

Epinephrine drives human M2a allergic macrophages to a regulatory phenotype reducing mast cell degranulation in vitro

To the Editor,

As the prevalence of allergies rises, the impact of social factors such as physiological stress has gained much attention. While stress is suggested to exacerbate allergic conditions, including asthma and atopic dermatitis, less is known about the effect of acute stress mediator epinephrine on allergic M2a macrophages in a Th2 environment. This study aimed to investigate whether human M2a macrophages express adrenergic receptors to respond to epinephrine and what effect epinephrine could exhibit on M2a macrophages in an in vitro Th2 environment. We further assessed whether epinephrine-treated M2a macrophages could affect IgE-mediated degranulation in human mast cells in vitro.

To study the effect of epinephrine on human M2a macrophages, we isolated monocytes from healthy donors and matured them in the presence of M-CSF according to a standard protocol¹ into monocyte-derived macrophages (M0). M0 were subsequently treated with IL-4 and IL-13 to differentiate them into M2a phenotype, which showed higher expression of CD206 marker and IL-10 production. Detailed information on this study is available in this article's online supplementary information. The presence of the β 2-adrenergic receptor (β 2-AR) was confirmed in the M2a subtype, but no expression of α 2A-AR, β 1-AR, and β 3-AR was detected (online repository; Figure S2D-E). The 16-hour treatment of M2a macrophages with 1 μ M epinephrine led to a significant upregulation of the cytokines IL-10 ($P = .0131$), TNF ($P = .0012$) and IL-6 ($P = .0001$), while no M1 marker IL-12 was detected (Figure 1A-D). This effect was not observed in the supernatants of M2a macrophages treated with the vehicle (negative control). Also, CD86 surface marker expression was significantly upregulated ($P = .0313$) (Figure 1G, Figure S3), indicating an antigen presentation capacity of this phenotype. Since epinephrine can induce cytokine production already after a few hours, we also observed the mRNA production of IL-10, IL-6, TNF, IL-1 β , and CCL1 after 2 hours. Other M2 markers, including CCL2, CCL22, CCL18, and TGF- β , were less affected, and expression of IFN- γ was not detected after epinephrine treatment (Figure 2A). The production of anti-inflammatory IL-10 cytokine alongside IL-6, TNF, and IL-1 β and upregulation of CD86 suggest that epinephrine can drive M2a macrophages towards an immunoregulatory M2b phenotype in vitro. Since the M2b phenotype is commonly induced by exposure to immune complexes and TLR ligands, which was not the case in

our study, and we did not observe CCL1 production in the supernatants of epinephrine-treated M2a macrophages,^{1,2} we termed this immunoregulatory phenotype "M2b-like." It is important to note that the immunoregulatory function of this phenotype was confirmed in vitro on human cord blood-derived mast cells (CBMCs), where treatment with supernatants from epinephrine-treated M2b-like macrophages significantly reduced the IgE-mediated β -hexosaminidase degranulation ($P = .0013$). Interestingly, this effect was significantly pronounced compared to treatment with epinephrine alone ($P \leq .05$) (Figure 2B).

To the best of our knowledge, this is the first report about the presence of the β 2-AR receptor on the M2a macrophage phenotype, which is an important player in allergy. We, however, acknowledge that our study has its limitations. Although μ M epinephrine in mouse cells can induce regulatory macrophages³ and dose-dependent studies of epinephrine on human monocytes revealed the strongest effect on chemokine/cytokine production in 1-10 μ M/L concentration range, often used to stimulate human monocytes in vitro,^{4,5} our results do not necessarily translate into real human setting. However, there is reason to believe that during stress the local epinephrine concentrations at the immunological synapse are higher than in circulation due to sympathetic neuronal discharge and local catecholamine production from neighbouring immune cells (previously termed "diffusely expressed adrenergic organ"⁶). Another limitation of results (Figure 2A) is the normalization against a single housekeeping gene. We acknowledge that under given conditions, using a second gene for normalization had been advisable. This was a study on epinephrine effect on in vitro Th2 inflammation. To translate these data and develop targeted therapies in the future, it would be important to obtain the information on the exact signalling pathway that epinephrine might have activated on M2a human macrophages and drive the M2b-like phenotype. Fc γ signalling known to induce the M2b phenotype by activating phosphoinositide 3-kinase (PI3K)², may be a possible pathway induced by epinephrine in our study; furthermore, catecholamine activation of a β 2-AR noncanonical pathway through phosphoinositide 3-kinase (PI3K) induced regulatory macrophages in mice.³

Even though M2b-like macrophages retain the ability to produce many pro-inflammatory cytokines including IL-6, TNF, and IL-1 β , the upregulation of IL-10 (IL-10^{high}/IL-12^{low}) is certainly a

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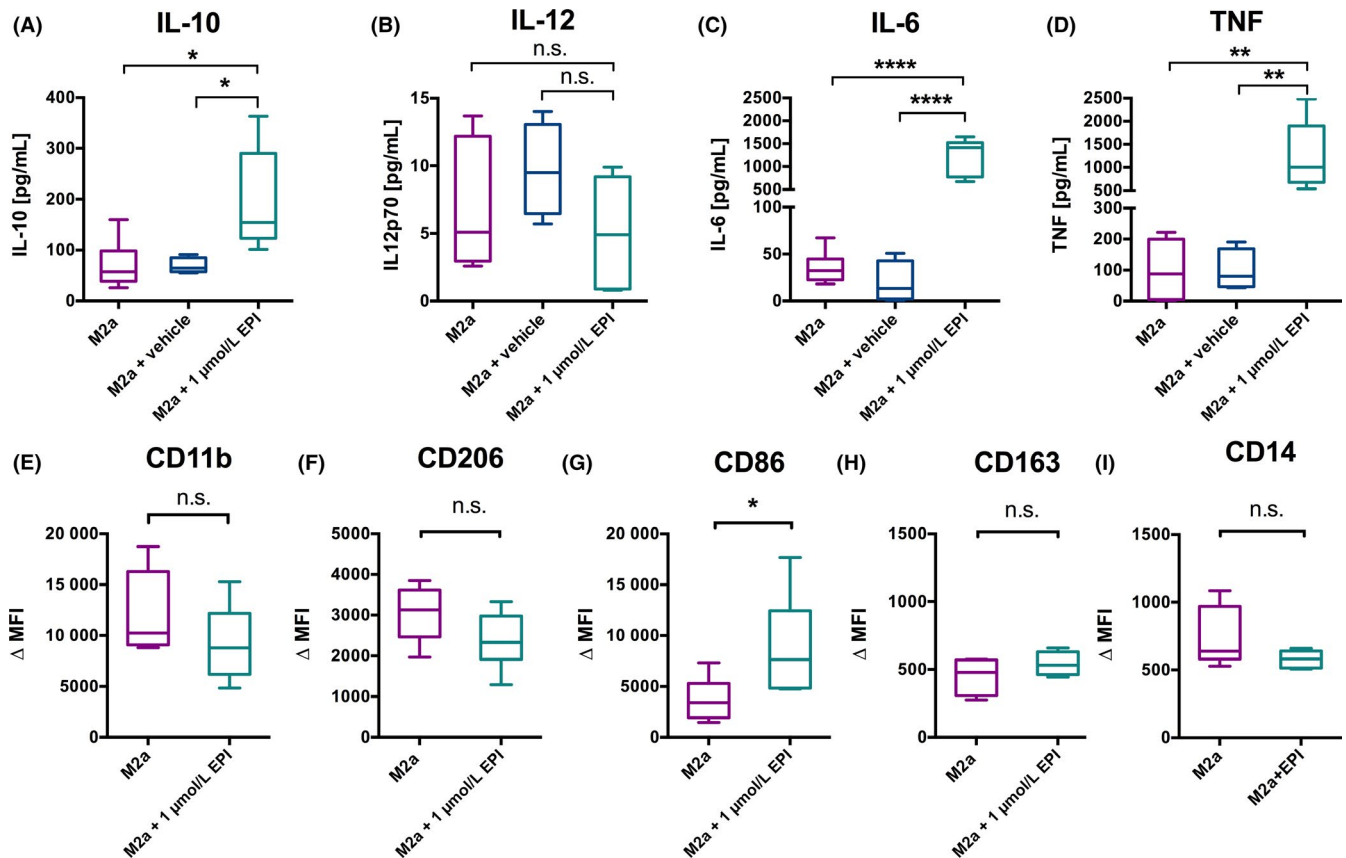


FIGURE 1 Epinephrine effect on M2a cytokine production assessed by ELISA and surface marker expression assessed by flow cytometry. M2a macrophages (purple) were incubated overnight (16 h) with 1 μmol/L epinephrine (EPI) (teal) or vehicle (blue). IL-10 (A), IL-12 p70 (B), IL-6 (C) and TNF (D) cytokines were assessed in supernatants (mean ± SD of six independent donors), and CD11b (E), CD206 (F), CD86 (G), CD163 (H) and CD14 (I) surface expression (mean ± SD of six independent donors) was assessed on M2a macrophages (purple) or EPI-treated M2a (teal). ΔMFI is calculated after isotype control MFI subtraction

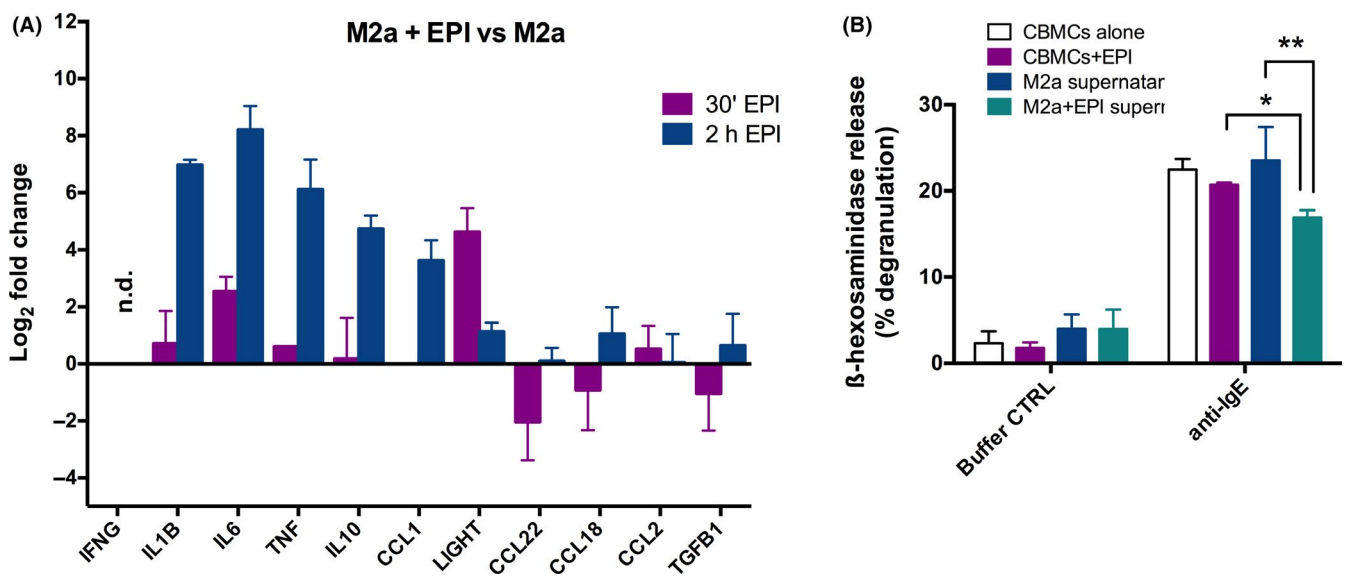


FIGURE 2 Transcriptional profiling of epinephrine-treated vs untreated M2a macrophage genes (A) and FcεRI-mediated β-hexosaminidase release in CBMCs. (A) Incubation with 1 μmol/L epinephrine (EPI) for 30 min (purple bars) and 2 h (blue bars) (mean ± SD of three independent donors). (B) FcεRI-mediated β-hexosaminidase release assessed in CBMCs after overnight incubation with supernatants from M2a macrophages (blue bars), epinephrine-treated M2a macrophages (teal bars) or 1 μmol/L epinephrine (purple bars) (two different CBMC batches and three different PBMC donors (n = 6))

central part of this phenotype and in the range reported in the previous studies.^{1,7} Future studies should address the involvement of IL-10, but also IL-6, TNF, and IL-1 β in the observed reduction of β -hexosaminidase production by CBMCs, as this was beyond the scope of this work. However, IL-10 could be a possible target, since it was shown to suppress the Fc ϵ RI signalling pathway and reduce histamine release in CBMCs⁸ or to directly affect the Fc ϵ RI expression and reduce degranulation in human skin mast cells.⁹ Although mast cells are known to express β 2-AR and can respond to epinephrine stimulation (control treatment; Figure 2B), the observed effect on degranulation of CBMCs with supernatants from M2b-like macrophages was significantly higher than the impact of epinephrine alone. Due to its short half-life and its instability under supernatant storage conditions (-20°C), epinephrine is not expected to be present in the supernatants of M2b-like macrophages.

In conclusion, the treatment of human allergic M2a macrophages with epinephrine led to a phenotypic switch to a macrophage subtype, which we term "M2b-like." In vitro data suggest that the M2b-like phenotype suppresses the IgE-dependent release of inflammatory mediators from mast cells. In allergic patients, acute stress may drive the plasticity of macrophages towards a regulatory M2b phenotype and reduce allergic symptoms, but further studies are needed to translate the results of this in vitro study into real life. However, as recently demonstrated in a clinical study in which the effects of acute stress on skin prick testing greatly varied among individuals,¹⁰ the net outcome of short-term acute stress in patients seems to be more complex and also depends on coping mechanisms. Together, our findings support further studies on the role of acute stress mediators in allergies.

KEYWORDS

allergy, beta2-adrenergic receptor, epinephrine, M2a macrophages, mast cell

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Online Repository Material

Supplementary results

MDMs purity

As reported in the previous study^{E1}, the purity of adherent monocyte-derived macrophages (CD3⁺CD11b⁺CD86⁺) after 72h in M-CSF-rich medium used in the experiments was $>85\pm5\%$ (**Fig. S1 a-f**). The majority of MDM contamination originates from CD3⁺ cells ($45-70\%^{E2}$), which are reported in our study to be 3% on average. Taking into consideration the frequency of total B cells within PBMCs of 5-15% (approx. 1/5 of CD3⁺ cells) and NK cells of 5-10% (approx. 1/7 of CD3⁺ cells^{E2}), we can assume that the total lymphocyte contamination in our study is $< 5\%$.

FcεRI expression on CBMCs

Enhancement of FcεRI expression on CBMCs cultured for 4 days in the presence of rhIL-4 and human myeloma IgE was confirmed by flow cytometry, as previously reported^{E3} (**Fig. S1 g**).

Phenotypic characterization of M2a macrophages

M2a macrophages produce IL-10 cytokine and show high expression of CD206 surface marker^{E4}. M2a phenotype further showed expression of CD11b pan-macrophage marker^{E5} as well as higher CD86 expression. We also observed lower expression of CD14 marker previously reported in M2a macrophages differentiated from MDMs cultured in XVivo medium^{E6} (**Fig. S2 a-c**).

β2-AR expression on M2a macrophages

M2a macrophages showed the expression of β2-AR both in RT-PCR and immunofluorescence staining (**Fig. S2 d-e**). We did not observe the mRNA expression of α2A-AR, β1-AR and β3-AR on human M2a macrophages (Fig. S2 d).

Materials and Methods

Human primary cells and antibodies

Human primary cells: MDMs: PBMCs were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, South Logan, UT) from ten healthy human donors (ICF obtained and the study approved by EC of the Medical University of Vienna (ECS2007/2016) and 2×10^6 cells/ml seeded into 6-well plates (Falcon, Corning, NY). Non-adherent cells were washed away after 2h with HBSS (GE Healthcare Life Sciences, South Logan, UT) and adherent cells (monocytes) kept in RPMI 1640 medium (Sigma-Aldrich, Saint Louis, MO) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Waltham, MA) and 1X P/S (Sigma-Aldrich, Saint Louis, MO) enriched with 20 ng/ml M-CSF (BioLegend, San Diego, CA) for 7-9 days to obtain M0. MDM cultures received a half medium change every 2-3 days^{E1}. **CBMCs:** Mononuclear cells from umbilical cord blood (ICF obtained from mothers and study approved by Research Ethics Board, IWK Health centre, Halifax, Nova Scotia) were cultured as previously reported^{E7} at 1×10^6 cells/ml and passaged once per week for 4 weeks in StemSpan SFEM medium (Stem Cell Technologies, Vancouver, CAN) supplemented with 10 ng/ml rhIL-3 (Peprotech, Rocky Hill, NJ) (present only for the first week of culture), 10 ng/ml rhIL-6 (Peprotech, Rocky Hill, NJ) and 100 ng/ml rhSCF (Peprotech, Rocky Hill, NJ) in 5% CO₂ at 37°C. At 5 weeks of culture, the medium was replaced by RPMI 1640 containing 10% FBS, 2 mM L-glutamine (GE Healthcare Life Sciences, South Logan, UT), 1X P/S, 50 μM 2-Mercaptoethanol (Sigma-Aldrich, Saint Louis, MO), 15 mM HEPES, 0.1 mM non-essential amino acids (GE Healthcare Life Sciences, South Logan, UT), 100 ng/mL rhSCF and 10 ng/mL rhIL-6. Mast cell purity (c-kit/CD117 expression at week 6) used in the experiments was $\geq 95\%$. CBMC cultures were devoid of dendritic cells and $>99\%$ positive for tryptase.

Antibodies: Following anti-human mAbs were purchased from BioLegend, San Diego, CA: CD3 (clone SK7, APC/CY7), CD11b (clone ICRF44, PE/CY7), CD86 (clone IT2.2, PE), CD163 (clone GHI/61, APC) CD206 (clone 15-2, APC/CY7). Anti-human mAb CD14 (clone 61D3, FITC) was purchased from Thermo Fisher Scientific, Waltham, MA. All respective mouse mAb ISO were purchased from BioLegend, San Diego, CA including, IgG1 (clone MOPC-21, APC/CY7), IgG1 (clone MOPC-21, FITC), IgG1 (clone MOPC-21, PE/CY7), IgG2b (clone MPC-11, PE) and IgG1 (clone MOPC-21, FITC), IgG1 (clone MOPC-21, APC). Anti-human mAb β 2-AR (clone R11E1) was purchased from Bio-Rad Laboratories, Hercules, CA, USA. Mouse IgG1 ISO mAb (clone MG1-45) and anti-mouse IgG mAb (clone RMG1-1) were purchased from BioLegend, San Diego, CA. Anti-human IgE Fc mAb (clone B3102E8, HRP) was purchased from Southern Biotech, Birmingham, AL, anti-human α FC ϵ RI mAb (clone AER-37, PE/CY7) from BioLegend, San Diego, CA and mouse IgG2b ISO mAb (clone eBMG2b, PE) used in CBMCs experiments from eBioscience, San Diego, CA.

Polarization of MDM toward the M2a macrophage phenotype and treatment with epinephrine

M0 were harvested using 2.5 mM EDTA (Sigma-Aldrich, Saint Louis, MO) in ice-cold PBS without Mg^{2+} and Ca^{2+} (GE Healthcare Life Sciences, South Logan, UT) and cultured in cRPMI with 20 ng/ml rhM-CSF, 20 ng/ml rhIL-4 and 20 ng/ml rhIL-13 (ImmunoTools, Friesoythe, Germany) for 72h to obtain M2a^{E1}. Cell images were acquired using a Primo Vert Microscope (Carl Zeiss, Germany) and the ToupCam camera system (ToupTek, Zhejiang, China). 72h-polarized M2a were stimulated overnight (16h) with 1 μ M EPI (Sigma-Aldrich, Saint Louis, MO) in TRIS buffer, pH 7.4 to assess cytokine production and surface marker expression and 48h-polarized M2a for 30 min or 2h to evaluate gene expression. Quantitative kinetic chromogenic LAL assay showed absence of bacterial endotoxin in EPI sample (<0.0210 EU/ml of LPS).

Cell staining and flow cytometry analysis

M2a and M2a $\pm$ EPI were detached using ice-cold 2.5 mM EDTA/PBS, washed twice with HBSS +3% FBS and incubated at 4°C for 30 min with multicolour mAb mixture (1:100 dilution) against CD14, CD11b, CD206, CD163, and CD86 or appropriate ISOs. For distinguishing dead cells from live cells, 2 drops of SYTOXTM Green Ready FlowTM Reagent (Thermo Fisher Scientific, Waltham, MA) were added directly on 1×10^6 cells, incubated for 15 min at RT and analyzed in flow cytometry. The cells were analyzed using dual laser FACSCanto II equipped with FACSDiva Software (Becton Dickinson, Franklin Lakes, NJ, USA) and geometric MFI was calculated for each fluorochrome.

Enzyme-linked immunoassay (ELISA)

The presence of IL-10 (detection range 2-300 pg/ml), IL-6 (2-200 pg/ml), TNF (4-500 pg/ml), IL-12(p70) (4-500 pg/ml), IFN- γ (Thermo Fisher Scientific, Waltham, MA) and CCL-1 (detection range: 3.9-1000 pg/ml; R&D Systems, Minneapolis, MN, USA) in the supernatants was assessed by ELISA kits according to manufacturer's instructions.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

48h-polarized M2a $\pm$ EPI 30 min/2h were harvested (150 000 cells) using ice-cold 2.5 mM PBS/EDTA, washed once with HBSS, pelleted and lysed in Trizol (Sigma-Aldrich, Saint Louis, MO). RNA was isolated, using Direct-zol RNA MiniPrep column system (Zymo Research, Irvine, CA) and 200 ng RNA transcribed using iScript cDNA Synthesis Kit (BioRad, Hercules, CA) according to manufacturer's instructions. RT-qPCR was performed using HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia). RT-PCR analysis was performed using duplicates (and technical replicates of each biological replicate). Primers used: GAPDH forward: TTTTGCGTCGCCAGCCGAG and reversed: ACCAGAGTTAAAAGCAGCCCTGG; TNF forward: CAGGCGGTGCTTGTTCTCTCA and reversed: GGCTTGTCACCTCGGGGTTTCG; IL6 forward: ATCCTCGACGGCATCTCAGC and reversed: TTCACCAGGCAAGTCTCCTCA; TGF β forward: GTTGTGCGGCAGTGTTGAG and reversed: AGTGAACCCGTTGATGTCCA; IL10^{E8} forward: GCCTAACATGCTTCGAGATC and reversed: TGATGTCTGGGTCTTGTTTC; CCL22 forward: TCCGTTACCGTCTGCCCTG and

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100 reversed: GGCACCTCTGGGATCGGCACA; IL1B^{E9} forward: CAGTGGCAATGAGGATGACTTG and
 101 reversed: AGTGGTGGTCGGAGATTCGT; CCL1 forward: TGTTGCTTCTCATTTGCGG and
 102 reversed: CTCTTTGCCTCTCTTCAGCTTG; LIGHT/TNFSF14 forward:
 103 CTCAGGCGTGTCTCTTCGGGTT and reversed: CACTCCTCATCCGTGGGTTGG; CCL18 forward:
 104 AAGCCCCAGCTCACTCTGACC and reversed: TGCCAGGAGGTATAGACGAGGC; CCL2^{E10}
 105 forward: CAGCCAGATGCAATCAATGCC and reversed: TGGAACTCCTGAACCCACTTCT; ADRB2
 106 forward: TAGAGATATGCAGAAGGAAGGGCAT and reversed:
 107 CCATGCAAAGAGGAAGTGAAGTGA and IFNG forward: TGGCTTTTCAGCTCTGCATCGT and
 108 reversed: TTTTCTGTCACTCTCCTCTTTCCA; ADRA2A forward: ACTGGACTACAAGGGCATGG
 109 and reversed: ACATCAAACCAAGGCCAAG; ADRB1 forward: GTGGCCCTACGCGAGCAGAAG
 110 and reversed: GCGTAGCCCAGCCAGTTGAAGA; ADRB3 forward: CCCAATACCGCCAACACCAGT
 111 and reversed: CGGCCAGCGAAGTCACGA. GAPDH was selected as a reference gene, as described
 112 elsewhere^{E11}. For transcriptional profiling of epinephrine-treated M2a macrophages for 30 min and 2h,
 113 $\Delta\Delta C_q$ levels were calculated after normalization with GAPDH and presented as a log2 fold change for
 114 M2a+epinephrine_{30min} treatment vs M2a and M2a+epinephrine_{2h} treatment vs M2a macrophages.

115 *Immunofluorescence staining of the β 2-AR*

116 1.5×10^4 M2a cells were left to adhere on 8-well chamber slide (μ -slide; Ibidi GmbH, Munich,
 117 Germany), washed twice with PBS and fixed using 4% paraformaldehyde (VWR International, Vienna,
 118 Austria) (30 min at RT). Cells were permeabilised with 0.2% Triton X-100 (Bio-Rad Laboratories,
 119 Hercules, CA) (2 min at RT), blocked with 10% FBS/PBS (30 min at RT) and incubated with a 1:25
 120 dilution of mouse anti-human β 2-AR 1°mAb (4 μ g/ml) or mouse IgG1 ISO (1h). Anti-mouse IgG was
 121 used as a 2°Ab (2.5 μ g/ml). DNA was visualized using DAPI (Bio-Rad Laboratories, Hercules, CA)
 122 (1:5000), slides mounted with fluoromount (Sigma-Aldrich, Saint Louis, MO) and analysed by
 123 fluorescence microscopy using Axio Observer Z1 inverted microscope with an AxioCam MRc5
 124 camera and AxioVision software (Carl Zeiss Meditec, Jena, Germany). Relative quantification of β 2-
 125 AR fluorescence intensity was performed using ImageJ software (version 1.52 e; National Institutes of
 126 Health, US).

127 *Stimulation of CBMC with supernatants from M2a-treated macrophages*

128 Two CBMC batches (1×10^6 cells/ml) were pre-sensitized for 72h in complete RPMI with 2.5 μ g/ml
 129 human myeloma IgE and 10 ng/ml rhIL-4 to upregulate Fc ϵ RI expression^{E3}. Half of the medium was
 130 supplemented with 1 μ M EPI or supernatants from M2a and EPI-treated M2a macrophages (n=3
 131 PBMC donors) for 24h. β -hexosaminidase release assay was carried out by previously reported
 132 method^{E12}.

$$\% \text{ release} = \frac{(O.D. \text{supernatant} - O.D. \text{HTB control})}{(O.D. \text{supernatant} - O.D. \text{HTE control}) + (O.D. \text{pellet} - O.D. \text{HTE control})} \times 100$$

133 *Statistical analysis*

134 The treatments M2a+EPI vs M2a and stimulation of CBMCs with macrophage supernatants were
 135 assessed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test.
 136 Differences between groups were considered significant at P value <0.05. Statistical analyses were
 137 performed with GraphPad Prism 6.0 for Macintosh (GraphPad Software, Inc., La Jolla, CA).

138 **List of abbreviations**

BSA	bovine serum albumin
CBMCs	cord blood-derived mast cells
cRPMI	complete RPMI
DAPI	4',6-diamidino-2-phenylindole
EDTA	ethylenediaminetetraacetic acid disodium salt dihydrate

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EPI	epinephrine	139	
FBS	fetal bovine serum	140	
FSC-A	forward scatter area	141	
FSC-H	forward scatter height	142	
HBSS	Hank's balanced salt solution	143	
ICF	informed consent form	144	
IL	interleukin	145	
ISO	isotype control	146	
mAb	monoclonal antibody	147	
M-CSF	macrophage colony-stimulating factor	148	
MDM	monocyte-derived macrophages	149	
MFI	mean fluorescence intensity	150	
PBMCs	peripheral blood mononuclear cells	151	
PBS	phosphate buffered saline	152	
rh	recombinant human	153	
P/S	penicillin-streptomycin	154	
RT	room temperature	155	
SCF	stem cell factor		References
SSC-H	side scatter height		
SSC-W	side scatter width		
TRIS	tris(hydroxymethyl)aminomethane		E1.
β2-AR	β2-adrenergic receptor		Bianchini

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Supplementary figure legend

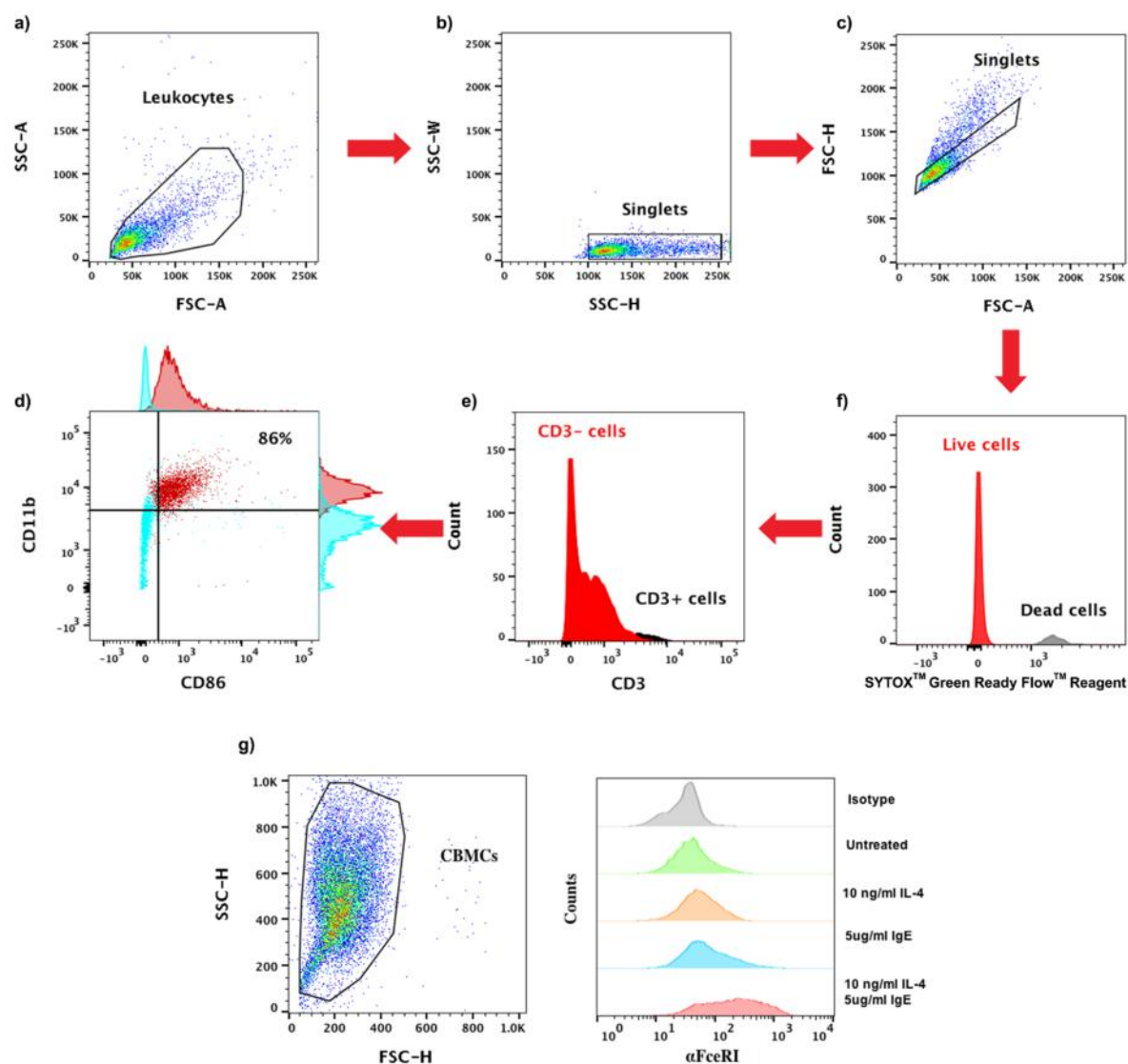


Figure S1. Flow cytometry analysis representing MDM purity (a-f) and FcεRI expression on CBMCs (g). Assessment of adherent monocyte-derived macrophages after 72h in M-CSF differentiation medium by flow cytometry by gating leukocytes (a), single cells SSC-W and SSC-H (b) and FSC-H and FSC-A (c). SYTOX™ Green Ready Flow™ Reagent was used to gate for live cells (red label) (d). The percentage of live cells was 87±2%. Live cells were gated using anti-CD3 (e) and CD3+ cells (red, 98%) were further analysed using anti-CD11b and anti-CD86 (f). Isotype control is represented in cyan. CBMCs were cultured for 4 days in the presence of rhIL-4 and human myeloma IgE and stained with monoclonal antibody against FcεRI before or after stimulation with rhIL-4 myeloma IgE or rhIL-4 and IgE (g).

Epinephrine drives human M2a allergic macrophages to a regulatory phenotype reducing mast cell degranulation in vitro

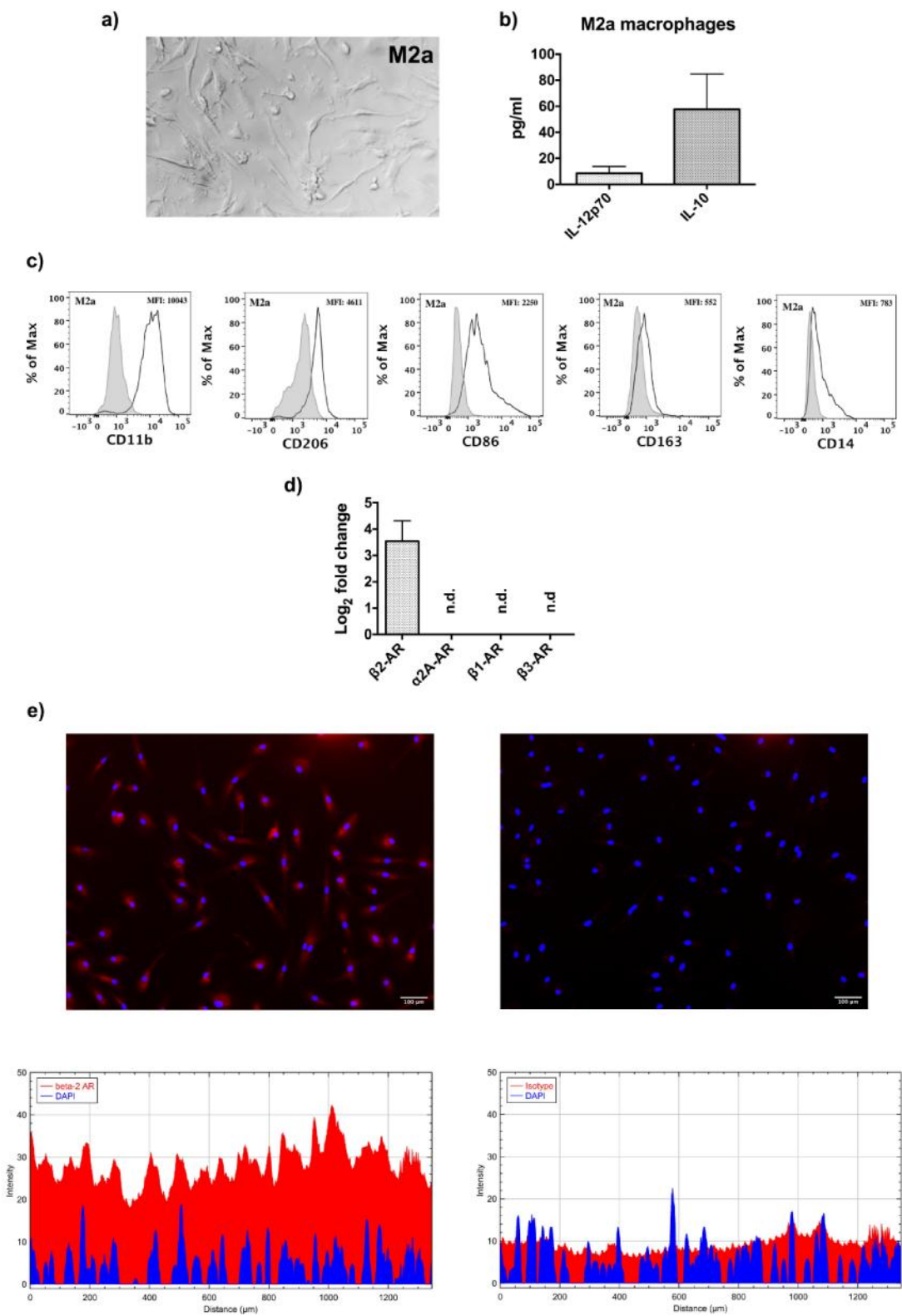
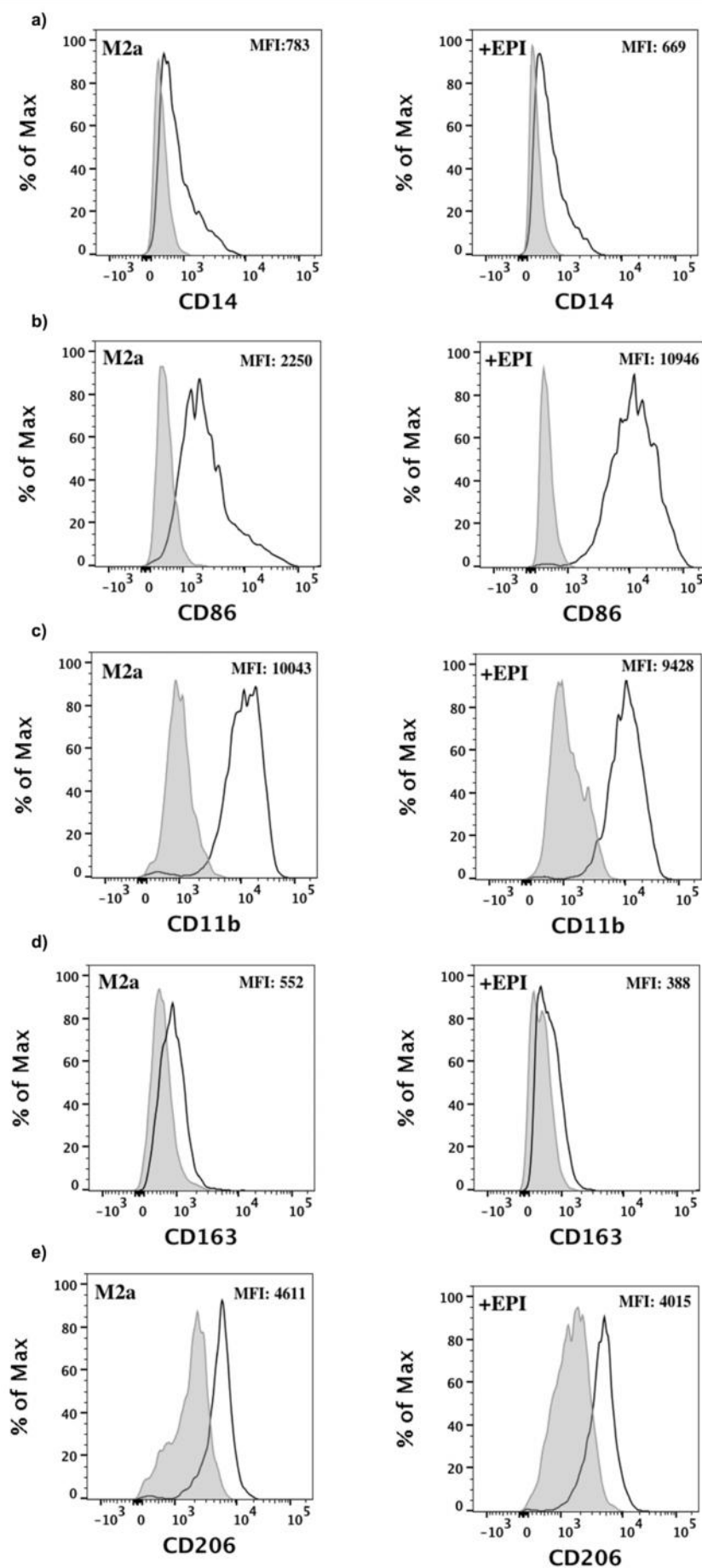


Figure S2. Phenotypic characterization of M2a macrophages and the β 2-adrenergic receptor expression. M2a macrophages were obtained after treatment with rhM-CSF for 9 days and further stimulation with rhIL-4 and rhIL-13 for 72h and characterized phenotypically in light microscopy (a). IL-12 and IL-10 cytokine production was assessed by ELISA (b) and surface marker expression in flow cytometry (c) (representative image of n=6 donors). The shaded gray histogram represents the relevant isotype control antibodies. RT-PCR revealed the presence of β 2-AR mRNA (mean $\pm$ SD, n=4 normalized to Caco-2 cell line used as a negative control) (d) and β 2-AR protein expression in M2a macrophages detected by immunofluorescence (representative confocal microscopy image (n=3) (e). β 2-AR expression was quantified using ImageJ software. The red peaks represent fluorescence intensity of M2a macrophages stained with the β 2-AR antibody or respective isotype control.

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242 **Figure S3.** Flow cytometry analysis of surface marker expression in the presence and absence of
243 epinephrine. M2a macrophages were incubated overnight (16h) with 1 μ M epinephrine (EPI) and
244 surface marker expression including CD11b (a), CD206 (b), CD86 (c), CD163 (d), and CD14 (e)
245 (representative image of six independent donors) was assessed on M2a macrophages or EPI-treated
246 M2a. The shaded gray histogram represents the relevant isotype control antibodies.