

Immunoglobulin M regulates airway hyperresponsiveness independent of T helper 2 allergic inflammation

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Abstract

Allergic asthma is a disease driven by T helper 2 (Th2) cells, eosinophilia, airway hyperresponsiveness (AHR) and IgE-secreting B cells. Asthma is largely controlled by corticosteroids and β_2 adrenergic receptor agonists that target and relax airway smooth muscle (ASM). Immunoglobulin M (IgM) isotype secreted by naïve B cells is important for class switching but may have other undefined functions.

We investigated the role of IgM in a house dust mite (HDM)-induced Th2 allergic asthma model. We sensitised wild-type (WT) and IgM-deficient (IgM^{-/-}) mice with HDM and measured AHR, and Th2 responses. We performed RNA sequencing on the whole lung of WT and IgM^{-/-} mice sensitised to saline or HDM. We validated our AHR data on human ASM by deleting genes using CRISPR and measuring contraction by single-cell force cytometry.

We found IgM to be essential in AHR but not Th2 airway inflammation or eosinophilia. RNA sequencing of lung tissue suggested that IgM regulated AHR through modulating brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1 (*Baiap2l1*) and other genes. Deletion of *BAIAP2L1* led to a differential reduction in human ASM contraction when stimulated with TNF- α and Acetylcholine, but not IL-13.

These findings have implications for future treatment of asthma beyond current therapies.

eLife assessment

Studying several allergens in different mouse strains, the authors assess the role of IgM in airway inflammatory responses and show that IgM deficient mice have reduced airway hyperresponsiveness. Although the findings, based on experimental evidence from a wide range of immunological and other assays, including the expression of a protein that regulates actin in smooth cells, are interesting and **useful**, the study is **incomplete** as the data and analyses do not support their primary claims.

Introduction

Immunoglobulin M (IgM) is the first antibody isotype expressed during B cell development and the first humoral antibody responder, conserved across all species from zebrafish to humans ¹. IgM can be divided into natural and antigen-induced IgM and can either be membrane-bound IgM-type B cell receptor (BCR) or secreted IgM ^{2,3}. Natural IgM plays multiple roles in homeostasis including scavenging and clearance of apoptotic cell debris in conjunction with phagocytic macrophages, B cell survival through tonic signals, lymphoid tissue architecture and prevention of autoimmune diseases ^{4–6}. At mucosal sites, both natural and antigen-induced IgM play a role in shaping healthy microbiota and IgM repertoire is also shaped by microbiota ^{7,8}. Secreted IgM antigen complexes can connect signals via unique and shared receptors, suggestive of a more pleiotropic role in homeostasis and disease states ^{9–11}.

Natural IgM and secreted IgM are essential in many infectious and non-infectious diseases including those induced by parasites, fungi, bacterial, viral and autoimmune diseases ¹¹. In *Plasmodium falciparum*, anti- α -gal IgM and anti-MSP1 directed antibodies are protective against primary and secondary infections ^{12,13}. Mice deficient of secreted IgM are susceptible to pulmonary *Cryptococcus neoformans* and *P. carinii* infection partly due to reduced activation of innate and adaptive responses ^{14,15}. At mucosal surfaces, IgM promotes healthy gut bacteria that is beneficial for homeostasis such as Firmicutes and Bacteroidetes ¹⁶. Natural and induced secreted IgM produced mainly by B1a cells is protective against *Streptococcus pneumoniae* and *Francisella tularensis* infection ^{17,18}. In these settings, the protective effects of sIgM depended on cytokines IL-1 γ and GM-CSF ^{17,18}. The involvement of natural or induced IgM in allergic asthma is unknown, despite selective IgM syndrome dominated by asthma patients ¹⁹.

Asthma is a T helper 2 (Th2) disease characterised by eosinophilic lung inflammation, mucus production, airway hyperresponsiveness (AHR), Th2 cytokines (interleukin-4 (IL-4), IL-5 and IL-13) and B cells producing IgE ²⁰. IgM is central to class switch recombination that results in IgE class switched B cells ²¹. We and others recently showed that the role of B cells in asthma is complex, where the load of the antigen is crucial in their function ^{22–24}. Mice deficient of B cells (μ MT^{-/-}) can mount exaggerated AHR when challenged with house dust mite (HDM) ^{24–26} or ovalbumin (OVA) ^{27–29} partly due to lack of regulatory B cells that dampen AHR ²³. Furthermore, interleukin 4 receptor alpha signalling in B cells is required for optimal Th2 allergic airway inflammation through regulation of AHR, germinal centre (GC) formation and B effector 2 function ²².

Interestingly, B cell isotypes show unique functions in allergic asthma, for example IgE and its high affinity receptor, Fc ϵ R are redundant in an HDM model ³⁰, whereas IgD plays an amplifying and regulatory role in various allergic models ³¹. In this context IgD activate basophils to secrete IL-4 and Th2 induction during the sensitisation stage through binding

basophils via galectin-9 and CD44. Once Th2 responses have been amplified, IgD ligation blocked IgE-mediated basophil degranulation through competing for antigen and inhibiting FcεR mediated signalling³¹. How IgM isotype contributes in the development of allergic asthma is unclear. It is also unclear whether secreted IgM plays different roles compared to membrane bound IgM, which is more likely to undergo class switching to IgE. We challenged mice lacking both membrane and secreted IgM with HDM and other allergens and found a profound reduction in AHR. RNA sequencing of lung tissue showed a downregulation of brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1 (*Baiap2l1*) and erythroid differentiation regulatory factor 1 (*Erd1*) genes associated with actin cytoskeleton and rearrangement smooth muscle contraction. As a proof of principle, we showed in human smooth muscle cell line that deletion of these genes via CRISPR-resulted in reduction in smooth muscle contraction at a single cell level. These are unexpected functions of secreted, and membrane bound IgM; namely, its involvement in modulating airway smooth muscle contraction.

Methods

Mice

IgM-deficient homozygotes mice on Balb/C background and on C57BL/6 background were backcrossed at least 10 generations³². Wild type on Balb/C and C57BL/6 backgrounds were used as a littermate control. Mice were housed in independently ventilated cages under specific pathogen-free conditions at the University of Cape Town Animal Facility. All mice were used at eight to 10 weeks of age and animal procedures were performed according to the strict recommendation by the South African Veterinary Council and were approved by the University of Cape Town Animal Ethics Committee (Reference number 014/019, 018/013 and 022/014).

House Dust-mite induced allergic airway disease

A high dose and a low dose HDM treatment schedule were used to induce symptoms of allergic asthma in mice²². Mice were anaesthetised with ketamine (Anaket-V; Centaur Labs, Johannesburg, South Africa) and xylazine (Rompun; Bayer, Isando, South Africa). For low-dose HDM, mice were sensitised intratracheally (i.t.) on day 0 with 1 µg of HDM (Stellergens Greer Laboratories, Lenoir, U.S.A.) and intranasally challenged with 3 µg HDM on days 8, 9, 10, 11 and 12. For high-dose HDM, mice were with HDM 100 µg and challenged with HDM 10 µg. AHR was measured on day 15. After the procedure, mice were euthanised and tissue samples were collected for analysis.

Adoptive transfer of naïve B cells

Spleens were collected from naïve congenic CD45.1 Balb/C mice and passed through 40µm strainer to obtain single-cell suspensions. Cells were stained with FITC-B220 and APC-CD19 for 30min at 4°C. A dead cell exclusion dye (7AAD) was added before sorting on BD FACS Aria I to at least 96% purity. 2.5×10^6 cells were adoptively transferred intravenously (i.v.) into IgM^{-/-} recipient mice a day before HDM sensitisation.

Adoptive transfer of naïve serum

Naïve wild-type mice were euthanised and blood was collected via cardiac puncture before being spun down (5500rpm, 10min, RT) to collect serum. Serum (200µL) was injected intraperitoneally into IgM-deficient mice, 1 day before sensitisation with HDM. In some experiments, serum was injected intraperitoneally at day -1, 0, and a day before challenges.

Ovalbumin-induced allergic airway inflammation

Mice were sensitised intraperitoneally with (50µg in 200µl) of ovalbumin (OVA) adsorbed to 0.65% alum (Sigma-Aldrich, Aston Manor, South Africa) on days 0, 7, 14. On days 23, 24, 25, mice were intranasally challenged with 100µg of OVA under anaesthesia with ketamine (Anaket-V; Centaur Labs, Johannesburg, South Africa) and xylazine (Rompun; Bayer, Isando, South Africa). AHR was measured on day 26. After the procedure, mice were euthanized with halothane and tissue samples collected for analysis.

Papain-induced lung inflammation

Mice were anaesthetized with isoflourane (3L/min) briefly before being challenged with 50µL of 25 µg of Papain (Sigma-Aldrich, Aston Manor, South Africa) on days 1, 2 and 3 or PBS. AHR was measured on day 4. After the procedure, mice were euthanized with halothane and tissue samples collected for analysis.

Airway Hyperresponsiveness

Airway resistance and elastance of the whole respiratory system (airways, lung chest wall) after intranasal challenge was determined by forced oscillation measurements as described previously [33](#) with the Flexivent system (SCIREQ, Montreal, Canada) by using the single compartment (“snapshot”) perturbation. Measurements were carried out with increasing doses of acetyl- γ -methylcholine (methacholine, Sigma-Aldrich, Aston Manor, South Africa) (0, 5, 10, 20 and 40 mg/mL) for Balb/C or (0, 20, 40, 80 160 and 320 mg/mL) for C57BL/6. Differences in the dose-response curves were analysed by repeated-measures Two-way ANOVA with the Bonferroni post-test. Only mice with acceptable measurements for all doses (coefficient of determination >0.90) were included in the analysis.

Flow cytometry

Single-cell suspensions were prepared from lymph nodes in Roswell Park Memorial Institute (RPMI) media (Gibco, Paisley, United Kingdom) by passing them through 100µm strainer. To obtain single cell suspensions from lung tissues, a left lobe was digested for 1 hour at 37°C in RPMI containing 13 mg/mL DNase I (Roche, Randburg, South Africa) and 50 U/mL collagenase IV (Gibco, Waltham, Massachusetts) and passed through 70µm strainer. Antibodies used in these experiments included, phycoerythrobilin (PE)-conjugated anti-Siglec-F (clone, E50-2440), anti-IL-5 (clone, TRFK5), anti-CD44 (clone, KM114), anti-T and B cell activation antigen (clone, GL7), anti-CD43 (clone, S7), FITC-conjugated anti-Ly6G (clone, 1A8), anti-IgD (clone, 11-26C2a), IL-4 (clone, 11B11), anti-PD-1 (clone, 29F.1A12), PerCP Cy5.5-conjugated anti-Ly6C (clone, AL-21), -CD45.1 (clone, A20), anti-IL-17 (clone, TC11-18H10), anti-mouse alpha muscle actin (Abcam, ab8211-500), Allophycocyanin (APC)-conjugated anti-CD11c (clone, HL3), anti-CD5 (clone, 53-7.3), BV421 conjugated anti-CD11b (clone, M1/70), anti-CD62L (clone, MEL-14), anti-IgG1 (clone, A110-1), AlexaFluor 700-conjugated anti-CD3 ϵ (clone, 145-2C11) -anti-IFN- γ (clone, XMG1.2), BV510-anti-CD4 (clone, RM4-5) and anti-B220 (clone, RA3-6B2), APC-Cy7-conjugated anti-CD19 (clone, 1D3) and anti-CD8 (clone, 53-6.7), BV786 conjugated anti-IgE (clone, R35-72) and anti-IL-33R (ST2) (clone, U29-93), biotin-conjugated anti-IgM (clone, AF-78), anti-CD95 (clone, Jo2), anti-CD249 (clone, BP-1), anti-CD45 (clone, 30-F11) were purchased from BD Pharmingen (San Diego, CA). PE-Cyanine7 anti-F4/80 (clone, BM8), anti-IL-13 (clone, eBio13A), anti-CXCR5 (clone, L138D7), AlexaFluoro 700-conjugated anti-MHC II (clone, M5/114), Rabbit anti-BAIAP2L1 (Abcam, PA554000), Live/dead Fixable Yellow stain (Qdot605 dead cell exclusion dye) were purchased from eBiosciences. Biotin-labelled antibodies were detected by Texas Red conjugated PE (BD Biosciences). PE-Goat anti-Rabbit IgG (Abcam, ab72465) was used to detect Rabbit anti-BAIAP2L1. For staining, cells (1×10^6) were stained and washed in PBS, 3% FCS FACS buffer. For intracellular cytokine staining, cells were restimulated with phorbol myristate acetate (Sigma-Aldrich) (50 ng/mL), ionomycin (Sigma-Aldrich) (250ng/mL), and monensin (Sigma-Aldrich) (200mM in IMDM/10% FCS) for 5h at 37°C then fixed in 2% PFA, permeabilised with Foxp3 transcriptional factor staining buffer kit

(eBioscience) before intracellular staining with appropriate cytokine antibodies and acquisition through LSR Fortessa machine (BD Immunocytometry system, San Jose, CA, USA) and data was analysed using Flowjo software (Treestar, Ashland, OR, USA).

Histology

Left upper lung lobes was fixed in 4% formaldehyde/PBS and embedded in paraffin. Tissue sections were stained with periodic acid-Schiff for mucus secretion, and haematoxylin and eosin (H&E) stain for inflammation. Slides were scanned at 20x magnification on the virtual slide VS120 microscope (Olympus, Japan). Downstream processing of images was done through Image J (Fiji) for image extraction at series 15 and Ilastik software was used for mucus area quantification on whole lung sections. The data shown are representative of 1 experiment of 3 independent experiments (n = 5-7 mice per experiment).

Antibody and cytokine ELISAs

Antibody ELISAs were carried out as previously described³³ using 10 µg/ml HDM to coat for specific IgGs. Total IgE in serum was measured using anti-mouse IgE (BD Biosciences, 553413) to coat, mouse IgE (κ, anti-TNP, BD Biosciences, 557079) as standard and biotin anti-mouse IgE (BD Biosciences, 553419) as a secondary antibody.

For *in vitro* cytokine production analysis, single-cell suspensions were prepared from mediastinal lymph nodes of HDM-treated and littermate control mice. Cells (2×10^5 cells, in 200 µL) were incubated for 5 days in RPMI/10% FCS (Delta Bioproducts, Kempton Park, South Africa) in 96-well plates. Cells were either stimulated with HDM (30 µg/mL) or plate bound anti-CD3 (10 µg/mL) and supernatants were collected after a 5-day incubation period. Concentrations of IL-4, IL-5 (BD Biosciences) and IL-13 (R&D Systems, Minneapolis, Minn), were measured using ELISA assays according to the manufacturer's protocol.

RNA Extraction

Small lung lobe was frozen in Qiazol (Qiagen, Germany) and stored at -80°C. Total RNA was isolated from the lysate using miRNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA quantity and purity were measured using the ND-1000 NanoDrop spectrophotometer (ThermoScientific, DE, USA).

cDNA Synthesis and RT-qPCR

For *Muc5a* gene expression analysis, 100 ng total RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR) was performed using LightCycler® 480 SYBR Green I Master (Roche, Germany) and *Muc5a* primers (IDT, CA, USA). Fold change in gene expression was calculated by the $\Delta\Delta C_t$ method and normalized to β -actin which was used as an internal control.

Whole lung RNA sequencing

Whole lung RNA was extracted using RNAeasy kit (Qiagen, Germany) according to the manufacturer's instructions. We used Agilent 2100 Bio analyzer (Agilent RNA 6000 Nano Kit) to do the total RNA sample QC: RNA concentration, RIN value, 28S/18S and the fragment length distribution. The first step in the workflow involved purifying the poly-A containing mRNA molecules using poly-T oligo attached magnetic beads. Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments had addition of a single 'A' base and subsequent ligation of the adapter. The

products were then purified and enriched with PCR amplification. PCR yields were quantified by Qubit and pooled samples together to make a single strand DNA circle (ssDNA circle), which gave the final library. DNA nanoballs (DNBs) were generated with the ssDNA circle by rolling circle replication (RCR) to enlarge the fluorescent signals at the sequencing process. The DNBs were loaded into the patterned nanoarrays and pair-end reads of 100 bp were read through on the DNBseq platform for the following data analysis study. For this step, the DNBseq platform combines the DNA nanoball-based nanoarrays and stepwise sequencing using Combinational Probe-Anchor Synthesis Sequencing Method.

Bioinformatics workflow

The ribosomal RNA (rRNA) was first removed using SortMeRNA. We then did the Fastq file quality control using Fastqc and multiqc software to assess the quality of the raw reads followed by adapter trimming using Trim galore. The reads were then aligned to the mouse reference genome (mm10_UCSC_20180903) using STAR aligner. The map read counts was then extracted using featurecounts. The gene differential analysis was conducted using the DESeq2. The genes with LFC ≥ 2 and adjusted p-value ≤ 0.05 was used to do the gene ontology over-representation analysis done using clusterProfiler³⁴ Bioconductor package. The Benjamini-Hochberg method was used for multiple test correction.

Human bronchial Airway Smooth Muscle cell culture

A healthy human bronchial smooth muscle cell line (BSMC, Lonza) was cultured in Smooth Muscle Growth Medium (Lonza) supplemented with Insulin (CC-4021D), human Fibroblastic Growth Factor-B (CC-4068D), Gentamicin sulfate-Amphotericin-1000 (CC-4081D), 5% Foetal Bovine Serum (CC-4102D), human Epidermal Growth Factor (CC-4230D) (all purchased from Lonza) in a T25 flask (Lonza) until confluence. After two passages in the T75 flask, confluent cells were seeded at 1.6×10^5 BSMC cells onto 24-well trays and immediately transfected with CRISPR/Cas9 single guide RNAs.

CRISPR/Cas9 single guide RNA transfections

single guide RNAs targeting human *BAIAP2L1* (# CD.Cas9.CXVQ6494.AA); *ERDR1*, (# CD.Cas9.YFVV2490.AA); HPRT Negative Control (#Alt-R® CRISPR-Cas9crRNA) were purchased (IDT, CA, USA via WhiteScientific PTY LTD). Single guide RNA (1 μ M) and Cas9 enzyme (1 μ M) in Opti-MEM medium (Life Technologies™, Carlsbad, CA, USA) were transfected using Lipofectamine RNAi Max 1000 (Thermo Scientific) into BSMC cells (1.6×10^5) per well and either stimulated with Acetylcholine (10 μ M), recombinant human IL-13 (100 ng/mL), recombinant human TNF- α (10 ng/mL) or left unstimulated for 48 hours at 37°C, 5% CO₂ incubator. Gene deletion was confirmed by DNA extraction (Wizard Genomic DNA Purif. Kit, Promega) and PCR amplification of target genes using *BAIAP2L1* forward (GTCCCGGGGGCCCGA) and reverse (AAGCGCCCAAGAATGTGGGG) primers, and product run on a 1.2% agarose gel.

Cytotoxic detection Assay

To measure cytotoxicity after single guide RNA transfection, lactate dehydrogenase (LDH) was measured in supernatants collected at 48 hours post transfection using cytotoxicity detection kit PLUS Assay (CYTODET-RO, Roche) according to manufacturer's instructions and plates were read at 490 nm.

Single-cell force cytometry using Fluorescently Labelled Elastomeric Contractible Surface (FLECS) technology

BSMCs were seeded on Fibronectin-coated elastomeric micropatterns (Forcyte Biotechnologies #F2AX0G03Y, 50 microns, Alexa Fluor 488 - bound Fibrinogen, 24-well plate) at a concentration of 75,000 cells per well in Smooth muscle growth medium (SmGM) (Lonza) and left to adhere and

spread for 90 minutes. Unattached cells were then removed and fresh SmGM medium supplemented with rhTNF- α (10 ng/mL)/rhIL-13 (100 ng/mL)/ACh (10 μ M) was added. BSMCs were stimulated for 3 hrs before being fixed in pre-warmed to 37°C 4% PFA. Fixed samples were washed and then stained with ATTO-565 phalloidin (ATTO-TEC) and DAPI (Life Technologies) and imaged on a StellarVision microscope using Synthetic Aperture Optics technology (Optical Biosystems). All images were analyzed using Fiji software by measuring the length of each micropattern per condition after stimulation and subtracting the length of the unstimulated micropattern of the same condition.

Immunofluorescent

Left upper lung lobes were fixed in 4% formaldehyde/PBS and embedded in paraffin. Tissue sections (5 μ m) were deparaffinised and hydrated in water before antigen retrieval in boiling 10 mM Citrate buffer (pH 6, pressure cooker for 2 mins). Tissue was incubated with Rabbit anti-BAIAP2L1 (Abcam, PA554000) in PBS-Tween (1:250, overnight, 4°C) and FITC anti-mouse alpha muscle actin (Abcam, ab8211-500, 1:100, overnight, 4°C). Tissues were washed with PBS-Tween before incubation with secondary antibody PE-Goat anti-Rabbit IgG (Abcam, ab72465) in PBS-Tween (1:500, RT in the dark for 30min). Tissues were washed with PBS-Tween before mounting in DAPI mounting media (Thermofisher). Fluorescent images were captured at 60x magnification using LSM880 Airy Scan (Zeiss, UK).

Western blotting

Protein harvest: RIPA lysis buffer supplemented with protease inhibitors was added to the lung tissue. The whole-cell lysates were incubated on ice for 20 min and vortexed every 5 min for 60 seconds. Then, the lysate was centrifuged at 3000 rpm for 15 minutes at 4°C, and the supernatant was collected. Total protein concentrations were determined using the Pierce BCA Assay kit.

SDS-PAGE: 20 μ g of each protein sample was mixed with Laemmli buffer and heated for 10 minutes at 100 °C. Equal amounts of protein samples were loaded into 5% acrylamide stacking gel and a 12% acrylamide separating gel along with 5 μ L PageRuler™ Plus Prestained protein ladder (catalogue #: 26619, Thermo Scientific™) and separated by electrophoresis at 100 V. Proteins were transferred onto nitrocellulose membranes (Bio-Rad) using the Bio-Rad Mini Trans-Blot® Cell according to the manufacturer's instructions.

After protein transfer, the nitrocellulose membranes were stained with Ponceau stain for approximately 1-3 minutes to visualize if the proteins had been transferred. Once the protein bands were identified, the membrane was washed with ddH₂O until 80% of the Ponceau staining is removed. The remaining 20% of the stain allowed the accurate segmentation of the membrane to separate high molecular weight proteins from low molecular weight proteins. The nitrocellulose membranes were blocked with 5 % (w/v) non-fat milk, 0.01% Tween-20 in TBST) on the shaker at room temperature for one hour. The low and high molecular weight membranes proteins were incubated with respective primary antibodies (Rabbit anti-human BAIAP2L1, PA5-54000, Invitrogen) or (Goat anti-mouse GAPDH, sc 365062, Santa Cruz) overnight shaking in the 4°C cold room. Membranes were washed 4 times with 1xTBST in 15-minute intervals. Horseradish peroxidase (HRP) secondary antibodies were added to their respective membranes and incubated for one hour at room temperature, with shaking. subsequently, membranes were washed 3 times in 1xTBST in 15 minutes intervals. To visualise the protein bands on autoradiographic film (Santa Cruz Biotechnology®), Lumino Glo® substrate kit was added to each membrane. Briefly, the substrate luminol was oxidised by hydrogen peroxide in the presence of the catalyst HRP to yield a chemiluminescent product.

Plating of faecal pellets to confirm a reduction in bacterial colonies following antibiotic treatment

The faecal pellet was collected from each individual mouse treated with antibiotics as well as control groups not treated with antibiotics. The faecal pellet was weighed and resuspended in sterile PBS at a concentration of 50 mg/mL. The sample was resuspended until homogenous, and 1/100 dilution was made in sterile PBS. 50µL of 1/100 dilutions was plated on tryptic soy agar plates and spread evenly across the plate before incubating at 37°C for 16 hrs. Individual colonies were counted manually.

Statistical analysis

P-values were calculated in GraphPad Prism 6 (GraphPad Software, Inc) by using nonparametric Mann-Whitney Student's t-test or Two-way ANOVA with Bonferroni's post-test for multiple comparisons, and results are presented as standard error of the mean (SEM) or mean of standard deviation (SD). Differences were considered significant if P was <0.05.

Results

IgM-deficient mice show profound airway hyperresponsiveness reduction when exposed to HDM

We sensitised and challenged IgM-deficient (IgM^{-/-}) and wild-type Balb/c (WT) mice with HDM intratracheally (i.t.) and analysed AHR, lung infiltrates and cytokines (**Fig. 1a** [↗](#), Supplementary Fig. 1a). We found moderately reduced resistance and elastance in IgM^{-/-} sensitised with a high dose of HDM (100µg) compared to WT mice (Supplementary Fig. 1b). Similarly, we also observed a profound reduction in AHR in IgM^{-/-} mice sensitised with a low dose of HDM (1µg) compared to WT (**Fig. 1b** [↗](#)). Interestingly, lung eosinophils were intact at both low dose (**Fig. 1c** [↗](#)) and high dose HDM (Supplementary Fig. 1d). We could also show that AHR reduction in IgM^{-/-} mice was not allergen-specific, as we observed similar findings using ovalbumin (OVA) complexed to alum adjuvant (Supplementary Fig. 2a-b) and acute papain-induced allergic inflammation (Supplementary Fig. 2c-d). We observed similar finding of profound AHR reduction in IgM^{-/-} mice in C57BL/6 background compared to WT mice (Supplementary Fig. 3a). These findings suggested that IgM-deficient mice have profound AHR reduction independent of allergen or mice background strain.

IgM-deficiency does not impact B cell subsets in primary and secondary organs, but class switching is impaired

We also observed no significant changes between IgM^{-/-} and WT mice in mucus production shown by *Muc5a* gene expression (**Fig. 1d** [↗](#)) or goblet cells in Balb/C (Supplementary Fig. 1f) and C57BL/6 mice challenged with HDM (Supplementary Fig. 3b). Accompanying reduction in AHR in IgM^{-/-} mice was low titers of total IgE, HDM-specific IgE, and mediastinal lymph node (mLN) B cell surface expression of IgM and IgG1 (**Fig. 1e-f** [↗](#)), owing to a lack of class switching. Interestingly, the numbers of lung B cells were normal (**Fig. 1c** [↗](#) and Supplementary Fig. 1c) and the frequencies of B cell subsets such as follicular, marginal zones and germinal centres (GCs) B cells were not affected by lack of IgM (**Fig. 1g** [↗](#) and Supplementary Fig. 1g). The lack in class switching in Balb/C mice was also consistent with what we found in IgM^{-/-} mice in the C57BL/6 background (Supplementary Fig. 3c). This suggested a normal interaction between B and T cells in GCs, but lack of AID-dependent class switching, despite increased expression of IgD (Supplementary Fig. 4a) [↗](#). We checked for natural IgM and antigen-induced IgM in multiple tissues. B cells expressing

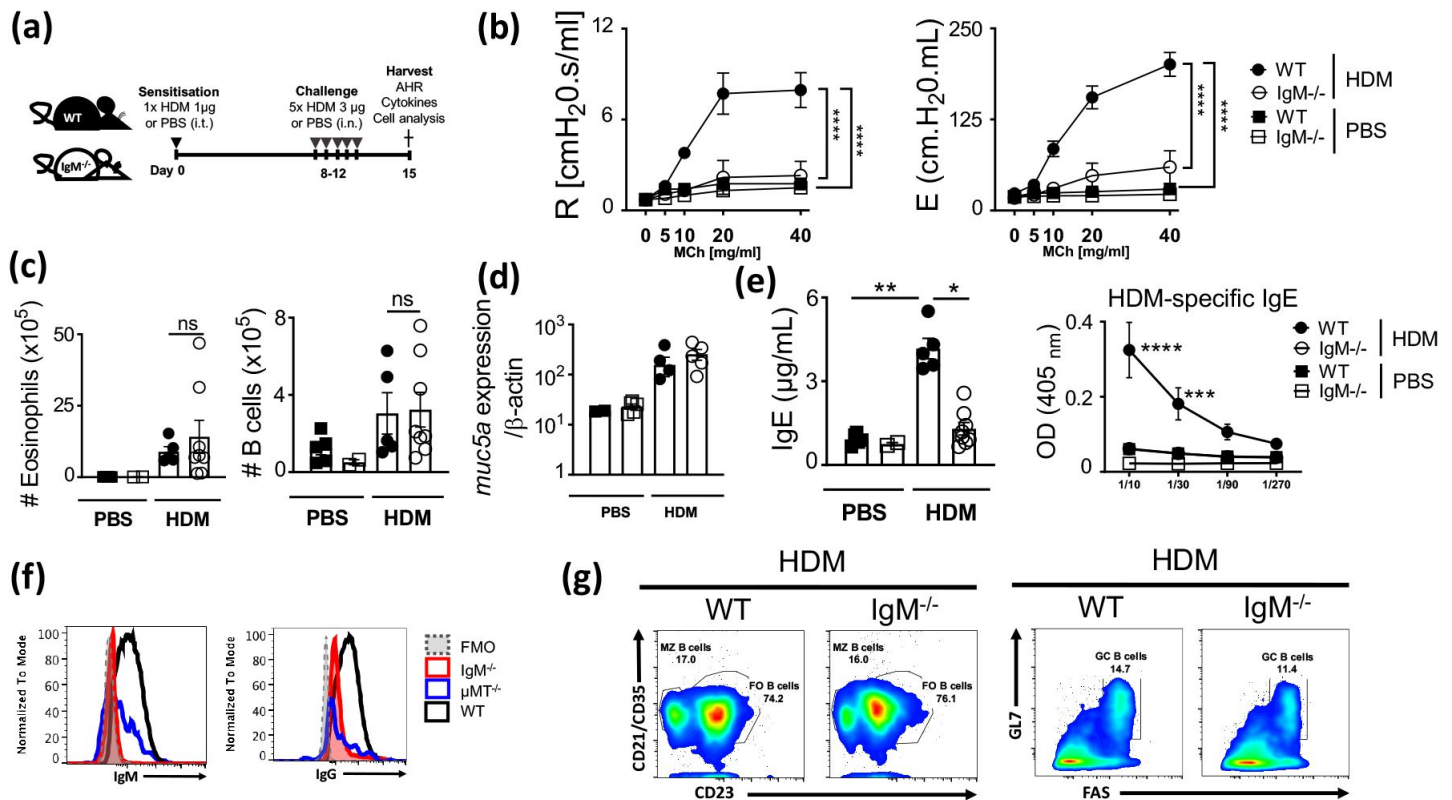


Fig. 1

IgM-deficiency leads to reduced airway hyperresponsiveness and class switching to IgE in HDM-induced asthma.

a Schematic diagram showing sensitisation and challenge protocol where mice (IgM^{-/-}) and wild type littermate control (WT) were sensitised with HDM 1 µg intra-tracheally on days 0 and challenged with HDM 3 µg on days 8-12. Analysis was done on day 15.

b Airway resistance and elastance were measured with increasing doses of acetyl methacholine (0 -40 mg/mL).

c Total lung eosinophil numbers (live⁺Siglec-F⁺CD11c⁻) and B cells (live⁺B220⁺CD19⁺MHCII⁺) were stained and analysed by Flow cytometry and enumerated from % of live cells.

d *Muc5a* gene expression in whole lung tissue.

e Total IgE and HDM-specific IgE in serum.

f IgG1 and IgM surface expression in mediastinal lymph node B cells of WT, IgM^{-/-} and µMT^{-/-} mice.

g Marginal Zone (live⁺B220⁺CD19⁺MHCII⁺CD21/CD35⁺CD23⁺), follicular (live⁺B220⁺CD19⁺MHCII⁺CD23⁺CD21/CD35⁺) and Germinal Centre (live⁺B220⁺CD19⁺MHCII⁺GL7⁺FAS⁺) B cells in the mediastinal lymph node of WT and IgM^{-/-} mice challenged with HDM.

Shown is mean ± SEM from two pooled experiments (n=7 - 10). Significant differences between groups were performed by Student t-test (Mann-Whitney) (c, d, e) or by Two-Way ANOVA with Benforroni post-test (b) and are described as: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

IgD were increased in all tissues including mLN, peritoneal cavity and spleen (Supplementary Fig. 4a-c) and pre-B cell subsets were normal in BM (Supplementary Fig. 4d) in the absence of IgM as previously reported ³².

T helper 2 cells are intact in the absence of IgM, and serum transfer can partially restore antibody function

To investigate whether the reduction in AHR was due to reduced Th2 cells and cytokines, we stimulated total mLN and lung cells with anti-CD3 for 5 days or with PMA/ionomycin for 5 hrs in the presence of monensin and measured secreted or intracellular IL-4 and IL-13 expression. We found no differences in CD4 T cells and T follicular helper cells (Fig. 2a) in mLN and in secreted or intracellular levels of IL-4 and IL-13 between IgM^{-/-} and WT mice challenged with HDM in both mLN and lungs (Fig. 2b-c). To investigate what drives this reduced AHR in the absence of IgM between secreted antibodies or the IgM B cell receptor on the surface of B cells, we transferred serum from naïve WT mice into IgM^{-/-} mice a day before sensitization, during sensitization and a day before challenge with HDM allergen (Fig. 2d), as previously described ³⁶. AHR in IgM^{-/-} was still reduced compared to WT mice even after the transfer of WT serum (Fig. 2e), but levels of total IgE, HDM-specific IgE and HDM-specific IgG1 were increased and comparable to those found in WT mice (Fig. 2f and Supplementary Fig. 5e). The redundant role of IgE was consistent with previous studies where IgE nor its high-affinity receptor (FcγRI) were essential in AHR in an HDM or OVA models ³⁰. The lack of functional role of serum-transferred IgE was consistent with earlier findings on *H. polygyrus* transfer of immune serum where IgE was found not to be essential in protection against *H. polygyrus* re-infection ³⁶. We also tried to restore IgM function through adoptive reconstitution with congenic CD45.1 BM or sorted B cells into IgM^{-/-} mice (Supplementary Fig. 5a). This approach did not replenish IgM B cells to levels observed in WT mice and as a result did not restore AHR, total IgE and IgM in these mice (Supplementary Fig. 5b-c). To understand why we could not reconstitute IgM, we checked for transferred congenic B cells in different organs after reconstitution with WT BM in IgM^{-/-} mice and found some engraftment in BM and spleen, but poor engraftment to lung and mLN which may partially explain our inability to restore AHR in IgM^{-/-} mice challenged with HDM (Supplementary Fig. 5d).

IgM is known to play key role in shaping gut microbiota and its diversity can be shaped by gut commensals ^{16, 37}. To understand whether microbiota could influence AHR in IgM-deficient mice, we treated WT and IgM^{-/-} with an antibiotic cocktail 3x per week for 2 weeks (Supplementary Fig. 6a). We confirmed the reduction of bacteria in the faecal pellet after 2 weeks of antibiotic treatment (Supplementary Fig. 6a). We then sensitised and challenged these mice with HDM and measure AHR (Supplementary Fig. 6b). We observed increased resistance and elastance in WT mice compared to IgM^{-/-} mice and pre-treatment with antibiotics did not influence AHR (Supplementary Fig. 6b). Treatment with antibiotics did increase total IgE, HDM-specific IgE and IgG1 in WT mice as expected ³⁸, but had no impact on IgM-deficient mice (Supplementary Fig. 6c), suggesting that overall antibiotics did not influence AHR.

RNA sequencing reveals enrichment of genes associated with skeletal muscle contraction and actin re-arrangement

Because we had found no other changes in asthmatic allergic features between WT and IgM^{-/-}, except for profoundly reduced AHR, we resorted to RNA sequencing to see if there were lung-specific factors that influenced AHR regulation in IgM-deficient mice. Principal component analysis (PCA) depicted distinct global transcriptional changes with PC1 and PC2 explaining most of the variation (Fig. 3a). We found a smaller variation in gene expression between WT and IgM^{-/-} in both HDM-challenged and PBS control mice (Fig. 3b). We could mainly detect downregulation of genes such as brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1 (*Baiap2l1*), erythroid differentiation regulatory factor 1 (*Erdr1*), chemokines such as *Ccl8*, *Ccl9*, *Ccl17* and *Ccl22* in IgM^{-/-} mice challenged with HDM (Fig. 3c-d). Interestingly, *Baiap2l1* and

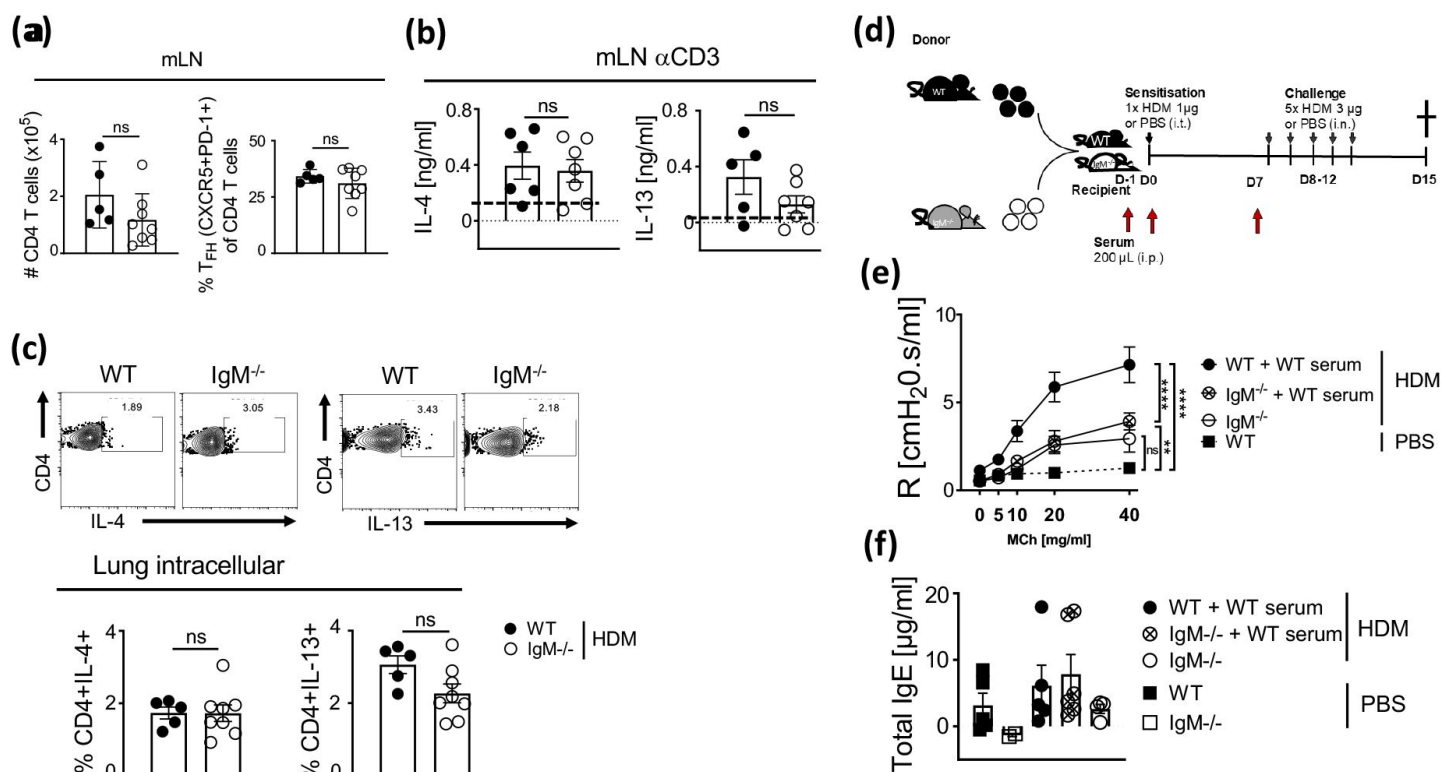


Fig. 2

IgM-deficiency does not lead to reduced Th2 allergic airway inflammation and serum transfer restores IgE, but not AHR.

Fig. a-c, mice treated as in [Fig. 1a](#).

a Total mediastinal lymph node CD4 T cell numbers (live⁺CD3⁺CD4⁺) and % of Follicular Helper T cells (live⁺CD3⁺CD4⁺PD-1⁺CXCR5⁺) of CD4 T cells were stained and analysed by Flow cytometry and enumerated from % of live cells.

b Mediastinal lymph nodes were stimulated with anti-CD3 (10 μ g/mL) for 5 days and supernatants were used to measure levels of IL-4 and IL-13. Cytokines were not detected in unstimulated or HDM (30 μ g) stimulated mLN.

c Representative FACS plots and frequencies of lung CD4 T cells (live⁺CD3⁺CD4⁺) producing IL-4 and IL-13 after 5 hr stimulation with PMA/ionomycin in the presence of monensin.

d Schematic diagram showing serum transfer from WT to IgM^{-/-} which were then sensitised as shown in [Fig 1,a](#).

e Airway resistance was measured with increasing doses of acetyl methacholine (0 - 40 mg/mL).

f Total IgE in serum of mice either transferred with WT serum, IgM^{-/-} serum or no serum.

Shown is the mean $\pm$ SEM from two pooled experiments (n=5 - 8). Significant differences between groups were performed by Student t-test (Mann-Whitney) (C, D, E) or by Two-Way ANOVA with Benfornoni post-test (B) and are described as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Erdr1 were also downregulated in IgM^{-/-} saline-treated control mice, suggesting an inflammation-independent effect (Fig. 3c and e). We also found the presence of the J-chain coding gene in WT mice which was absent in IgM^{-/-} mice, confirming a deletion in genes associated with holding IgM monomers together and thus IgM³⁹ (Fig. 3e). Gene set enrichment analyses (GSEA) confirmed that the genes contributing to changes in AHR between WT and IgM^{-/-} were associated with muscle system processes and skeletal muscle contraction (Fig. 3f). There was also an over-representation of gene ratios associated with skeletal muscle development, differentiation and contraction, and suppression of genes associated with chemotaxis and plasma membrane-bounded cell projection (Supplementary Fig. 7).

BAIAP2L1 is expressed in lung cells in contact with airway smooth muscle cells

We decided to focus on *Baiap2l1* also known as Inverse-bin-amphiphysin-Rvs (I-BAR)-domain-containing protein insulin receptor tyrosine kinase substrate (IRTKS), which has been shown to promote actin polymerisation and microvilli length in the intestinal epithelial cells⁴⁰. We first verified whether BAIAP2L1 was also downregulated at the protein level in IgM-deficient mouse lungs challenged with HDM (Fig. 4a). We found lower expression levels of BAIAP2L1 in IgM-deficient mice compared to WT mice challenged with HDM by western blotting, although this was not significant (Fig. 4a). Human Protein Atlas search suggested that BAIAP2L1 is expressed by multiple cell types including fibroblasts, skeletal muscle, smooth muscle and macrophages⁴¹. We then investigated by immunofluorescence which cell types within the lung could be expressing BAIAP2L1 (Fig. 4b). We found BAIAP2L1 to be closely expressed within structural cells in close contact to smooth muscle cells (less than 10µm), a cell type known to be essential in bronchoconstriction. Airway smooth muscle together with the extracellular matrix contributes to smooth muscle hypertrophy which causes the narrowing of the airways. These cells by histology appear to be separated due to fixation methods but are mashed together, especially during the remodelling of the airways⁴². IgM deficiency did not impact this expression in cells in close contact with smooth muscle (Fig. 4b). Because both western blot and immunofluorescent were inconclusive, we also verified the expression of BAIAP2L1 via flow cytometry (gating in Supplementary Fig. 8a) and found it to be expressed mainly by alpha-smooth muscle cells and increased in expression in HDM treated mice compared to PBS control mice (Fig. 4c). Furthermore, we showed a reduction in BAIAP2L1 expression amongst alpha-smooth muscle cells but not other cells (CD45 positive or alpha-smooth muscle negative cells, Supplementary Fig. 8b) in IgM-deficient mice compared to WT mice treated with HDM, although this did not reach significance (Fig. 4c). Overall, this data suggested that BAIAP2L1 is reduced at RNA and protein level and likely influenced the reduction of AHR in IgM-deficient mice. This was consistent with gene set enrichment in our RNA seq data where there was a dominance of genes associated with muscle contraction which regulates AHR.

CRISPR-Cas9 deletion of BAIAP2L1 leads to reduced airway smooth muscle contraction at a single cell

To understand how *Baiap2l1*, one of the genes downregulated in IgM-deficient mice, could influence AHR, we resorted to an *in vitro* model that allowed us to measure contraction using fluorescently labelled elastomeric contractile surface (FLECS) technology⁴³ (Fig. 5a). We opted for airway smooth muscle as it has been shown to contribute to the narrowing of the airway during an allergic attack and together with neighbouring cells such as extracellular matrix and immune cells are critical in hypertrophy⁴². We chose the human bronchial smooth muscle cell (BSMCs) line as *BAIAP2L1* is expressed in structural cells including muscle and epithelial cells, and actin together with myosin is essential in muscle contraction⁴¹. To this end, we used CRISPR-Cas9 technology to knock down *BAIAP2L1* in BSMCs (Supplementary Fig. 9a-b). We also included ERDR1 as one of the genes that were also downregulated in IgM-deficient mice. BSMCs (160

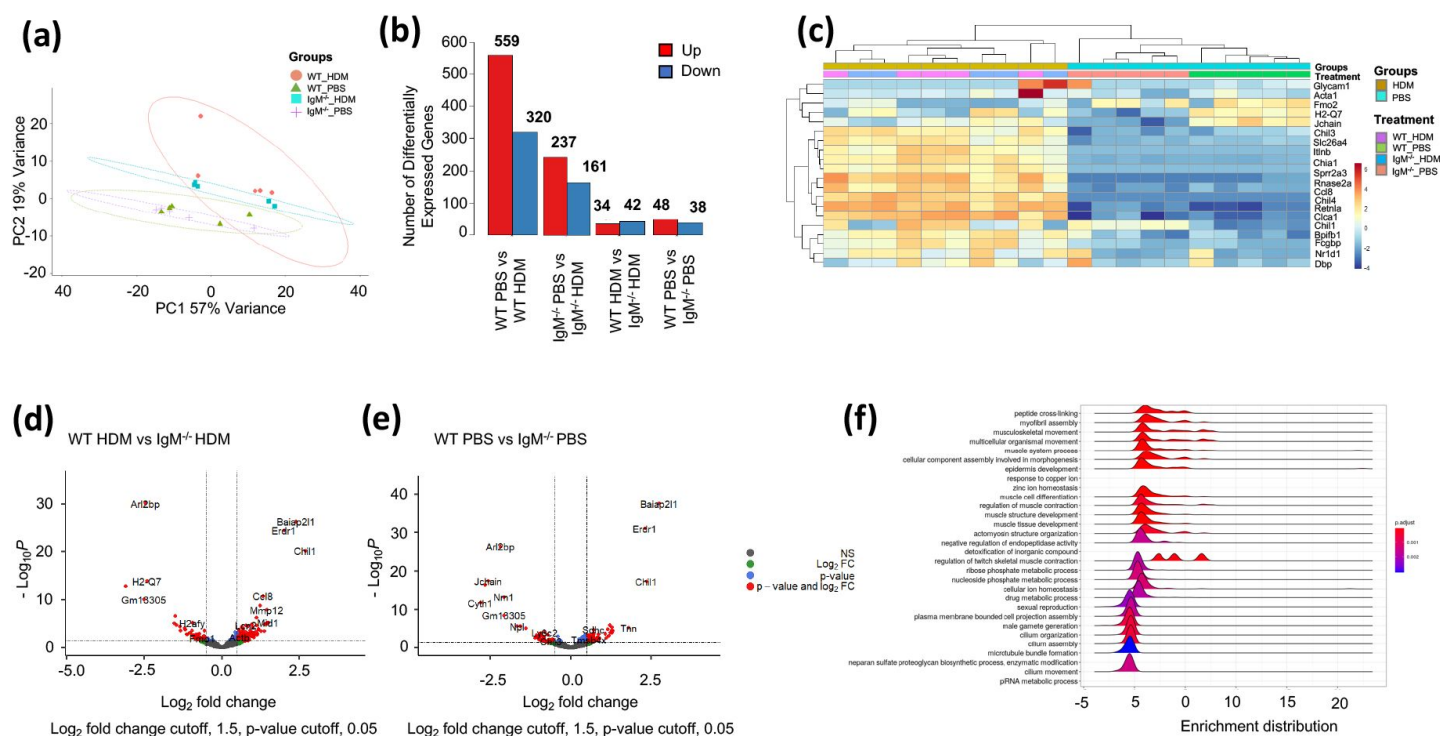


Fig. 3

Genes associated with muscle contraction are downregulated in IgM-deficient mice.

WT and IgM^{-/-} mice were treated as in [Figure 1A](#) and RNA was collected from the whole lung for RNA sequencing.

a Principal-component (PC) analysis showing variation in the global gene expression profiles across the different groups. PC1 (60%) and PC2 (18%), which capture the greatest variation in gene expression, are shown. Orange colour represents WT HDM, green colour represents WT PBS, Blue colour represents IgM^{-/-} HDM and purple crosses represent IgM^{-/-} PBS. Each dot represents an individual mouse.

b Number of differentially expressed genes between groups.

c Heatmaps depicting the differentially expressed genes between WT and IgM^{-/-} samples from HDM-treated and PBS mice ranked based on hierarchical clustering.

d-e Volcano plots: numbers and colour relate to genes that have an adjusted *p* value <0.05. Blue, significantly downregulated; red, significantly up regulated; grey, non-differentially expressed. P values were adjusted for multiple testing using the Benjamini-Hochberg method. (D) represent changes between WT and IgM^{-/-} treated with HDM and (E) represents changes between WT and IgM^{-/-} treated with saline.

f Gene set enrichment analysis (GSEA) of hallmark gene sets from the Molecular Signatures Database of the Broad Institute, showing the normalized enrichment scores (NES) for lung RNA-Seq data from WT mice.

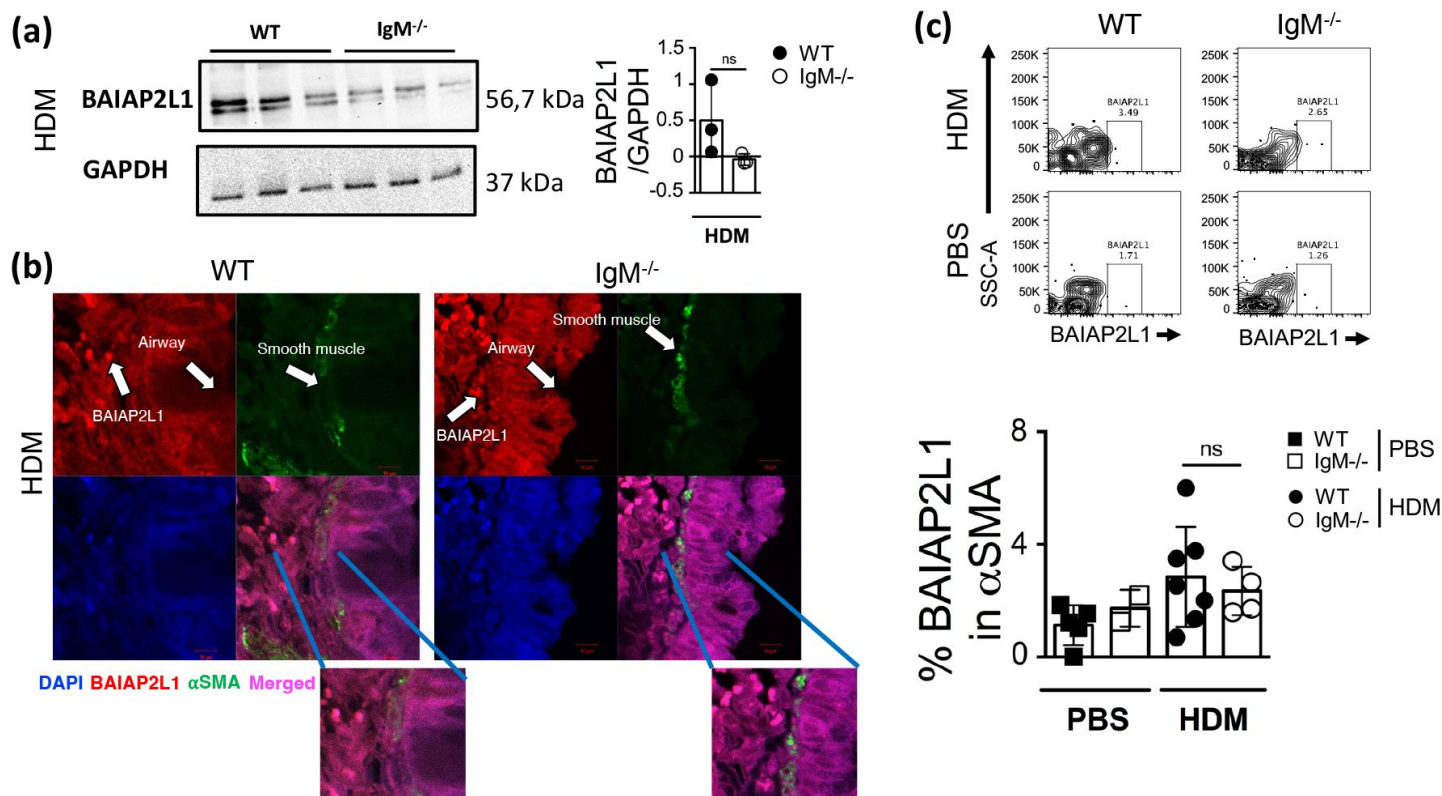


Fig. 4

***BAIAP2L1* is expressed in close contact to smooth muscle.**

a Mouse lungs were homogenised in RIPA buffer and blotted on nitrocellulose. Rabbit anti-human BAIAP2L1 and mouse anti-GAPDH was used as primary antibody. Lines 1-3 is WT mice, lines 4-6 is IgM^{-/-} mice sensitised and challenged with HDM.

b Lung sections from WT and IgM^{-/-} mice sensitised and challenged with HDM were immunostained for nuclei stained DAPI (Blue), anti-BAIAP2L1 (red), α smooth muscle actin (green) and merged images (Magenta). Insert below shows zoomed in image of merged BAIAP2L1 and α smooth muscle actin.

c Representative flow cytometry plots showing BAIAP2L1 expression (Live⁺ Singlets⁺ CD45⁻ αSMA⁺ BAIAP2L1⁺) in WT and IgM^{-/-} treated with HDM or PBS. Quantification of % BAIAP2L1 in αSMA is shown.

Shown are representative images from 2 independent experiments (n= 3 mice per group).

000/well) were stimulated with 10 μ M acetylcholine (ACh), 10 ng/mL of human TNF- α and 100 ng/mL IL-13, followed by transfection with ribonucleoprotein (RNP) complexes containing single guide RNA targeting *BAIAP2L1* and Cas9 for 48h (**Fig. 5b**). Transfected cells were seeded onto elastomeric patterns, stimulated with the same stimulants for 3h and fixed in 4% paraformaldehyde, followed by staining with DAPI and Phalloidin before imaging on Stellarvision microscope and quantification (**Fig. 5a**). We validated the deletion of *BAIAP2L1* by PCR and showed reduced expression of *BAIAP2L1* in ACh and TNF- α stimulated cells transfected with *BAIAP2L1* sgRNA when compared to scramble sgRNA (Supplementary Fig. 9c, line 3 and 4 compared to line 5 and 6). We also detected low expression of *BAIAP2L1* in unstimulated sgRNA scramble and sgRNA *BAIAP2L1* transfected cells (Supplementary Fig. 9c, lines 1 and 2). Transfections with sgRNA did not impact cell viability (Supplementary Fig. 9d). As expected, BSMCs displayed a higher level of contraction in TNF- α or IL-13 stimulated cells compared to unstimulated cells (**Fig. 5b**-d). In BSMCs transfected with sgRNA-*BAIAP2L1* and -*ERDR1*, we saw a significant reduction in contraction levels in TNF- α stimulated cells when compared to scramble sgRNA transfected and stimulated cells (**Fig. 5b**, c). We saw no differences in contraction between scramble sgRNA and sgRNA-*BAIAP2L1* and -*ERDR1* stimulated with IL-13 (**Fig. 5d**).

***BAIAP2L1* deletion but not *ERDR1* is essential in acetylcholine-induced smooth muscle contraction**

Contraction of the ASM is central to AHR as it controls airway diameter and airflow which can increase resistance²⁰. Airway smooth muscle contractility is induced when extracellular factors such as ACh bind through muscarinic 3 acetylcholine receptor (m3AChR), activating a signalling cascade leading to calcium accumulation and contraction⁴⁴. We transfected BSMCs with sgRNA-*BAIAP2L1*, -*ERDR1* or scrambled and stimulated cells with 10 μ M ACh (**Fig. 6a**). We saw a significant reduction in BSMC contraction transfected with sgRNA-*BAIAP2L1* when compared to scramble sgRNA transfected and ACh stimulated (**Fig. 6b-c**). We saw no significant differences in BSMC contraction between sgRNA-*ERDR1* transfected and scramble sgRNA transfected and ACh stimulated cells (**Fig. 6b-c**). Taken together, this data indicates that *BAIAP2L1* a gene that was downregulated in IgM-deficient mice has a stimulant-specific role in inducing airway contraction of bronchial smooth muscle cells during asthma.

Discussion

Here, we described an unexpected function of IgM in the regulation of bronchoconstriction. We show by RNA sequencing that in the lung tissue of IgM-deficient mice, there is a reduction in genes (*Baiap2l1* and *Erdr1*) associated with actin cytoskeleton and re-arrangement, a key factor in smooth muscle contraction and AHR. We used single-cell force cytometry and CRISPR-Cas9 technology to validate these genes in the human BSM cell line and show as a proof of concept that deletion of *BAIAP2L1* reduced muscle contraction in a stimulant-specific manner.

B cells play a complex role in allergic asthma, earlier studies using B cell-deficient mice showed a redundant role of B cells in OVA-induced allergic airway inflammation and airway hyperreactivity²⁷⁻²⁹. We and others have recently shown using a more complex allergen HDM relevant to human asthma that antigen load is crucial in B cell function²²⁻²⁴. We explored these possibilities in the context of IgM deficiency and observed a profound reduction in AHR. This was in complete contrast to what has been observed in B cell-deficient mice and in IgE or Fc ϵ RI-deficient mice^{23, 24, 30}. We also found a similar reduction in AHR at higher doses of HDM, albeit less pronounced.

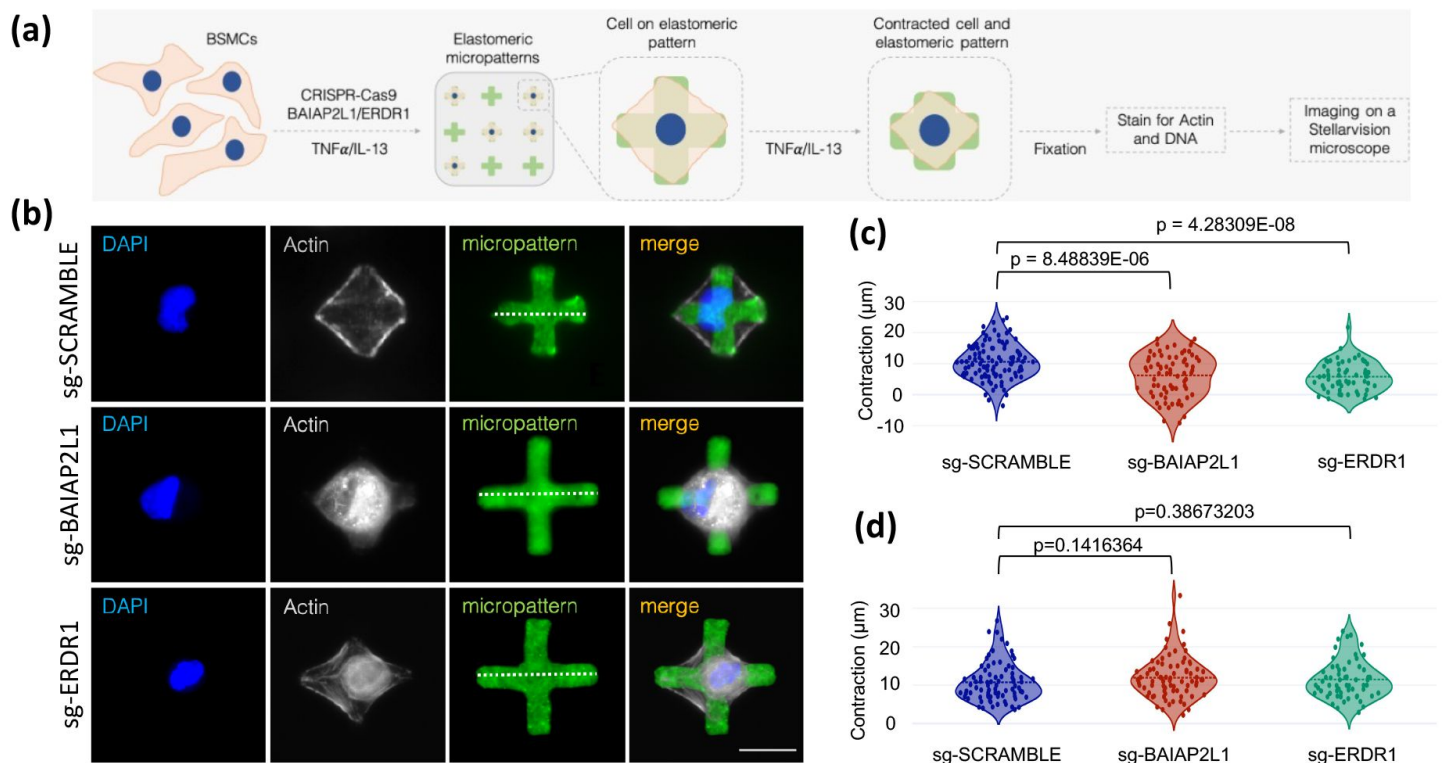


Fig. 5

CRISPR-based deletion of *BAIAP2L1* leads to reduced smooth muscle contraction at a single-cell level.

a, Bronchial smooth muscle cells (1.6×10^5 cells/well) were transfected with CRISPR-Cas9 single guide RNAs (scramble, *BAIAP2L1* and *ERDR1*), stimulated with recombinant human IL-13 (100 ng/mL) and TNF- α (10 ng/mL) for 48 h. Cells were then transferred to elastomeric micropatterns, stimulated again with rIL-13 and rTNF- α and fixed before imaging on a StellarVision microscope.

b, Representative images of single BSMCs on micropatterns from scramble, *BAIAP2L1* and *ERDR1* stimulated with 10 ng/mL TNF- α . DNA was stained with DAPI, actin fibers with Phalloidin-565 and elastomeric micropatterns are coated in Fibronectin-488. Merged images are shown on the right.

c, Violin plots showing contraction of 50-100 cells/condition stimulated with 10 ng/mL TNF- α , individual dots represent a single cell contraction, where blue is scramble sgRNA, red is *BAIAP2L1* sgRNA and green is *ERDR1* sgRNA.

d, Violin plots showing contraction of 50-100 cells/condition stimulated with 100 ng/mL IL-13, individual dots represent a single cell contraction, where blue is scramble sgRNA, red is *BAIAP2L1* sgRNA and green is *ERDR1* sgRNA.

Shown is mean $\pm$ SEMs from two pooled experiment ($n = 50 - 100$). Significant differences between groups were performed by student t-test (Mann-Whitney) and p value is shown.

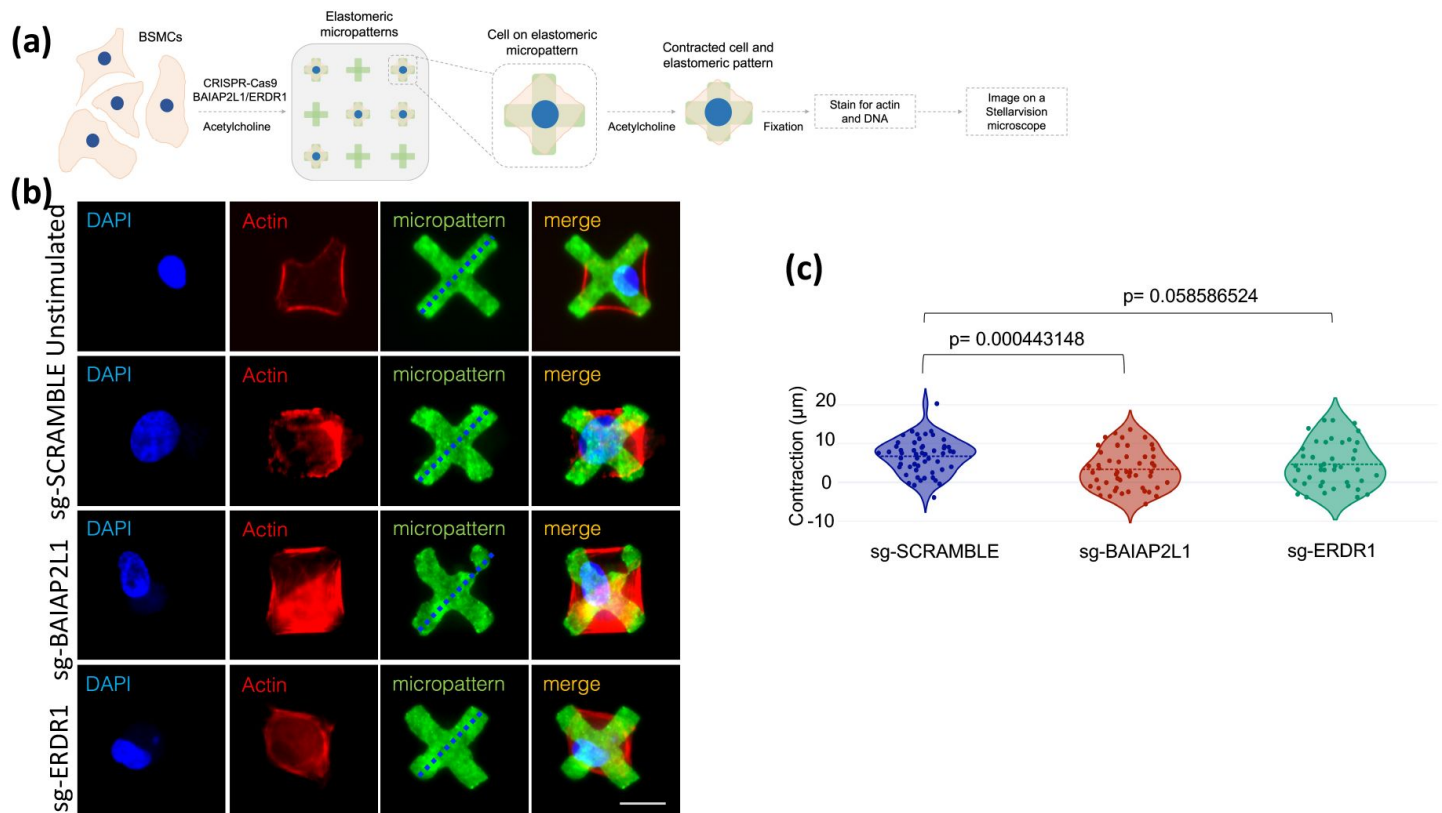


Fig. 6

CRISPR-based deletion of *BAIAP2L1* reduces smooth muscle contraction upon stimulation with acetylcholine.

a Bronchial smooth muscle cells (1.6×10^5 cells/well) were transfected with CRISPR-Cas9 single guide RNAs (scramble, *BAIAP2L1* and *ERDR1*), stimulated with Acetylcholine ($10 \mu\text{M}$) for 48 h. Cells were then transferred to elastomeric micropatterns, stimulated again with ACh ($10 \mu\text{M}$) and fixed before imaging on a StellarVision microscope.

b, Representative images of single BSMCs on a single micropattern from unstimulated, scramble, *BAIAP2L1* and *ERDR1* stimulated with ACh ($10 \mu\text{M}$). Shown are DAPI, actin (red), a green fluorescent micropattern and merged images.

c, Violin plots showing contraction of 50-100 cells/condition stimulated with ACh ($10 \mu\text{M}$) individual dots represent a single cell contraction, where blue is scramble sgRNA, red is *BAIAP2L1* sgRNA and green is *ERDR1* sgRNA.

Shown is mean $\pm$ SD from 1 representative experiment of 2 independent experiments ($n = 50 - 100$). Significant differences between groups were performed by student t-test (Mann-Whitney) and p value is shown.

Reduction in AHR was not only specific to HDM, but we also found reduced AHR in IgM-deficient mice sensitised with OVA complexed to alum and challenged with OVA, although less pronounced. AHR reduction in IgM-deficient mice was also seen in the acute papain model which only activates innate responses including innate lymphoid cells ⁴⁵. Interestingly, this reduction in AHR was not associated with reduced allergic airway inflammation including eosinophilia, mucus production or Th2 cells in all models tested, which was unexpected considering these features are key in AHR. B cell-deficient mice show a reduction in eosinophilia and antigen-specific Th2 cells at low doses of HDM, which leads to reduced AHR ²⁴. Similar findings are observed in mice lacking IL-4Rα specifically in B cells when challenged with HDM ²².

IgM deficiency did not impact B cell development in primary and secondary lymphoid tissues and accumulation of follicular and germinal centre B cells. Interestingly, there was a lack of class switching to IgG1 and IgE isotypes despite increased expression of IgD. The lack of class switching to IgG1 and IgE contrasts with earlier reports, which suggested that IgD can largely replace IgM for class switching to other isotypes, resulting in delayed neutralising IgG1 against vesicular stomatitis virus (VSV) ^{32, 46}. To get a better understanding of which IgM function was responsible for AHR, we showed that the transfer of naïve wild-type mice serum to IgM-deficient mice could restore IgE production and HDM-specific IgE and IgG1. This is consistent with what has been observed in the context of influenza viral infections, where the transfer of purified or serum IgM into B cell-deficient mice restored IgM-induced viral neutralisation ⁴⁷. We believe that naïve sera transferred to IgM-deficient mice were able to bind to the surface of B cells via IgM receptors (FcμR / Fcα/μR), which are still present on IgM-deficient B cells, and this signalling is sufficient to facilitate Class Switch Recombination (CSR). This was also confirmed by the ability of B cells to reach germinal centres in IgM-deficient mice during HDM exposure. Our IgM^{-/-} mouse lacks both membrane-bound and secreted IgM, and transferred serum contains at least secreted IgM which can bind to B cell surfaces. Of course, we can't rule out that transferred sera from WT mice also contains some IgG1 which can facilitate class switching to IgE when transferred to IgM-deficient mice. Despite the ability of wild-type serum to restore IgE, it was not enough to restore AHR, which may be due to the difficulties in restoring IgM to normal levels (200-800 μg). Our findings on the redundant role of IgE were consistent with previous studies where IgE nor its high-affinity receptor (FcγRI) were essential in AHR in an HDM or OVA model ³⁰. The lack of functional role of serum-transferred IgE was consistent with earlier findings on *H. polygyrus* transfer of immune serum where IgE was found not to be essential in protection against *H. polygyrus* re-infection ³⁶. Our efforts to adoptively transfer wild-type bone marrow or sorted B cells into IgM-deficient mice were also largely unsuccessful partly due to poor engraftment of wild-type B cells into secondary lymphoid tissues. Natural secreted IgM is mainly produced by B1 cells in the peritoneal cavity, and it is likely that any transfer of B cells via bone marrow transfer would not be sufficient to restore soluble levels of IgM ^{3, 10}.

B cell isotype IgD has been shown to promote Th2 cells and OVA-, papain- and NP-specific humoral responses specifically IgG1 and IgE through basophil activation ³¹. IgD has also been shown to inhibit IgE-mediated basophil activation through downregulation of genes associated with cytoskeleton organisation such as signal transducers phosphoinositide-3 kinase, RAS, and RHO ³¹. Our RNA sequencing data showed downregulation of *Baiap211* and *Erdr1* in IgM-deficient mice, genes which have been shown to associate with actin cytoskeleton and re-arrangement, ^{40, 41, 48}. Contraction of the ASM is central to AHR as it controls airway diameter and airflow which can increase resistance ⁴⁹. ASM activated by cytokines such as IL-13 or TNF-α or bronchoconstrictor ACh induces cytosolic calcium accumulation ^{49, 50}. Ca²⁺ release results in a cascade of events that involves kinases and phosphorylation of key molecules such as actin and myosin involved in smooth muscle contraction ⁴⁴. Gene set enrichment analysis suggested that genes downregulated in IgM-deficient mice were involved in processes such as muscle contraction, muscle twitching, skeletal muscle movement. As a proof of concept we validated the involvement of *BALAP2L1* in bronchial smooth muscle contraction using a high throughput method that allows us to measure contraction of 1000s of single cells ⁴³. When we deleted

BAIAP2L1 using CRISPR-Cas9, we showed that deletion of *BAIAP2L1* in BSMCs reduced smooth muscle contraction when stimulated with TNF- α and ACh, but not IL-13. Deletion of ERDR1 had minor impact on muscle contraction across different stimulants suggesting a stimulant and gene-specific role. IRTKS/*BAIAP2L1* has been shown to regulate microvilli elongation by forming a complex with actin-regulating protein EPS8⁴⁰. In this setting IRTKS elongate microvilli via distinct mechanisms that require functional WH2 and SH3 domains, which binds EPS8, an F-actin capping and bundling protein⁴⁰. We have not fully dissected how *BAIAP2L1* could be regulated by IgM, but it is clear that through its actions to regulate actin bundling, it is involved in muscle contraction which requires actin and myosin head interaction (summarised in **Fig. 7**).

We speculate that IgM can directly activate smooth muscle cells by binding a number of its surface receptors including Fc μ R, Fc α / μ R and pIgR^{51–53}. IgM binds to Fc μ R strictly, but shares Fc α / μ R and pIgR with IgA^{53, 51, 53}. Both Fc α / μ R and pIgR can be expressed by non-structural cells at mucosal sites^{53, 54}. We would not rule out that the mechanisms of muscle contraction might be through one of these IgM receptors, especially the ones expressed on smooth muscle cells^{53, 54}. Certainly, our future studies will be directed towards characterizing the mechanism by which IgM potentially activates the smooth muscle.

We believe these findings report for the first time an independent function of IgM from its natural class switching and non-specific binding to microbial components. We believe that IgM function in regulating ASM may be indirect through other unknown factors but does not involve microbiota as treatment of mice with a mixture of antibiotics did not restore AHR. Early vaccination with bacterial species, such as group A *streptococcus* containing GlcNAc or γ -1,3-glucans can protect adult mice against *A. fumigatus*-induced allergic asthma⁵⁵. This is mainly through conserved germline-encoded IgM antibodies, which have broad specificities to common allergens containing GlcNAc moieties such as Dermatophytes^{8, 56}. Together these findings demonstrate for the first time an important function of IgM in regulating airway hyperresponsiveness independent of the presence of T helper 2 allergic inflammation. These findings have implications for future treatment of allergic asthma through bronchodilators.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Conceived and supervised study: SH FB. Performed the experiments: SH AFS JK KJ NM MS AN FM HN WM. Analysed the data: SH AFS KJ JO NM HN FM. Wrote the paper: SH FB. All authors discussed the results and commented on the manuscript.

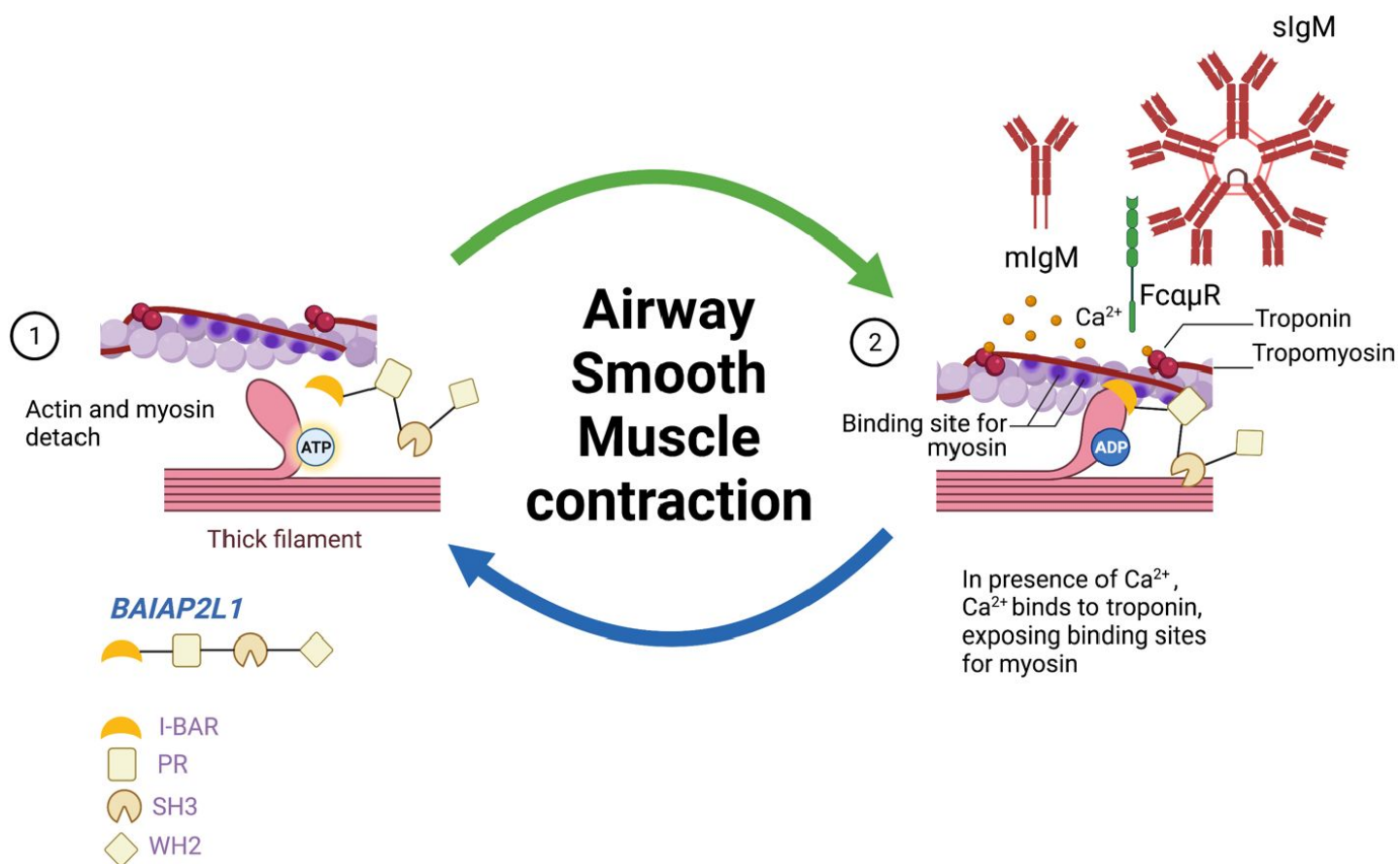


Fig. 7

Working model showing how *Baiap2l1* could influence muscle contraction through influencing myosin and actin filament interaction. Image adapted from Biorender.

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Reviewer #1 (Public Review):

Summary: The authors of this study sought to define a role for IgM in responses to house dust mites in the lung.

Strengths:

Unexpected observation about IgM biology
Combination of experiments to elucidate function

Weaknesses:

Would love more connection to human disease

Reviewer #2 (Public Review):

Summary:

The manuscript by Hadebe and colleagues describes a striking reduction in airway hyperresponsiveness in Igm-deficient mice in response to HDM, OVA and papain across the B6 and BALB-c backgrounds. The authors suggest that the deficit is not due to improper type 2 immune responses, nor an aberrant B cell response, despite a lack of class switching in these mice. Through RNA-Seq approaches, the authors identify few differences between the lungs of WT and Igm-deficient mice, but see that two genes involved in actin regulation are greatly reduced in IgM-deficient mice. The authors target these genes by CRISPR-Cas9 in in vitro assays of smooth muscle cells to show that these may regulate cell contraction. While the study is conceptually interesting, there are a number of limitations, which stop us from drawing meaningful conclusions.

Strengths:

Fig. 1. The authors clearly show that IgM KO mice have strikingly reduced AHR in the HDM model, despite the presence of a good cellular B cell response.

Weaknesses:

Fig. 2.

The authors characterize the cd4 t cell response to HDM in IGM KO mice. They have restimulated medLN cells with antiCD3 for 5 days to look for IL-4 and IL-13, and find no discernible difference between WT and KO mice. The absence of PBS-treated WT and KO mice in this analysis means it is unclear if HDM-challenged mice are showing IL-4 or IL-13 levels above that seen at baseline in this assay. The choice of 5 days is strange, given that the response the authors want to see is in already primed cells. A 1-2 day assay would have been better. It is concerning that the authors state that HDM restimulation did not induce cytokine production from medLN cells, since countless studies have shown that restimulation of medLN would induce IL-13, IL-5 and IL-10 production from medLN. This indicates that the sensitization and challenge model used by the authors is not working as it should. The IL-13

staining shown in panel c is also not definitive. One should be able to optimize their assays to achieve a better level of staining, to my mind.

In d-f, the authors perform a serum transfer, but they only do this once. The half life of IgM is quite short. The authors should perform multiple naïve serum transfers to see if this is enough to induce FULL AHR.

The presence of negative values of total IgE in panel F would indicate some errors in calculation of serum IgE concentrations.

Overall, it is hard to be convinced that IgM-deficiency does not lead to a reduction in Th2 inflammation, since the assays appear suboptimal.

Fig. 3. Gene expression differences between WT and KO mice in PBS and HDM challenged settings are shown. PCA analysis does not show clear differences between all four groups, but genes are certainly up and downregulated, in particular when comparing PBS to HDM challenged mice. In both PBS and HDM challenged settings, three genes stand out as being upregulated in WT v KO mice. these are *Baiap2l1*, *erdr1* and *Chil1*.

Fig. 4. The authors attempt to quantify BAIAP2L1 in mouse lungs. It is difficult to know if the antibody used really detects the correct protein. A BAIAP2L1-KO is not used as a control for staining, and I am not sure if competitive assays for BAIAP2L1 can be set up. The flow data is not convincing. The immunohistochemistry shows BAIAP2L1 (in red) in many, many cells, essentially throughout the section. There is also no discernible difference between WT and KO mice, which one might have expected based on the RNA-Seq data. So, from my perspective, it is hard to say if/where this protein is located, and whether there truly exists a difference in expression between wt and ko mice.

Fig. 5 and 6. The authors use a single cell contractility assay to measure whether BAIAP2L1 and ERDR1 impact on bronchial smooth muscle cell contractility. I am not familiar with the assay, but it looks like an interesting way of analysing contractility at the single cell level. The authors state that targeting these two genes with Cas9gRNA reduces smooth muscle cell contractility, and the data presented for contractility supports this observation. However, the efficiency of Cas9-mediated deletion is very unclear. The authors present a PCR in supp fig 9c as evidence of gene deletion, but it is entirely unclear with what efficiency the gene has been deleted. One should use sequencing to confirm deletion. Moreover, if the antibody was truly working, one should be able to use the antibody used in Fig 4 to detect BAIAP2L1 levels in these cells. The authors do not appear to have tried this.

Other impressions:

The paper is lacking a link between the deficiency of IgM and the effects on smooth muscle cell contraction.

The levels of IL-13 and TNF in lavage of WT and IGMKO mice could be analysed.

Moreover, what is the impact of IgM itself on smooth muscle cells? In the Fig. 7 schematic, are the authors proposing a direct role for IgM on smooth muscle cells? Does IgM in cell culture media induce contraction of SMC? This could be tested and would be interesting, to my mind.

Reviewer #3 (Public Review):

Summary:

This paper by Sabelo et al. describes a new pathway by which lack of IgM in the mouse lowers bronchial hyperresponsiveness (BHR) in response to metacholine in several mouse models of allergic airway inflammation in Balb/c mice and C57/Bl6 mice. Strikingly, loss of IgM does not lead to less eosinophilic airway inflammation, Th2 cytokine production or mucus metaplasia, but to a selective loss of BHR. This occurs irrespective of the dose of

allergen used. This was important to address since several prior models of HDM allergy have shown that the contribution of B cells to airway inflammation and BHR is dose dependent.

After a description of the phenotype, the authors try to elucidate the mechanisms. There is no loss of B cells in these mice. However, there is a lack of class switching to IgE and IgG1, with a concomitant increase in IgD. Restoring immunoglobulins with transfer of naïve serum in IgM deficient mice leads to restoration of allergen-specific IgE and IgG1 responses, which is not really explained in the paper how this might work. There is also no restoration of IgM responses, and concomitantly, the phenotype of reduced BHR still holds when serum is given, leading authors to conclude that the mechanism is IgE and IgG1 independent. Wild type B cell transfer also does not restore IgM responses, due to lack of engraftment of the B cells. Next authors do whole lung RNA sequencing and pinpoint reduced BAIAP2L1 mRNA as the culprit of the phenotype of IgM^{-/-} mice. However, this cannot be validated fully on protein levels and immunohistology since differences between WT and IgM KO are not statistically significant, and B cell and IgM restoration are impossible. The histology and flow cytometry seems to suggest that expression is mainly found in alpha smooth muscle positive cells, which could still be smooth muscle cells or myofibroblasts. Next therefore, the authors move to CRISPR knock down of BAIAP2L1 in a human smooth muscle cell line, and show that loss leads to less contraction of these cells in vitro in a microscopic FLECS assay, in which smooth muscle cells bind to elastomeric contractile surfaces.

Strengths:

1. There is a strong reduction in BHR in IgM-deficient mice, without alterations in B cell number, disconnected from effects on eosinophilia or Th2 cytokine production
2. BAIAP2L1 has never been linked to asthma in mice or humans

Weaknesses:

1. While the observations of reduced BHR in IgM deficient mice are strong, there is insufficient mechanistic underpinning on how loss of IgM could lead to reduced expression of BAIAP2L1. Since it is impossible to restore IgM levels by either serum or B cell transfer and since protein levels of BAIAP2L1 are not significantly reduced, there is a lack of a causal relationship that this is the explanation for the lack of BHR in IgM-deficient mice. The reader is unclear if there is a fundamental (maybe developmental) difference in non-hematopoietic cells in these IgM-deficient mice (which might have accumulated another genetic mutation over the years). In this regard, it would be important to know if littermates were newly generated, or historically bred along with the KO line.
2. There is no mention of the potential role of complement in activation of AHR, which might be altered in IgM-deficient mice
3. What is the contribution of elevated IgD in the phenotype of the IgM-deficient mice. It has been described by this group that IgD levels are clearly elevated
4. How can transfer of naïve serum in class switching deficient IgM KO mice lead to restoration of allergen specific IgE and IgG1?
5. Alpha smooth muscle antigen is also expressed by myofibroblasts. This is insufficiently worked out. The histology mentions "expression in cells in close contact with smooth muscle". This needs more detail since it is a very vague term. Is it in smooth muscle or in myofibroblasts.
6. Have polymorphisms in BAIAP2L1 ever been linked to human asthma?
7. IgM deficient patients are at increased risk for asthma. This paper suggests the opposite. So the translational potential is unclear.