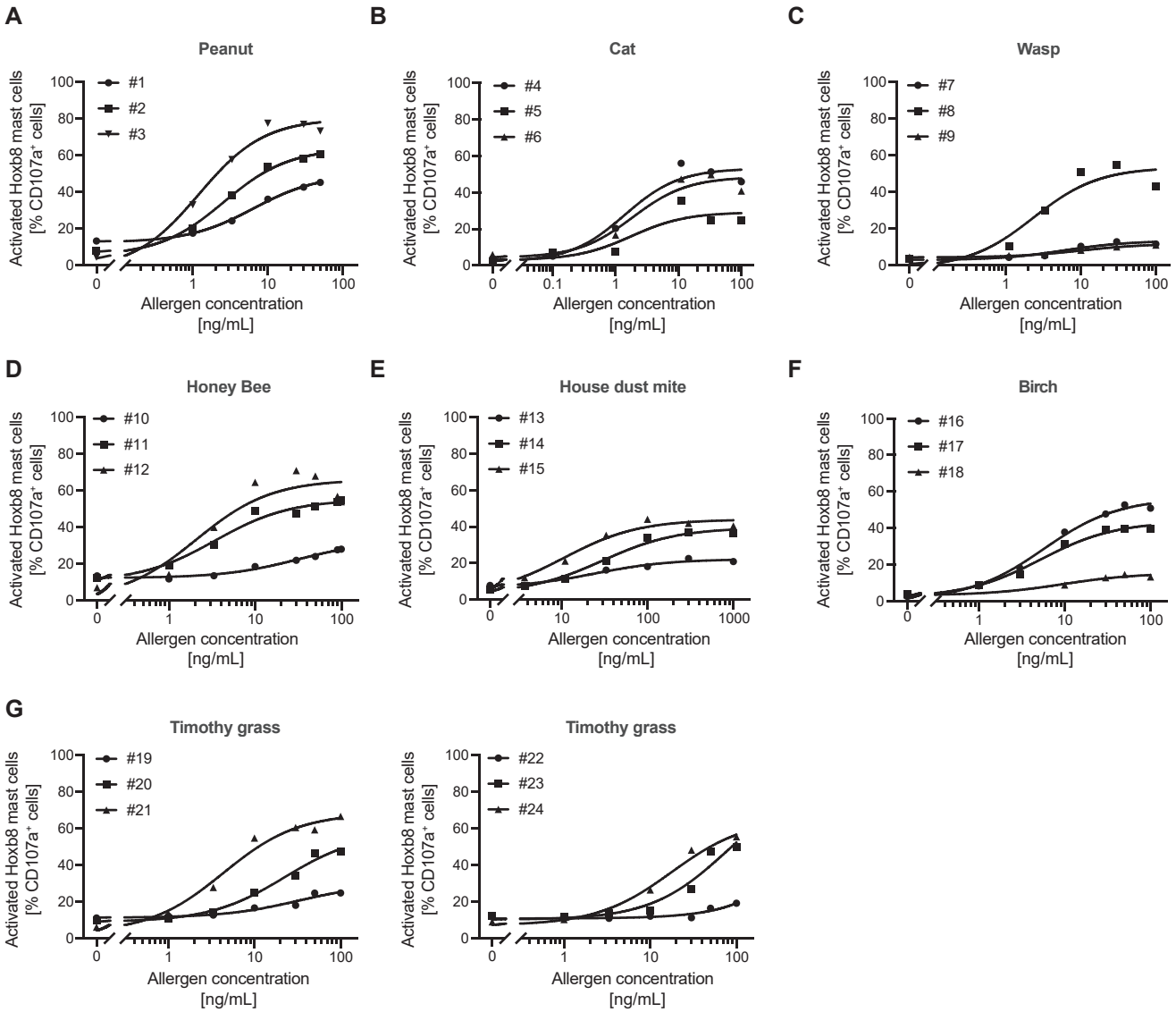

FIG 3. Testing of allergic patient sera on Hoxb8 MCs. Predefined sera of patients with allergy were used to sensitize cells overnight. Dose-dependent activation as measured by flow cytometry is shown for different allergen sources: peanut extract (**A**), recombinant Fel d1 (**B**), yellow jacket wasp venom (**C**), honeybee venom (**D**), house dust mite extract (**E**), common birch pollen extract (**F**), and timothy grass extract (**G**). Nonlinear regression curves were fitted to measured data points.

a placebo-treated control patient showed no obvious change in reactivity over the time frame of 36 months. To assess whether unresponsiveness of patient sera post-SCIT was due to the presence of protective allergen-specific IgG, we depleted IgG from 12-month post-SCIT sera and compared them with untreated 12-month post-SCIT sera. The untreated SCIT sera induced as expected no activation, whereas the IgG-depleted sera became highly reactive and dose-dependently activated the Hoxb8 MCs on timothy grass allergen challenge (Fig 4, *C*). Because our findings reemphasized the importance of allergen-specific IgG induction during SCIT, we further checked whether Hoxb8 MCs express the inhibitory IgG-Fc-receptor FcγRIIb (ie, CD32b), which could potentially be engaged by human IgG:allergen immune complexes with similar affinity than by mouse IgG[44] and might thereby inhibit Hoxb8 MC activation. Flow cytometric analysis showed a clear shift for the entire cell population when stained with an anti-CD32b antibody as compared with its isotype control, confirming the presence of FcγRIIb on the surface of Hoxb8 MCs (Fig 4, *D*). Together, these data strongly indicated that the functional Hoxb8 MC assay might be helpful in longitudinal monitoring of patients undergoing AIT treatment and for the clinical interpretation of therapy outcome. However, further studies with larger patient cohorts will be required to make generalized statements about the predictive value of this test for clinical outcomes.

Barcoded Hoxb8 MCs can be used for high-throughput allergy screening

In a next step, we investigated whether an allergy screening assay based on activation of Hoxb8 MCs could be adapted onto a high-throughput format.[45] For this purpose, we labeled

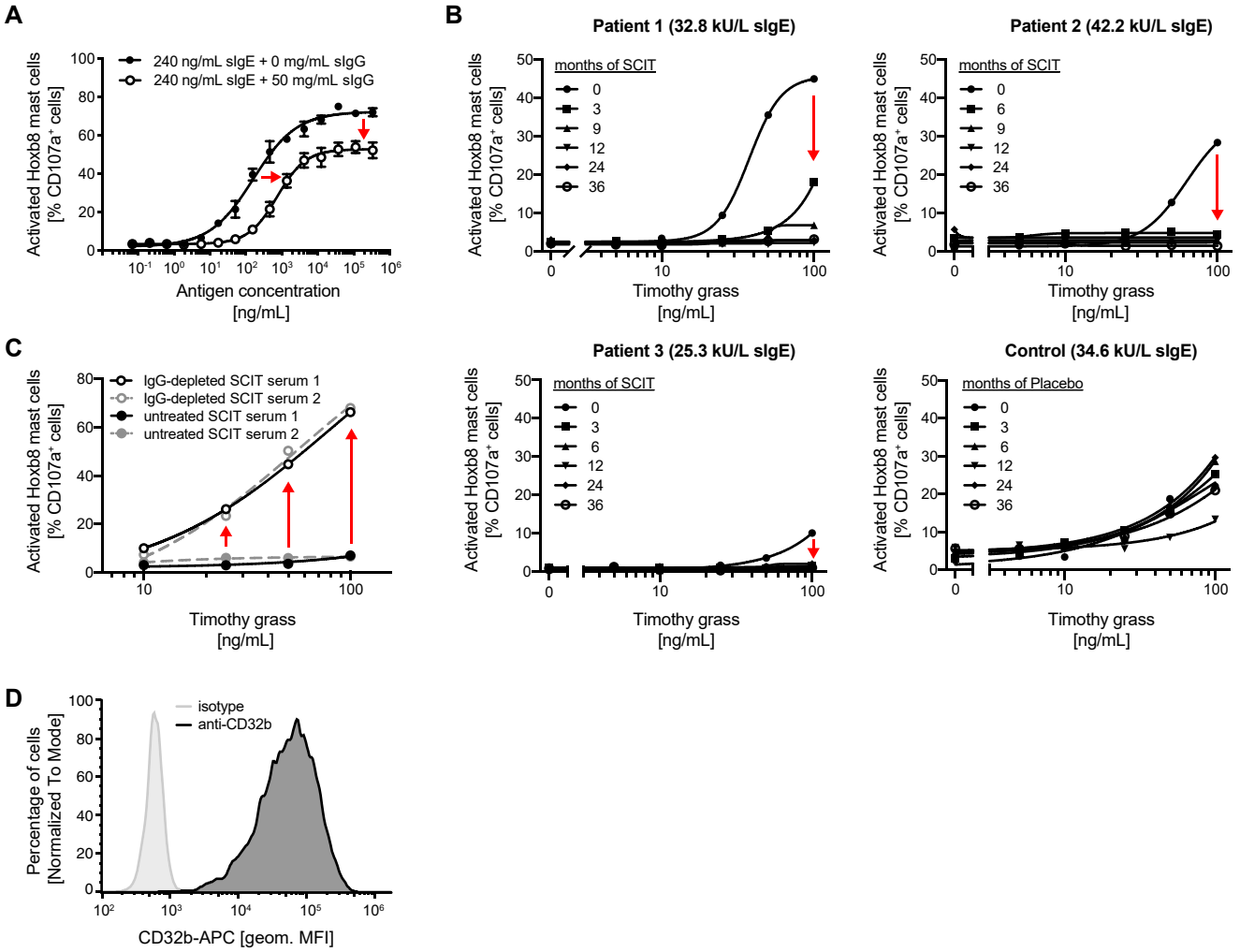

FIG 4. AIT monitoring with Hoxb8 MCs. **A,** Cells were sensitized overnight with an artificial serum containing either only human recombinant NIP-specific IgE (sIgE) or a combination of human recombinant NIP-specific IgE (sIgE) and IgG (sIgG). Dose-dependent activation of the cells as measured by flow cytometry is shown. **B,** Cells were sensitized overnight with sera from 3 patients with allergy to timothy grass who had undergone AIT for at least 36 months and 1 serum of a patient undergoing placebo treatment. Dose-dependent activation of the cells as measured by flow cytometry is shown. **C,** Cells were sensitized overnight with either untreated or IgG-depleted sera from 2 patients with allergy to timothy grass (gray and black) at 12 months post-AIT. Nonlinear regression curves were fitted to measured data points. Red arrows indicate shift of the curves. **D**, Differentiated Hoxb8 MCs were stained either with a control antibody (isotype in light gray) or with an anti-FcγRIIb antibody (anti-CD32b in dark gray). Flow cytometric analysis is shown in histogram representation with geometric mean fluorescence intensity (geom. MFI).

differentiated Hoxb8 MCs with varying concentrations of fluorescent dyes (ie, Pacific Blue, Alexa Fluor 488, Alexa Fluor 647) using covalent amine-coupling protocols (Fig 5, *A*). As previously described, each batch of cells received a unique cellular barcode based on a particular fluorescent label.[45] Flow cytometric analysis of the Hoxb8 MCs revealed a nice separation of the differentially labeled cell populations (Fig 5, *B*). As a gating strategy, we chose to first separate side scatter^{high}/forward scatter^{high} viable cells according to their Pacific Blue fluorescence intensity and subsequently to plot Alexa Fluor 488 against Alexa Fluor 647 signals. In a proof-of-concept experiment, we used different concentrations of recombinant human NIP-specific human IgE-JW8 to sensitize individually labeled cell populations before pooling all of them in a single tube to which we subsequently added a fixed concentration of NIP_{24}-BSA antigen for activation. Flow cytometric acquisition and analysis allowed us to retrieve the individual cell populations and monitor their activation status (Fig 5, *C*). The percentage of activation as determined by CD107a cell surface positivity of each identified cell population nicely correlated with the amount of sensitization (Fig 5, *C-D*). In a next step, we used 8 predefined sera (Table E3) from patients with allergy to timothy grass to sensitize individually labeled cell populations before pooling those in 4 tubes. To each of the 4 tubes, a certain concentration of timothy grass extract that has previously been established on nonbarcoded cells (ie, 0, 10, 50, 100 ng/mL) was added for activation. All cells were pooled for acquisition and subsequently deconvoluted to identify individual cell populations based on their fluorescence barcode (Fig 5, *E*). Each serum sample except for the no serum control

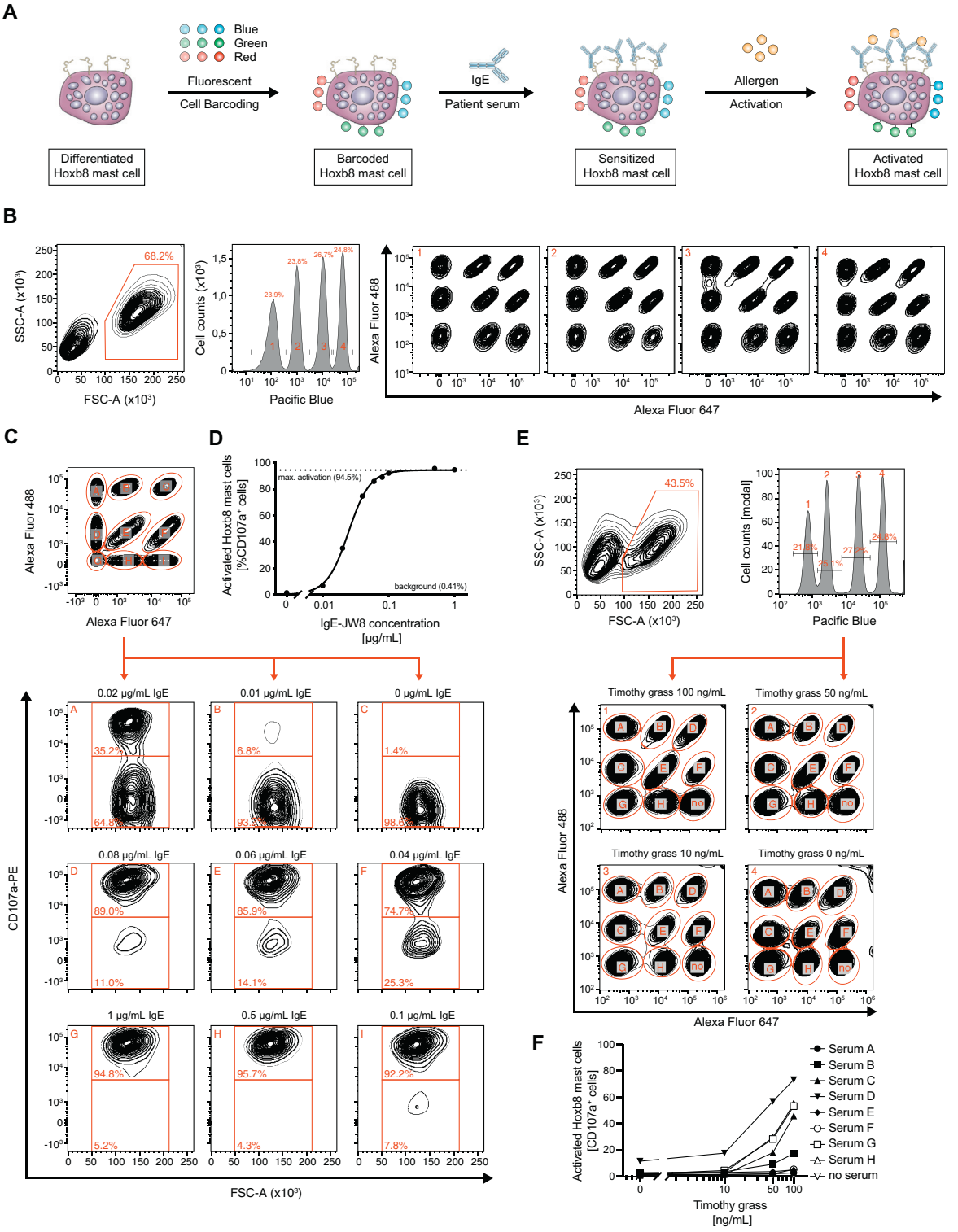

FIG 5. High-throughput screening using cellular barcoding. **A,** Schematics of basic workflow. **B,** Deconvolution gating strategy after acquisition of barcoded and pooled cells. Initial gating on side- and forward-scatter. Subsequent identification of 4 different cell populations (red 1-4). Further subdivision of populations into 9 individual subpopulations. **C,** Individually barcoded cell populations (red A-F) sensitized with a different concentration of human recombinant NIP-specific IgE were pooled and activated with NIP_{24}-BSA antigen. Deconvolution analysis to identify each individual cell subpopulation and to assess the activation status. **D,** Quantification of deconvoluted antigen-mediated activation of Hoxb8 MCs in an IgE dose-dependent manner for this high-throughput approach. A nonlinear regression curve to measured data points. **E,** Individually barcoded cell populations (red 1A-4H) sensitized with sera from 8 different patients with allergy to timothy grass were pooled in 4 separate tubes and activated with 4 allergen

shows allergen dose-dependent cell activation (Fig 5, *F*), demonstrating that the fluorescent cell barcoding-based high-throughput approach is suitable to screen multiple sera in an experimental run.

Next, we investigated whether an individual patient serum could be screened against multiple allergens in such a high-throughput approach. To do so, we incubated barcoded cells with processed sera from 2 polysensitized patients (Table E4) and separately stimulated them with various allergens. After activation, cells from an individual patient serum were pooled for acquisition and subsequently deconvoluted to identify individual cell populations on the basis of their fluorescence barcode. While we detected reactivity against timothy grass as well as cat and birch allergens in serum of patient 1 (Fig 6, *A* and *C*), the serum of patient 2 reacted against house dust mite and cat allergens (Fig 6, *B* and *D*). These data clearly indicated that the established high-throughput format is a suitable and rapid setup to functionally screen and identify polysensitized patients.

DISCUSSION

In this study, we describe the establishment and basic validation of a new functional allergy screening assay with remarkable diagnostic potential. The test is based on passive sensitization of human high-affinity IgE receptor transgenic and conditional Hoxb8-immortalized progenitor-derived mouse mast cells (Hoxb8 MCs) with patient serum. The progenitor cells have a high replicative rate and a near-unlimited renewal potential, allowing on-demand *in vitro* differentiation of large numbers of functional Hoxb8 MCs within a few days. Our study reveals that this test provides comprehensive information on the allergic status of the patient and overcomes many challenges and limitations associated with current diagnostic tools. The assay is useful to screen patient sera for reactivity against different allergens (eg, air-borne, food, and venoms) as well as to determine total IgE levels. Furthermore, it might offer the possibility to assess whether and when patients respond to AIT through longitudinal tracking of serum reactivity. Finally, the use of a cellular barcoding strategy allows simultaneous testing of multiple allergens or patient sera in a high-throughput manner, resulting in an unprecedented rapid and standardized diagnostic value as it is often required in large clinical trials.

The cells can be cryopreserved by standard protocols, and early passages of these cells were frozen in liquid nitrogen for storage, ensuring a steady stock of progenitors to generate a virtually unlimited number of differentiated Hoxb8 MCs. In contrast to other mast cell lines and primary bone marrow– or blood-derived mast cells,[42] these progenitor cells can be kept in culture for at least 4 months without losing self-renewal or differentiation potential. As shown here, with a doubling time of approximately 1 day, Hoxb8 MCs feature faster growth than previously described mast cell lines. We further report that differentiated Hoxb8 MCs express high levels of huFcεRIα and readily bind human recombinant IgE and IgE in sera of patients with allergy. IgE-sensitized Hoxb8 cells immediately degranulate on challenge with the cognate antigen, confirming functional activity of the chimeric huFcεRI, mFcεRIβ, mFcεRIγ tetramer on these cells. Overall, our data provide strong evidence that passive sensitization of Hoxb8 MCs with sera of patients with allergy is an ideal tool for both the identification of culprit allergens and longitudinal assessment of treatment response (eg, AIT or anti-IgE therapy) in terms of efficacy and duration.

The basophil activation test currently represents the only functional *in vitro* assay that is occasionally used for allergy diagnosis by experts.[10] However, broad clinical implication of this test has been hampered by the fact that it relies on fresh whole blood samples, which have to be immediately processed and analyzed by specialized laboratories. Previous studies have reported that basophil reactivity already declines after 4 hours of sample storage.[26,27] Others, however, have argued that the reactivity does not change within the first 24 hours after sampling.[12] Either way, a functional assay that is based on serum instead of whole blood such as the one presented in this study has the advantage that patient samples can be frozen and stored for later analysis without losing biological activity, even when it still requires specialized equipment and personnel to perform the test. This tremendously facilitates sample handling and allows for pro- as well as retrospective analysis of individual patient samples or entire sample cohorts (eg, from clinical trials). We have optimized the test for relatively small sample volumes of 25 μL serum so that a single standard blood draw of 2 to 3 mL into a serum preparation tube would allow more than 100 testing conditions.

Different to what has been described in most protocols for functional testing of patient sera in various basophil or mast cell systems,[29-32] we did not wash the cells before allergen challenge. We found that this modification of the protocol has several advantages. First of all, the nonwash setup closely mimics *in vivo* conditions in which basophils are constantly exposed to serum and at the same time it allows to assess the role of allergen-specific IgG[46,47] or other unknown modulatory factors in serum samples upon allergen challenge. Second, we have observed that the maximal activation of the cells is significantly higher when cells were not washed after overnight sensitization (Table I). We speculate that IgE:allergen complex formation occurs more efficiently in nonwash conditions, leading to enhanced cross-linking of FcεRI on the cell surface. However, this hypothesis warrants further investigation.

In comparison to previously published allergic effector cell lines or bone marrow– and blood-derived mast cells,[33] the Hoxb8 MCs feature many favorable characteristics. The fact that they derive from the same progenitor cells in a standard operating procedure makes the cells remarkably homogeneous and stable and the assay highly reproducible. In case of the monoclonal progenitor cell NT-1, the differentiated cells even derive all from this single clone. In addition, the cells feature an unprecedented signal-to-noise ratio upon allergen-mediated activation. While maximal activation of most previously

concentrations. After pooled acquisition, the deconvolution analysis was performed to identify each individual cell subpopulation and to assess the activation status. **F,** Quantification of deconvoluted dose-dependent allergen-mediated activation of Hoxb8 MCs for individual patient. *FSC-A,* Forward scatter-area; *SSC-A,* side scatter-area.

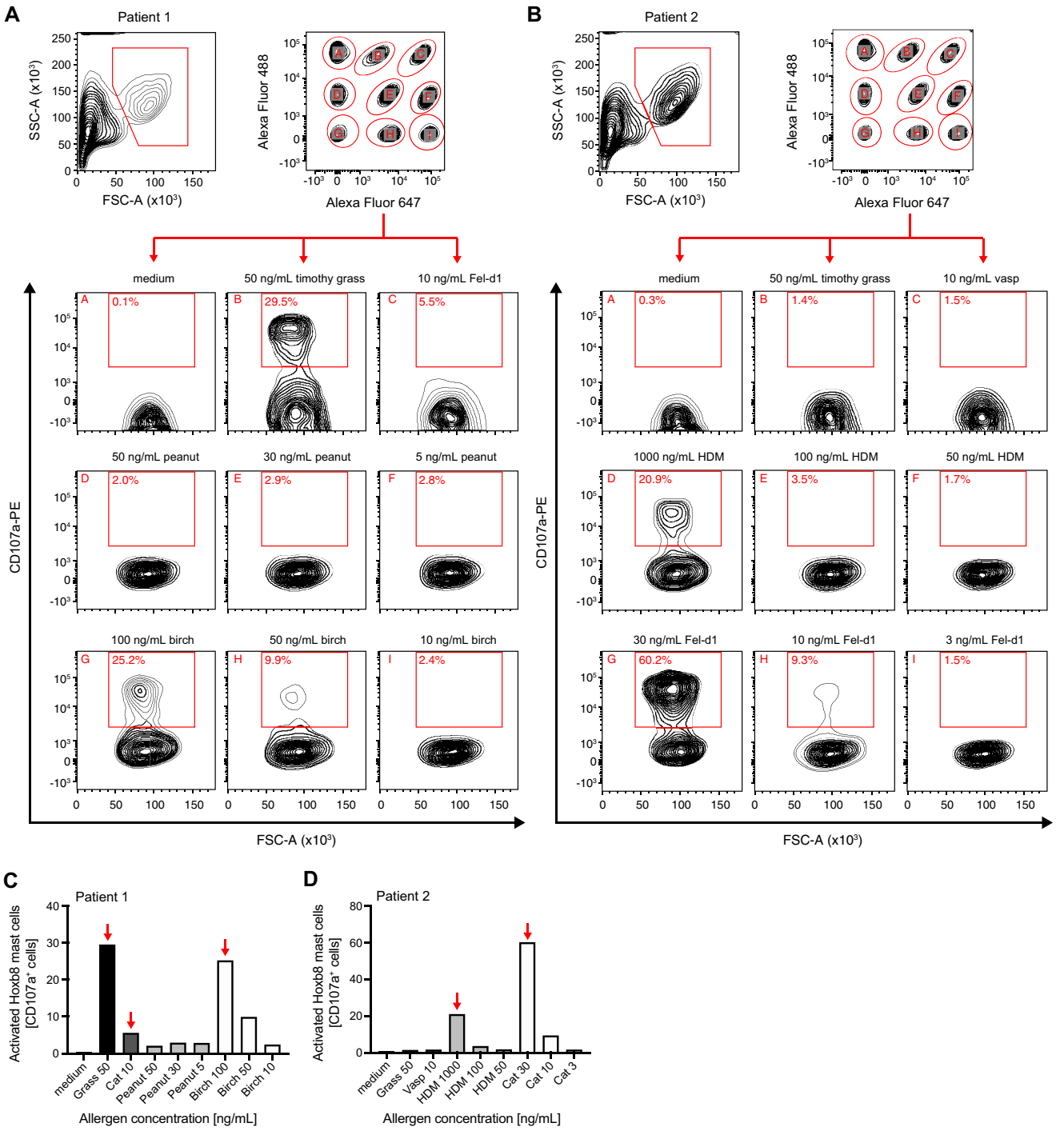

FIG 6. High-throughput screening of multiple allergens using cellular barcoding. Individually barcoded cell populations sensitized with 2 sera (**A** and **B**) from polysensitized patients. Cells were stimulated with different recombinant allergens or allergen extracts. After pooled acquisition, the deconvolution analysis was performed to identify each individual cell subpopulation and to assess the activation status by quantifying CD107a as a cell surface activation marker. **C** and **D**, Quantification of deconvoluted dose-dependent allergen-mediated activation of Hoxb8 MCs for individual allergens with serum from patient 1 (**C**) and patient 2 (**D**) is shown. Red arrows indicate the identified allergens that led to activation of the cells. *FSC-A*, Forward scatter-area; *SSC-A*, side scatter-area.

described allergic effector cell lines as well as of primary mast cell cultures lies between 40% and 60%,[31,48,49] Hoxb8 MCs can be activated to almost 100%, indicating the remarkable dynamic range and sensitivity of the system (Table I). Hoxb8 MCs even show higher maximal activation than LAD2 cells. Importantly, these activation parameters remain constant over multiple months

of progenitor cell culture, and prolonged use of the cells does not affect their phenotype or viability. Thus, in line with previous reports on conditional immortalization of myeloid progenitors by Hoxb8,[27] we conclude that Hoxb8 MCs and their immortalized progenitors feature a high genetic stability and a robust phenotype. Of note, the differentiated Hoxb8 MCs also feature an exceptional survival and maintenance of reactivity in the assay of at least 7 days in differentiation medium at room temperature in close tubes without O_2 or CO_2 supplementation (Fig E4). This finding clearly underscores the robustness of these cells.

A limitation of the here-described assay is that it is restricted to the detection of humoral components in patient sera contributing to the allergic reaction. This is the case for all assays that are based on passive sensitization including the previously described mast cell activation test.[13-15] As such, it will not reveal potential cell-intrinsic aspects including sensitivity and releasability of the patient's own cells (ie, basophils or mast cells), which might aggravate or mitigate the allergen-induced response. However, to this date, it remains rather elusive to what extent purely cell-intrinsic parameters might influence an allergic reaction. This-being said, the described assay will be helpful to discriminate humoral from cell-intrinsic contributions and to circumvent the diagnostic challenge of nonreleaser basophils.[50-52]

AIT is an important disease-modifying approach for the treatment of patients with allergy. Various mechanisms underlying AIT-mediated reestablishment of tolerance including the induction of protective allergen-specific IgG have been described.[16,18,53] Here, we demonstrated that the Hoxb8 MC assay might represent a suitable tool to functionally follow and quantify the treatment response of patients with allergy undergoing AIT. Not only does this test bear the potential to support clinicians in discriminating responders from nonresponders early on during treatment but it might also help to determine the time point of tolerance induction. The assay even allows to examine the induction of allergen-specific IgG during AIT, which has previously been reported as a potential predictor of treatment outcome,[54,55] and to assess its protective function. However, extensive follow-up studies with large patient cohorts are required to assess its full diagnostic utility. With such a functional test, it is likely that the need for risk associated *in vivo* allergen provocations to prove treatment success might become redundant. We further envision that the persistence of allergen tolerance after completing the treatment can be followed using the Hoxb8 MC assay.

The high-throughput approach based on fluorescent cell barcoding presented here additionally opens up the possibility to measure large sample cohorts in minimal time, which is often required in clinical research settings. Thus, the assay might be useful to assess the efficacy of drug candidates modifying serum IgE levels. Two other important areas in which this functional assay could be of interest are in quality control for the standardization of allergen preparations to be used in AIT (ie, assessment of batch-to-batch variations) and in nutritional science for allergenicity screening of novel food additives.[56,57] Taken together, our results strongly suggest that this novel functional Hoxb8 MC–based assay bears a significant diagnostic potential for many IgE-mediated hypersensitivity reactions. How it will be implemented and who might benefit from such an approach still has to be carefully evaluated and warrants further studies.

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Clinical implications: We developed a novel cell-based assay to accurately identify IgE-mediated allergies and quantify treatment responses. This test might significantly facilitate the clinical management of patients with allergy.

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METHODS

Reagents (ie, antibodies, recombinant proteins, and chemicals)

Human chimeric NIP-specific JW8-IgE was purchased from NBS-C BioScience (Vienna, Austria). Human chimeric NIP-specific JW183-IgG was purchased from BioRad Laboratories (Cressier, Switzerland). Isolated human Immunoglobulins IVIG Hizentra has been received from CSL Behring (King of Prussia, Pa). Recombinant murine IL-3 was purchased from Peprotech (London, UK). Allergens NIP_7-BSA and NIP_{24}-BSA were purchased from LGC Biosearch Technologies (Hoddesdon, UK). Various CAST allergens in the following were received from Bühlmann Laboratories AG (Schönenbuch, Switzerland): peanut extract (BAG-F13), cat recombinant Fel d 1 (BAG2-FELD1), wasp yellow jacket venom (BAG2-I3), honeybee venom (BAG2-I1), house dust mite extract (BAG-D1), common birch extract (BAG-T3), and timothy grass extract (BAG-G6). VivaSpin 2-mL ultrafiltration spin columns and 100-kDa MWCO PES membrane were purchased from Sartorius (Göttingen, Germany). IgG depletion columns NAb Protein G Spin Columns, 0.2 mL, were purchased from Thermo Fisher Scientific. Pacific Blue succinimidyl ester (NHS), Alexa Fluor 488 succinimidyl ester (NHS), and Alexa Fluor 647 succinimidyl ester (NHS) were all purchased from Thermo Fisher Scientific. Hoxb8 progenitor cells were cultured in RPMI-1640 medium AQmedia (Sigma-Aldrich, St Louis, Mo) complemented with 10% FCS Sera Pro (Pan Biotech, Aidenbach, Germany), 10% WEHI-3b supernatant (self-made), 100 U/mL penicillin, 100 μg/mL streptomycin (100× penicillin/streptomycin, Gibco by Sigma-Aldrich), and 100 nM 4-OHT (Sigma). Sensitization and activation of the cells was performed in activation medium (bone marrow–derived mast cell [BMMC] medium) composed of RPMI-1640 w/ stable glutamine, 2.0 g/L $NaHCO_3$ (Seraglob, Bioswisstec AG, Schaffhausen, Switzerland) complemented with 10% Hyclone FCS (Fisher Scientific, Hampton, NH), 100 U/mL penicillin, 100 μg/mL streptomycin (100× penicillin/streptomycin, Gibco), 10 mM HEPES buffer solution (stock-solution 1 mol, Gibco), 1 mM sodium pyruvate (stock-solution 100 mM, 100×, Gibco), 4 mM L-glutamine (stock-solution 200 mM, 100×, Gibco), 1× nonessential amino acids (stock-solution 100×, Gibco), 30 ng/mL mouse recombinant IL-3 (Peprotech), and 50 μM 2-mercaptoethanol (stock-solution 14.3 mol, Merck, Darmstadt, Germany). Tyrode's buffer used for β-hexosaminidase release assay was composed of 10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.9 mM $CaCl_2$, 2.1 mM $MgCl_2$, 5.6 mM L-glucose, and 0.1% BSA endotoxin-free dissolved in distilled H_2O. For flow cytometry, we used the following antibodies: anti-human IgE fluorescein isothiocyanate (FITC) (clone Ige21, Thermo Fisher Scientific), monoclonal mouse antihuman FcεRIα allophycocyanin (APC) (clone AER-37, Thermo Fisher Scientific), monoclonal rat anti-mouse CD200R FITC (clone OX-110), AbD Serotec (Bio-Rad), monoclonal rat ant-imouse CD117 c-KIT phycoerythrin (PE) (clone 2B8, Thermo Fisher Scientific), monoclonal rat anti-mouse CD107a (lysosome-associated membrane protein-1) APC and PE (clone 1D4B), IgG2aκ (BioLegend, San Diego, Calif), monoclonal mouse antihuman CD107a (lysosome-associated membrane protein-1) PE (clone H4A3), IgG1κ (BioLegend), monoclonal mouse anti-human CD117 c-KIT PE (clone A3C6E2), monoclonal rat anti-mouse CD63 (LAMP-3) APC (clone NVG-2), IgG2aκ (BioLegend), and monoclonal rat anti-mouse CD32 (Fcgr2) APC (clone S17012B), IgG2bκ (BioLegend), and the appropriate isotype controls: mouse IgG1κ isotype control FITC (clone P3.6.2.8.1, Thermo Fisher Scientific), mouse IgG2b isotype control APC (clone eBMG2b, Thermo Fisher Scientific), rat IgG2bκ isotype control PE (clone eB149/10H5; eBioscience, San Diego, Calif), mouse IgG1κ isotype control PE (MOPC-21, BioLegend), and rat IgG2bκ isotype control APC (clone RTK4530, BioLegend). Flow cytometry was performed using a BD FACSCanto device (BD Bioscience, Franklin Lakes, NJ) or a CytoFLEX S 4L 13C (B2-R3-V4-Y4) plus 96 DW plate loader (Beckman Coulter Life Sciences, Brea, Calif), and results were evaluated with FlowJo Version 10.1 (Ashland, Ore) unless stated otherwise.

Cell culturing of different allergic effector cells

Bone marrow–derived mast cells. Mice lacking the murine FcεRIα but transgenic for the human FcεRIα (sTG) and mice double-transgenic for human IgE and human FcεRIα (dTG) were euthanized by CO_2 asphyxiation. Femur, tibia, and humerus were removed, and the isolated bone marrow cells were grown in BMMC medium made up of RPMI-1640 w/ stable glutamine, 2.0 g/L $NaHCO_3$ (Seraglob) complemented with 10% Hyclone FCS (Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 10 mM HEPES buffer solution (stock-solution 1 mol, Life Technologies), 1 mM sodium pyruvate (Gibco), 4 mM L-glutamine (Gibco), 1× nonessential amino acids (Gibco), 30 ng/mL mouse recombinant IL-3 (Peprotech), and 50 μM 2-mercaptoethanol (Merck). For the first 2 weeks, the medium was changed every second day and cells were cultured at a concentration of 2×10^6 cells/mL in a T75 cell culture flask (Greiner Bio One, Kremsmünster, Austria) and kept in a humidified 37°C incubator with 5% CO_2. Afterwards, the medium was changed twice a week and the cells diluted to 1×10^6 cells/mL.

Human mast cell line 1. Human mast cell line 1 cells were cultured at 37°C and 5% CO_2 in filter-sterilized (0.22 μm, Sartorius) Iscove's modified Dulbecco's medium (1×) (Sigma) + GlutaMAX-I + 25 mM HEPES (Gibco) supplemented with 10% Hyclone FCS (Fisher Scientific) and 1.2 mM 1-thioglycerol (Sigma). The cells were passaged every 3 days to be diluted to 3.5×10^5 cells/mL and kept in a T25 cell culture flask (Greiner Bio One).

RBL-2H3α cells. RBL-2H3α cells were cultured at 37°C and 5% CO_2 in filter-sterilized (0.22 μm, Sartorius) RPMI-1640 medium w/ stable glutamine, 2.0 g/L $NaHCO_3$ (Seraglob) complemented with 10% Hyclone FCS (Fisher Scientific, NH) and 500 μM geneticin G-418 sulfate (Gibco). Cells were passaged every 3 days by removing the culture medium, 2 rinsing steps with 1× PBS, pH 7.4 (Insel Group), and detaching the cells for 5 minutes at 37°C and 5% CO_2 with trypsin-EDTA solution 0.25% (Sigma). Trypsin-EDTA was quenched by addition of culture medium, and the cells were diluted to 2×10^5 cells/mL and kept in a T75 cell culture flask (Greiner Bio One).

RBL SX-38 cells. RBL SX-38 cells were cultured at 37°C and 5% CO_2 in filter-sterilized (0.22 μm, Sartorius) Eagle's Minimal Essential Medium (Sigma-Aldrich, St Louis, Mo) complemented with 10% Hyclone FCS (Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 1× nonessential amino acids (Gibco), and 1.2 mg/mL geneticin G-418 sulfate (Gibco). Cells were passaged every 2 to 3 days by removing the culture medium, 3 rinsing steps with 1× PBS, pH 7.4 (Insel Group), and detaching the cells for 10 minutes at 37°C and 5% CO_2 with trypsin-EDTA solution 0.25% (Sigma-Aldrich). Trypsin-EDTA was quenched by addition of culture medium, and the cells were diluted to 2×10^5 cells/mL and kept in a T25 cell culture flask (Greiner Bio One) and kept in a humidified 37°C incubator with 5% CO_2.

Laboratory of Allergic Diseases 2 mast cell line. LAD2 medium was composed of StemPro-34 SFM medium (Gibco) complemented with StemPro-34 nutrient supplement (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 100 ng/mL recombinant hSCF (Peprotech). LAD2 cells stored in liquid nitrogen in pZerve (Sigma) were slowly equilibrated to room temperature (RT) before adding 0.2 mL culture medium w/ 200 ng/mL recombinant hSCF (Peprotech). The cells were transferred to a 6-well culture plate (Greiner Bio One) and oscillated on a plate shaker for 6 hours at RT before adding another 0.5 mL medium w/ 200 ng/mL recombinant hSCF (Peprotech). Twenty-four hours later, the cells could be diluted to the normal culture density of 2×10^5 cells/mL, transferred to a T25 cell culture flask (Greiner Bio One), and kept in a humidified 37°C incubator with 5% CO_2. In the following, the cells were passaged once a week by hemidepletion.

Human peripheral $CD34^+$ stem cell–derived mast cell. Peripheral $CD34^+$ stem cells were isolated from Buffy Coats by use of the EasySep Human Whole Blood CD34 Positive Selction Kit II (StemCell Technologies, Saint-Egrève, France) according to the manufacturer's instructions. The stem cells were cultured in serum-free medium containing human LDL (StemCell Technologies) modified after Schmetzer et al.[E1] Medium: StemSpan serum-free medium for expansion of hematopoietic cells (SFME) (StemCell Technologies), 50 μg/mL human LDL (StemCell Technologies), 50 μg/mL gentamycin (Gibco), 50 μM 2-mercaptoethanol (Merck), recombinant hSCF, and recombinant hIL-3 and recombinant hIL-6 (Peprotech). The medium was changed twice a week, and the cells were cultured at a minimal

concentration of 5×10^5 cells/mL in T25 and T75 cell culture flasks (Greiner Bio One) and kept in a humidified 37°C incubator with 5% CO_2.

Laboratory of University of Virginia mast cell line. Laboratory of University of Virginia mast cell line cells were cultured at 37°C and 5% CO_2 in filter-sterilized (0.22 μm, Sartorius) StemPro-34 SFM medium (Gibco) complemented with StemPro-34 nutrient supplement (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 100 μg/mL primocin (Invivo Gen, San Diego, Calif). The cells were passaged every 2 to 3 days to be diluted to 5×10^5 cells/mL and kept in a T25 cell culture flask (Greiner Bio One).

To assess the expression of hFcεRIα receptor on the above-mentioned cells, 5×10^4 cells were washed twice with 200 μL of PBS, pH 7.4, at 600*g* for 5 minutes at 4°C. Subsequently, the cells were stained with the antihuman FcεRIα antibody or its corresponding isotype control antibody for 15 minutes, light protected at RT. Flow cytometry was performed using a BD FACSCanto device (BD Bioscience), and results were evaluated with FlowJo Version 10.1.

To assess IgE-mediated activation of the above-mentioned cells, 5×10^4 cells per well were seeded in a 96-well round-bottom plate. Washing was performed once with 200 μL of PBS, pH 7.4, at 600*g* for 5 minutes at 4°C and the cells were resuspended in 25 μL corresponding medium containing increasing concentrations (0.005-10 μg/mL) of JW8-IgE and incubated overnight at 37°C, 5% CO_2. Subsequently, 25 μL of 2× antigen NIP_{24}-BSA diluted in medium and containing either the staining antibody anti-CD107a or anti-CD63 was added to the cells, yielding a total activation volume of 50 μL and an antigen concentration for challenge of 100 ng/mL. The cells were incubated for 25 minutes at 37°C, 5% CO_2. The cells were washed twice with PBS, pH 7.4, at 600*g* for 5 minutes at 4°C and measured by flow cytometry. The IgE-mediated activation of LAD2 cells was performed with a slightly adapted protocol. The cells were resuspended in 50 μL medium containing JW8-IgE and incubated overnight at 37°C, 5% CO_2. Subsequently, 25 μL of 3× antigen NIP_{24}-BSA diluted in medium and containing the staining antibody anti-CD107a was added to the cells, yielding a total activation volume of 75 μL for challenge.

Receptor quantification and IgE-binding capacity of differentiated mast cells

To quantitatively determine the surface density of the human FcεRIα on Hoxb8 MCs, the QIFIKIT (BIOCYTEX) (Code K0078, Dako, Denmark/Aligent, Santa Clara, Calif) was used according to the manufacturer's instructions. Therefore, 1×10^5 differentiated Hoxb8 MCs with average viability of 95% as determined by trypan blue staining were seeded in 96-well culture plates and incubated overnight with 5 μg/mL JW8-IgE or no IgE before they were incubated with 1 μg of the unconjugated monoclonal mouse antihuman FcεRIα antibody CRA-1 (Abnova, Taipeh, China). Subsequently, the cells were stained with $F(ab')_2$ fragment of FITC-conjugated goat antimouse immunoglobulins provided with the kit. Set-Up Beads and Calibration Beads from the kit were prepared as recommended by the manufacturer. The amount of rpc was deduced from the calibration curve established with the mean fluorescence intensity (MFI) values of calibration bead sample. For all other above-mentioned cell types, the same method was applied to determine the amount of hFcεRIα rpc. Only exception was the RBL SX-38 cells, which needed to be detached from the 96-well flat-bottom plate after overnight incubation with or without 5 μg/mL JW8-IgE using trypsin-EDTA solution 0.25% applied analogously to the normal culture handling described above.

To assess the IgE binding to the human FcεRI receptor by flow cytometry, differentiated Hoxb8 MCs were incubated overnight with increasing concentrations (0.1-500 μg/mL) of JW8-IgE. Subsequently, the cells were stained with the αhu-IgE.

To deduce IgE concentrations from human serum samples by flow cytometry, differentiated Hoxb8 MCs were incubated overnight with 25 different human serum samples used undiluted and in dilutions of 1:2, 1:5, and 1:10. Afterwards, cells were proceeded as described above. IgE concentrations in the serum samples were calculated from data interpolation into the standard curve established with IgE-JW8 using GraphPad, Prism 8 (GraphPad Holdings LLC, San Diego, Calif).

Activation of differentiated mast cells with recombinant proteins and patient sera

For the determination of the Hoxb8 MC activation by flow cytometry, differentiated Hoxb8 MCs with average viability of 95% as determined by trypan blue staining were seeded in 96-well culture plates and incubated overnight with increasing concentrations (0.01-5 μg/mL) of JW8-IgE for sensitization of cells. Subsequently, the cells were stimulated by direct addition of 100 ng/mL of the antigen NIP_{24}-BSA and the staining antibody anti-CD107a. Only now, the cells were washed to prepare for acquisition. Differentiated Hoxb8 MCs were treated as described above for overnight IgE-JW8 sensitization, washed, and then stimulated with 100 ng/mL of the antigen NIP_7-BSA diluted in Tyrode's buffer (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.9 mM $CaCl_2$, 2.1 mM $MgCl_2$, 5.6 mM L-glucose, 0.1% BSA endotoxin-free dissolved in distilled H_2O). The cells were incubated for 60 minutes at 37°C, 5% CO_2. Subsequently, the cells were centrifuged at 600*g* for 5 minutes at 4°C. Fifty microliter of the supernatants was transferred to a 96-well flat-bottom plate, whereas the remaining supernatants were removed from the cells. Then, the cells were lysed by addition of 20 μL 0.5% Triton-X (Sigma) made up in Tyrode's buffer and resuspended thoroughly. Again, 10 μL of the pellet lysate was transferred to the 96-well flat-bottom plate. Fifty microliter of the substrate pNAG solution (4 mM 4-nitrophenyl *N*-acetyl-B-D-glucosaminidase in substrate buffer containing 201 mM Na_2HPO_4 and 438 mM citric acid in ddH_2O, pH 4.5, all from Sigma) was added to the supernatants and pellet lysates and incubated for 60 minutes at 37°C (no CO_2). The enzyme-substrate reaction was stopped by addition of 150 μL 0.2 mol glycine (Sigma), pH 10.7, to each well, and the absorbance at 405-nm wavelength was measured on a standard ELISA plate reader SpectraMax M5 (Molecular Devices LCC, San José, Calif). The percentage of the net release of β-hexosaminidase from the Hoxb8 MCs on challenge was calculated by dividing the amount of released by the total amount of β-hexosaminidase (released plus leftover in the cells after challenge).

Serum samples from patients with defined allergies against peanut, cat, wasp, honeybee, house dust mite, birch, and timothy grass were prepared for Hoxb8 MC sensitization using VivaSpin 2 mL ultrafiltration spin columns (MWCO 100kDa). The columns were used according to the manufacturer's instructions to perform a buffer exchange of the human serum samples with BMMC medium. To determine the activation of Hoxb8 MCs passively sensitized with human patient serum by flow cytometry, 5×10^4 differentiated Hoxb8 MCs per condition were resuspended in processed serum samples and incubated overnight for IgE sensitization of the cells. Subsequently, the cells were washed before the addition of the cognate antigen and the staining antibody anti-CD107a. The allergens were titrated and the cells challenged with 1 to 50 ng/mL allergen for peanut, 0.1 to 100 ng/mL for cat, 1.1 to 100 ng/mL for wasp, 1 to 100 ng/mL for honeybee, common birch, and timothy grass, and 3.7 to 1000 ng/mL for house dust mite. The cells were incubated for activation, then washed and acquired.

To assess the effect of allergen-specific IgG possibly present in patients with allergy after AIT, an artificial serum has been assembled by mixing 240 ng/mL JW8-IgE with 10 mg/mL IVIG human immunoglobulins, either in presence or in absence of 50 μg/mL JW183-IgG. These artificially assembled serum samples were added to differentiated Hoxb8 MCs and incubated overnight. Subsequently, the cognate antigen NIP_7-BSA and the staining antibody anti-CD107a were added to the cells for stimulation. The antigen was added in increasing concentrations of 0.07 ng/mL to 333.33 μg/mL to assess dose-dependent activation. Following the stimulation, the cells were washed and cell activation was assessed by flow cytometry using a CytoFLEX S 4L 13C (B2-R3-V4-Y4) plus 96 DW plate loader (Beckman Coulter Life Sciences, Brea, Calif). Results were evaluated with FlowJo Version 10.1.

Serum or plasma samples from patients with SCIT at time points 0 (pretreatment) as well as at 3, 6, 9, 12, 24, and 36 months of therapy (posttreatment) were prepared for Hoxb8 MC sensitization with VivaSpin 2 mL ultrafiltration spin columns (MWCO 100kDa) as described above. To determine the effect of SCIT on the activation of Hoxb8 MCs passively sensitized with human patient serum, differentiated Hoxb8 MCs were incubated overnight with the corresponding processed serum samples. Subsequently, the cognate antigen timothy grass was added to the cells for

stimulation in increasing concentrations in the range of 5 to 100 ng/mL together with the staining antibody anti-CD107a. Only after stimulation, the cells were washed and acquired.

To investigate the effect of IgG in the serum of patients with SCIT, IgG was removed from serum samples of patients after 12 months of therapy using NAb Protein G Spin Columns (Thermo Fisher Scientific) according to the manufacturer's instructions. Two runs of IgG depletion were performed on 400 μL of serum before proceeding with the buffer exchange step using the VivaSpin 2 mL ultrafiltration spin columns as described above. Sensitization and activation of differentiated Hoxb8 MCs was performed as before.

Fluorescent cell barcoding of differentiated mast cells

To design a setup to allow a high-throughput format for the Hoxb8 MC activation, differentiated Hoxb8 MCs were labeled with 36 (4 x 3 x 3) unique fluorescent barcodes. Therefore, all possible concentration combinations of the fluorescent dyes Pacific Blue succinimidyl ester (40, 6, 0.5, 0 μg/mL), Alexa Fluor 488 succinimidyl ester (40, 2, 0 μg/mL), and Alexa Fluor 647 succinimidyl ester (8, 0.5, 0 μg/mL) were prepared in PBS, pH 7.4, and added to the cells. For covalent amine-coupling, the cells were incubated for 25 minutes at RT according to the manufacturer's instructions. Subsequently, the cells were washed before being pooled in a 5-mL FACS tube. Flow cytometry measurements were performed using a BD FACS LSR II SORP device (BD Bioscience). Single-cell populations were deconvolved using FlowJo, Version 10.1.

To assess activation of Hoxb8 MCs in the high-throughput format, 5×10^4 differentiated Hoxb8 MCs per well were washed before labeling with 9 (3 x 3) unique fluorescent barcodes was performed as described above using the fluorescent dyes Alexa Fluor 488 succinimidyl ester (40, 2, 0 μg/mL) and Alexa Fluor 647 succinimidyl ester (8, 0.5, 0 μg/mL). Following the covalent amine-coupling, the cells were washed before the overnight incubation with increasing concentrations (0.01-2 μg/mL) of JW8-IgE for sensitization. Then, without washing, the cognate antigen NIP_{24}-BSA at 100 ng/mL and the staining antibody anti-CD107a were added to the cells. After activation of the single-cell conditions, the cells were washed, pooled in a 5-mL FACS tube, and flow cytometry was performed using a BD FACS LSR II SORP device (BD Bioscience). The barcoded cell populations were deconvolved, and activation of each population was determined with FlowJo Version 10.1.

To demonstrate the high-throughput format for the activation of Hoxb8 MCs after passive sensitization with serum samples from human patients with allergy, differentiated Hoxb8 MCs were incubated overnight with VivaSpin 2 mL ultrafiltration spin columns (MWCO 100kDa)-processed serum samples from 8 defined patients with timothy grass allergy. Then, washing was performed before labeling the Hoxb8 MCs with 36 (4 x 3 x 3) unique fluorescent barcodes with the fluorescent dyes Pacific Blue succinimidyl ester (40, 6, 0.5, 0 μg/mL), Alexa Fluor 488 succinimidyl ester (40, 2, 0 μg/mL), and Alexa Fluor 647 succinimidyl ester (8, 0.5, 0 μg/mL). Subsequently, the cells were washed and the 9 conditions with the same Pacific Blue barcode were pooled into one 5-mL FACS tube. For activation, 1 concentration of the cognate antigen timothy grass (0, 10, 50, 100 ng/mL) and the staining antibody anti-CD107a were separately added to 1 of the 4 FACS tubes. After activation, washing was performed for the 4 FACS tubes and once more after pooling the cells of all 4 tubes into a single one. Flow cytometry was performed using a BD FACS LSR II SORP (upgrade) device (BD Bioscience) and results were evaluated with FlowJo Version 10.1. The barcoded cell populations were deconvolved, and activation of each population was determined with FlowJo, Version 10.1.

REFERENCE

E1. Schmetzer O, Valentin P, Smorodchenko A, Domenis R, Gri G, Siebenhaar F, et al. A novel method to generate and culture human mast cells: peripheral CD34+ stem cell-derived mast cells (PSCMCs). J Immunol Methods 2014; 413:62-8.

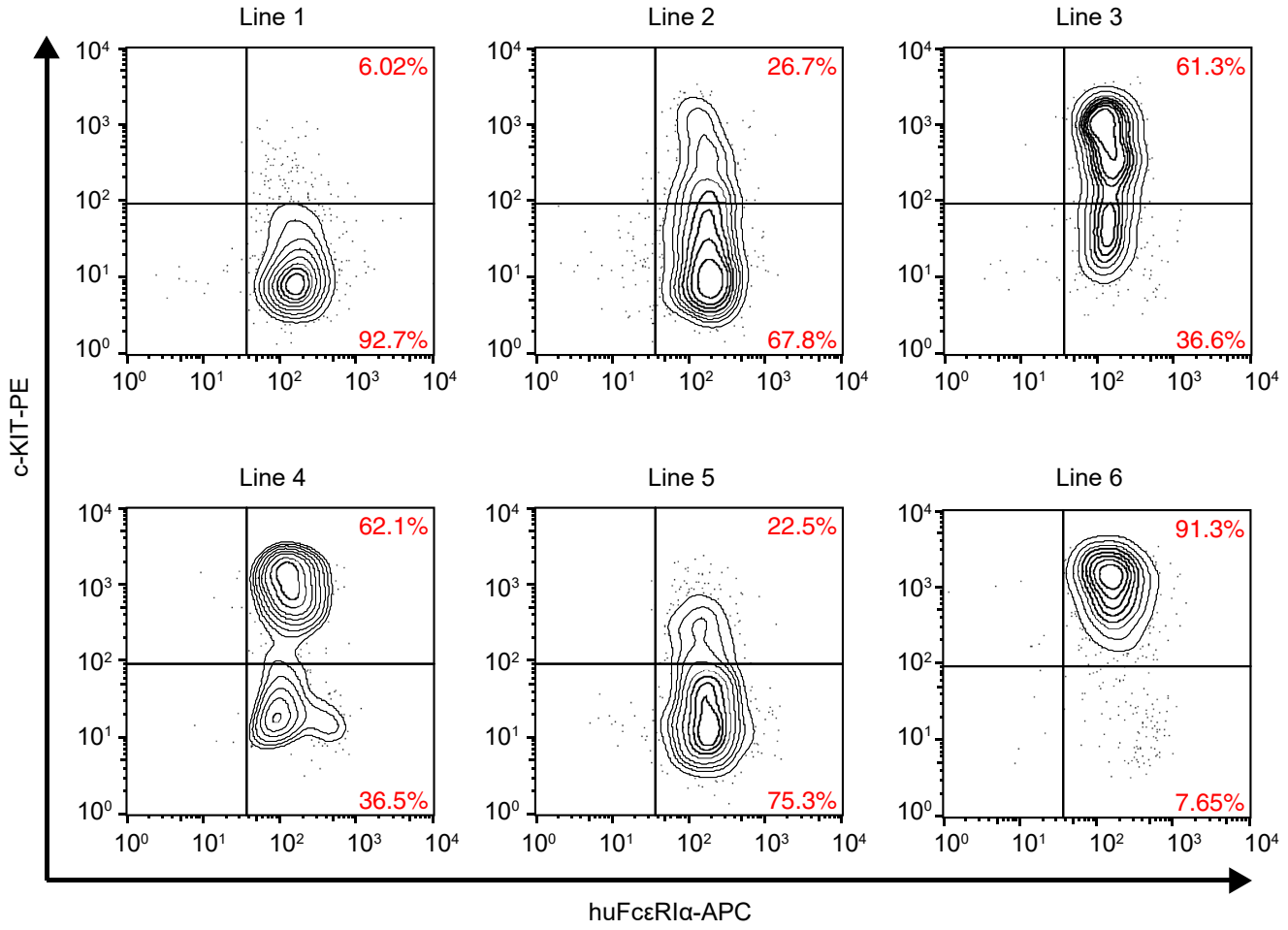

FIG E1. Different progenitor lines generated on ^{cond}Hoxb8 immortalization. Hematopoietic progenitors from the bone marrow of huFcεRIα mice were conditionally immortalized. Resulting lines from individual experiments were differentiated for 5 days and assessed by flow cytometry. Cells were pregated on side- and forward-scatter. Representative contour plots are depicted for the analysis of c-KIT/huFcεRIα expression.

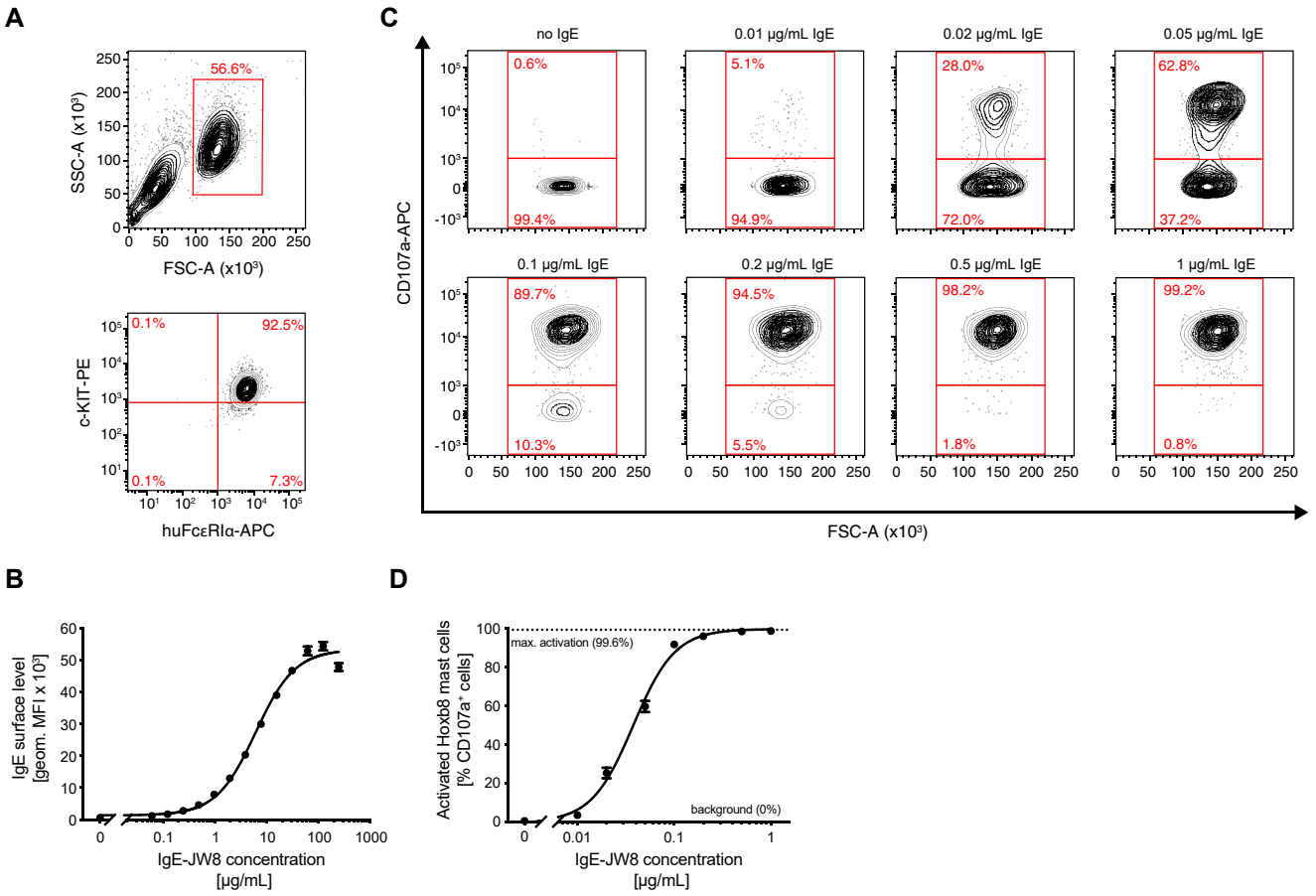

FIG E2. Characterization of monoclonal progenitor line NT-1. **A,** Flow cytometric assessment of the monoclonal NT-1 progenitor line 5 days after removal of 4-OHT. Representative contour plots are shown. Cells were gated on side- and forward-scatter (*top panel*) and subsequently analyzed for c-KIT/huFcεRIα expression (*bottom panel*). **B,** Dose-dependent binding of human recombinant IgE to differentiated NT-1 Hoxb8 MCs as assessed by flow cytometry. **C,** Representative contour plots for antigen-mediated activation of NT-1 Hoxb8 MCs in an IgE dose-dependent manner is depicted as measured by flow cytometry. **D,** Quantification of antigen-mediated activation of Hoxb8 MCs in an IgE dose-dependent manner is shown. In *B* and *D*, nonlinear regression curves were fitted to measured data points. Data in *B* and *D* are shown as mean ± SEM. *APC*, Allophycocyanin; *FSC-A*, forward scatter-area; *MC*, mast cell; *PE*, phycoerythrin; *MFI*, mean fluorescence intensity; *SSC-A*, side scatter-area.

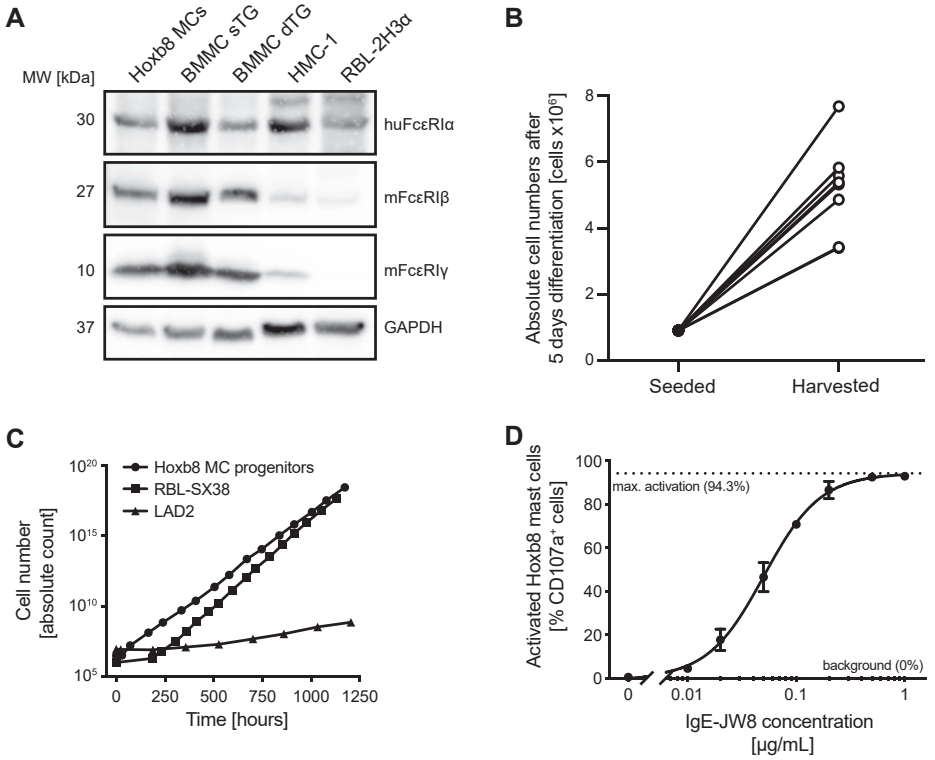

FIG E3. Properties of Hoxb8 progenitor line and differentiated Hoxb8 MCs. **A,** Representative western blot for the assessment of human FcεRIα as well as murine FcεRIβ and γ-chain expression in different allergic effector cells (Hoxb8 MCs, BMMCα-sTG, BMMCα-dTG, HMC-1, and RBL-2H3α). GAPDH is shown as loading control. Molecular weight of each band is indicated on the left. **B,** Absolute cell counts of seeded progenitor cells after 5 days of differentiation into harvested Hoxb8 MCs are shown. **C,** A comparison of cell growth for different allergic effector cells is shown over time. **D,** Quantification of antigen-mediated activation of Hoxb8 MCs in an IgE dose-dependent manner after 5 weeks of progenitor cell culture is depicted. A nonlinear regression curve was fitted to measured data points. Data in *D* are shown as mean ± SEM. *BMMCα-dTG*, Bone marrow–derived mast cells from mice double-transgenic for human IgE and the human high-affinity IgE receptor; *BMMCα-sTG*, bone marrow–derived mast cells from mice transgenic for the human high-affinity IgE receptor; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *HMC-1*, human mast cell line 1; *MC*, mast cell; *MW*, molecular weight.

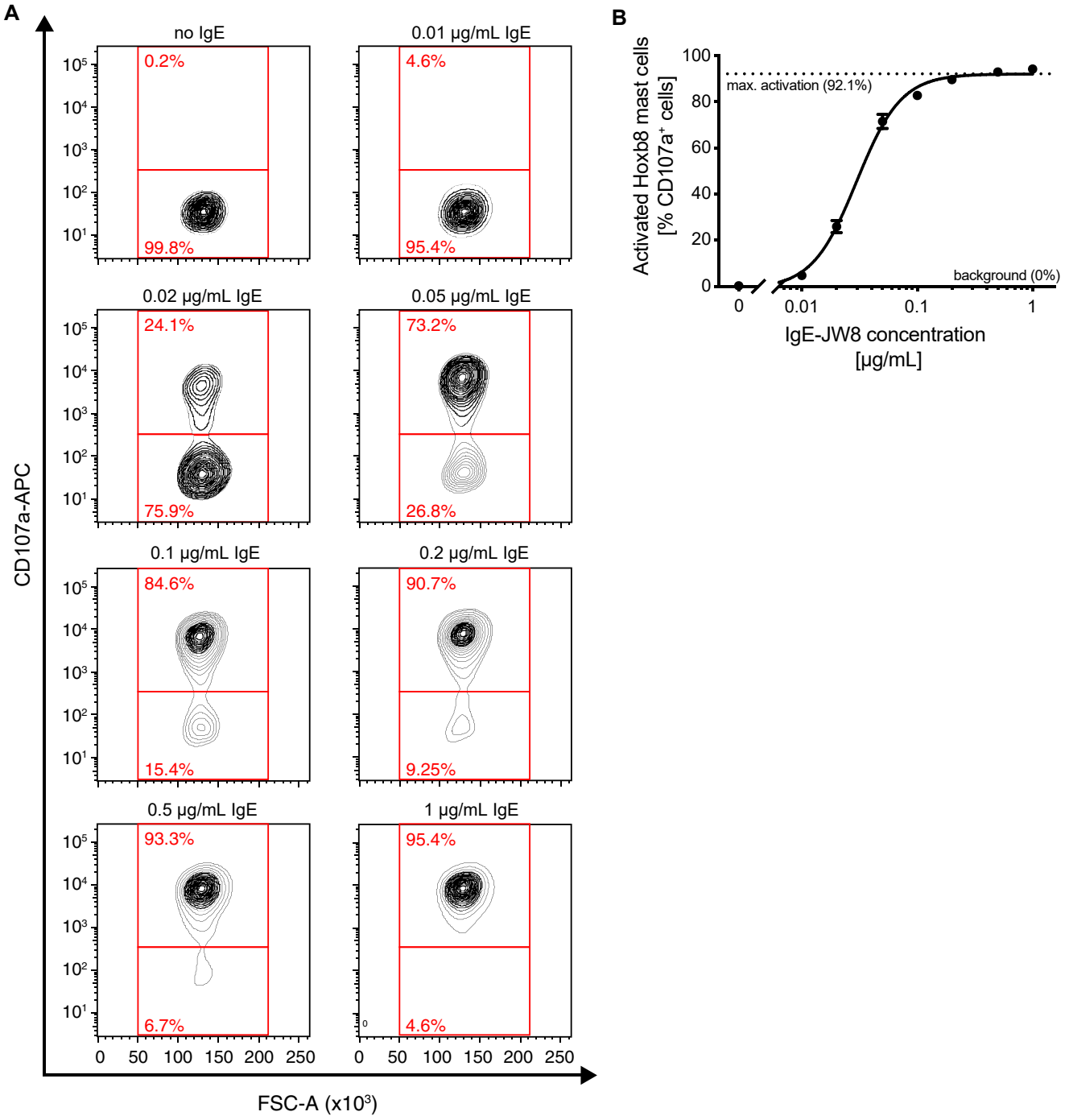

FIG E4. Activation of Hoxb8 MCs after 7 days of storage. **A,** Representative contour plots for antigen-mediated activation of Hoxb8 MCs in an IgE dose-dependent manner is depicted as measured by flow cytometry. **B,** Quantification of antigen-mediated activation of Hoxb8 MCs in an IgE dose-dependent manner is shown. Data of triplicates are shown as mean ± SEM. *FSC-A*, Forward scatter-area; *MC*, mast cell.

TABLE E1. Samples for allergy screening

Sample	Total IgE	Specific IgE	Ratio %	Allergen
1	91	11.5	12.637	rAra h2 (peanut)
2	259	37.8	14.595	rAra h2 (peanut)
3	565	61.7	10.920	rAra h2 (peanut)
4	851	100	11.751	rFel d1 (cat)
5	409	33.6	8.215	rFel d1 (cat)
6	385	58.7	15.247	rFel d1 (cat)
7	200	34.34	17.170	rVes v1 and rVes v5 (wasp)
8	151	51.92	34.384	rVes v1 and rVes v5 (wasp)
9	62	16.21	26.145	rVes v1 and rVes v5 (wasp)
10	83	11.3	13.614	rApi m1 (honey bee)
11	258	48.4	18.760	rApi m1 (honey bee)
12	434	59.9	13.802	rApi m1 (honey bee)
13	132	11.5	8.712	nDer p1 and nDer p2 (house dust mite)
14	708	99.1	13.997	nDer p1 and nDer p2 (house dust mite)
15	1339	155.3	11.598	nDer p1 and nDer p2 (house dust mite)
16	405	100	24.691	rBet v1 (birch)
17	118	26.3	22.288	rBet v1 (birch)
18	145	6.34	4.372	rBet v1 (birch)
19	146	16.8	11.507	rPhl p1 and rPhl p5b (timothy grass)
20	764	100	13.089	rPhl p1 and rPhl p5b (timothy grass)
21	494	100	20.243	rPhl p1 and rPhl p5b (timothy grass)
22	198	34.2	17.273	rPhl p1 and rPhl p5b (timothy grass)
23	883	100	11.325	rPhl p1 and rPhl p5b (timothy grass)
24	845	100	11.834	rPhl p1 and rPhl p5b (timothy grass)

TABLE E2. Samples for SCIT screening

Sample	Specific IgE (pretreatment)	Specific IgE (posttreatment)	Specific IgG_4 (pretreatment)	Specific IgG_4 (posttreatment)	Allergen
1	32.8	48.5	0.01	0.7	rPhl; all major and minor (timothy grass)
2	42.2	35.5	0.1	6.8	rPhl; all major and minor (timothy grass)
3	25.3	201.2	0.1	8.1	rPhl; all major and minor (timothy grass)
Ctrl	34.6	41.2	0.01	0.01	rPhl; all major and minor (timothy grass)

Ctrl, Control.

TABLE E3. High-throughput screening samples

Sample	Total IgE	Specific IgE	Ratio %	Allergen
A	146	16.8	11.507	rPhl p1 and rPhl p5b (timothy grass)
B	198	34.2	17.273	rPhl p1 and rPhl p5b (timothy grass)
C	385	72.1	18.727	rPhl p1 and rPhl p5b (timothy grass)
D	477	66.6	13.962	rPhl p1 and rPhl p5b (timothy grass)
E	764	100	13.089	rPhl p1 and rPhl p5b (timothy grass)
F	883	100	11.325	rPhl p1 and rPhl p5b (timothy grass)
G	494	100	20.243	rPhl p1 and rPhl p5b (timothy grass)
H	845	100	11.834	rPhl p1 and rPhl p5b (timothy grass)

TABLE E4. High-throughput polysensitization samples

Sample	Total IgE	Specific IgE	Allergen	Specific IgE	Allergen
Sample 1	1044	>100	rBet V1 (birch)	24.6	rAra h2 (peanut)
Sample 2	626	>100	rFel d1 (cat)	28.9	rDer p2 (house dust mite)