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Research article

Sex-dependent effect of aging on calcium signaling and expression of TRPM2 and CRAC channels in human neutrophils

María de los Ángeles Vázquez-Prieto [a], Nallely Lascurais-Santamaría [a], Nilda Belén Fernández-Eufrasio [a], Dvorak Montiel-Condado [b], Adriana Garibay-Escobar [c], Genaro Patiño-López [d], Reinhold Penner [e], Adriana Sumoza-Toledo [a,*]

[a] Instituto de Investigaciones Médico Biológicas, Universidad Veracruzana, Agustín de Iturbide S/N, 91700 Veracruz, Ver., México
[b] Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Av. Universidad, Cd. Universitaria, 66455, San Nicolás de los Garza, Nuevo León, México
[c] Departamento de Ciencias Químico Biológicas, Universidad de Sonora, Rosales y L. Encinas, 83000, Hermosillo, Sonora, México
[d] Laboratorio de Investigación en Inmunología y Proteómica, Hospital Infantil de México Federico Gómez, Calle Doctor Márquez 162, 06720, Ciudad de México, México
[e] The Queen's Medical Center & John A. Burns School of Medicine & University of Hawaii Cancer Center. 1356 Lusitana St., Honolulu, HI 96813, USA

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ABSTRACT

The vulnerability of older adults to bacterial infections has been associated with age-related changes in neutrophils. We analyzed the consequences of aging on calcium (Ca^{2+}) mobilization and TRPM2 and CRAC channels expression in human neutrophils. The percentages of granulocytes, mature neutrophils, and neutrophil precursors were equivalent between young and older adults. However, neutrophil chemotaxis towards IL-8, C5a, or fMLP was lower in older adults of both sexes. Interestingly, a stronger Ca^{2+} transient followed by an identical Ca^{2+} influx to IL-8 was observed in older adult females. In addition, the Ca^{2+} response to LPS was delayed and prolonged in neutrophils of older adult males. There was no significant difference in Ca^{2+} response to fMLP, C5a, or store-operated Ca^{2+} entry in the older adults. There were also no differences in the expression of CXCR2, CD88, FPLR1, and TLR4. Interestingly, TRPM2- and *ORAI1*-mRNA expression was lower in neutrophils of older adults, mainly in females. Both channels were detected intracellularly in the neutrophils. TRPM2 was in late endosomes in young adults and in lysosomes in older adult neutrophils. In summary, defective neutrophil chemotaxis in aging seemed not to stem from alterations in Ca^{2+} signals; nevertheless, the low TRPM2 and ORAI1 expression may affect other functions.

1. Introduction

Neutrophils are the most abundant and shortest-lived leukocytes in circulation, known as the first line of defense against bacterial and fungal infections [1]. They migrate to sites invaded by a pathogen following inflammatory signals recognized by chemokine receptors (e.g., CXCR1 and CXCR2), formyl peptide receptors (FPR), complement receptors (e.g., C5aR), and other receptors, expressed on their surface [1,2]. Neutrophil direct migration to sites of inflammation, phagocytosis, and secretion of antimicrobial agents rely on precise Ca^{2+} signaling [1,3]. Increased cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in neutrophil is caused by a rapid release of Ca^{2+} from intracellular

Abbreviations: BD FACS, Beckton Dickinson fluorescence-activated single cell sorting; Ca^{2+}, Calcium; $[Ca^{2+}]_i$, Intracellular free calcium concentration; C5a, Complement fragment 5a; CD11b APC, Cluster of differentiation 11b Allophycocyanin; CD15 PE-CF594, Cluster of differentiation 15 Phycoerythrin-CF594; CD16 FITC, Cluster of differentiation 16 Fluorescein IsoTioCyanate; CD284(TLR4), Cluster of differentiation 284; CD32, Cluster of differentiation 32; CD45 PerCP, Cluster of differentiation 45 Peridinin-Chlorophyll-protein; CD88(C5aR), Cluster of differentiation 88; CRAC, Ca^{2+} release-activated Ca^{2+} channel; CTCF, Corrected total cell fluorescence; CXCR1, CXC chemokine receptor 1; CXCR2, CXC chemokine receptor 2; EDTA, Ethylene diamine tetra-acetic acid; ER, Endoplasmic reticulum; F, Fluorescence; F0, Basal fluorescence; Fmax, Maximum fluorescence; fMLP, N-formyl-methionyl-leucyl-phenylalanine; FPLR1, Formyl peptide receptor like 1; FPR, Formyl peptide receptors; FPRL1 PE, Formyl peptide receptor like 1 Phycoerythrin; HBSS, Hanks' Balanced Salt Solution; IIMB, Instituto de Investigaciones Médico Biológicas; IL-8, Interleukin-8; IP3, Inositol 1,4,5-trisphosphate; LPS, Lipopolysaccharide; NET, Neutrophil extracellular traps; ORAI1, ORAI calcium release-activated calcium modulator 1; PBS, Phosphate buffer solution; PLCβ, Phospholipase Cβ; qRT-PCR, Quantitative real time-polymerase chain reaction; RNA, Ribonucleic acid; ROS, Reactive oxygen species; RT, Room temperature; SD, Standard deviation; SEM, Standard error of the mean; SERCA, Sarco-ER Ca^{2+}-ATPase; SOCE, Store-operated Ca^{2+} entry; STIM1, Stromal interaction molecule 1; TRPM2, Transient Receptor Potential Melastatin-2 channel.

* Corresponding author.
E-mail address: asumoza@uv.mx (A. Sumoza-Toledo).

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stores via inositol 1,4,5-trisphosphate (IP3) receptors in the endoplasmic reticulum (ER), followed by influx of extracellular Ca^{2+} via plasma membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels [3,4]. ORAI1 is the predominant CRAC channel in neutrophils and is essential for store-operated Ca^{2+} entry (SOCE) [3]. Other ion channels, such as Transient Receptor Potential Melastatin channel member 2 (TRPM2), also permeate extracellular Ca^{2+} in murine neutrophils and regulate their migration [5–7].

Aging of the immune system increases morbidity and mortality of individuals due to infectious diseases [8]. Aging is also associated with developing of a low-grade chronic inflammation termed "inflammaging" [1,8]. Neutrophils in older individuals show reduced bactericidal activity, phagocytosis and reactive oxygen species (ROS) production [9,10]. Moreover, directional chemotactic migration and NET formation are impaired [9,10]. In aged mice, intratracheally infected with *Pseudomonas aeruginosa,* it was observed that fewer neutrophils migrated to the lungs compared to infected young mice, despite having a higher level of CXCL-1 chemokine; increasing their risk of pulmonary infections and recurrences [11,12]. Neutrophils are also thought to contribute to "inflammaging" [1]. Excessive secretion of neutrophil-derived granule proteins and cytokines also contributes to tissue damage and the development of many chronic inflammatory disorders [1,13,14]. However, the molecular bases leading to defective neutrophil functions and "inflammaging" in aging remain poorly understood [8]. In addition, it has been well established that there are differences in the immune response based on sex. Innate immune disorders, such as sepsis, and post-surgery infections display a higher incidence and severity in males [15]. Here, we analyzed the impact of aging in human neutrophils of both sexes, on their calcium response to inflammatory stimuli, and CRAC and TRPM2 expression.

2. Material and methods

2.1. Participants

Twenty-three young adults (20–35 years old) and twenty older adults (60–85 years old) were randomly recruited for this study, by invitation through posters published at the Instituto de Investigaciones Médico Biológicas (IIMB) of the Universidad Veracruzana (UV), Veracruz, Ver., México. The study was approved by the Ethics Committees and Research Committees of the Instituto de Ciencias de la Salud (No. 007/2018) and IIMB (No. IIMB-016–018), of the UV. Participants provided written informed consent, completed a questionnaire and underwent medical and physical examination by a specialist at the IIMB. None of the participants had clinical evidence of infections, chronic inflammation, arteriosclerosis, diabetes Mellitus, malignancy, autoimmune disease, or immunodeficiency. Controlled blood pressure, use of contraceptive pills, supplements, antidepressants, or non-daily taking anti-allergic drugs were not considered exclusion criteria. Young adults and older adults were paired by sex in each experiment, which were performed in accordance with approved institutional and national guidelines for research involving human subjects and with the Helsinki declaration. The demographic data of the participants are described in Table 1.

Table 1
Study cohort characteristics.

	Young adult	Older adult
Recruited	23	20
Mean age	25	65.3
Age SD	3.46	5.01
Excluded	none	none
Sex (Male/Female)	12 (52.1%)/11 (47.8%)	9 (45%)/11 (55%)
Comorbidities		
High blood pressure	0 (0%)	3 (15%)
Anxiety	0 (0%)	1 (5%)
Allergy/asthma	1 (4.3%)	2 (10%)
Gonarthrosis	0 (0%)	1 (5%)
Medications		
Losartan/felodipine	0 (0%)	2 (10%)
Escitalopram	0 (0%)	1 (5%)
Anti-histamine/montelukast	0 (0%)	2 (10%)
Glucosamine/clonixin of lysine	0 (0%)	1 (5%)
Ciranizina	0 (0%)	1 (5%)
Miscellaneous (supplements, vitamins, teas)	0 (0%)	4 (20%)

2.2. Granulocyte and neutrophil population analysis by flow cytometry.

50 μl EDTA blood samples were treated with 10 μl purified mouse anti-human CD32 (BioLegend, Cat. #303202) diluted 1:100 in PBS for 10 min at room temperature (RT) to block FcγRs. Granulocytes were identified by cell size and complexity. Neutrophils were detected with mouse anti-human CD16 FITC (BD Pharmigen, Cat. #555406), and CD15 PE-CF594 (BD Horizon, Cat. #562372) antibodies [16]. For neutrophil precursors, samples were stained with mouse anti-human CD45 PerCP (BD Biosciences, Cat. #347464), CD16 FITC (BD Pharmigen), and CD11b APC (BioLegend, Cat. #301310) antibodies [17]. In addition, these samples were stained with the following antibodies: anti-human FPRL1 PE (R&D Systems, Cat. #FAB3479P), CD182 (CXCR2) Alexa fluor 647 (Biolegend, Cat. # 320714), CD88 (C5aR)-biotin (Miltenyi Biotec, Cat.# 130–104-332) or mouse anti-human CD284 (TLR4)-biotin (BioLegend, Cat. # 312804) followed by APC-conjugated streptavidin (eBioscience, Cat. #17–4317-82). Samples were incubated for 15 min at RT. Red cells were eliminated with 800 μl of Lysing Solution 10X Concentrate (BD FACS, Cat. #349202), diluted 1:10 with RT deionized water, for 10 min at RT, rinsed and resuspended in 600 μl PBS. Finally, 30,000 events were recorded using the BD Accuri C6 flow cytometer and BD Accuri C6 Software version 1.0.264.21. Data were analyzed with FlowJo software version X.0.7 (Tree Star, Inc., Ashland, OR, USA).

2.3. Neutrophil purification

Neutrophils were purified from 10 ml of heparinized venous blood sample by a gradient of two Ficoll-Hystopaque densities (1119 and 1077; Sigma Aldrich) according to the manufacturer's instructions. Neutrophil purity was 95–98% by flow cytometry. Cells were maintained in Hank's Balanced Salt Solution containing 1.26 mM Ca^{2+} (1.26 mM Ca^{2+}-HBSS; Hyclone).

2.4. Neutrophil chemotaxis assay

96-well MultiScreen chemotaxis plates (Merck) were sensitized with 10 μg/ml bovine collagen type 1 (Corning, Cat. #354231) for 3 h at RT and rinsed three times with PBS before its use [18,19]. Chemoattractants diluted in 150 μl 1.26 mM Ca^{2+}-HBSS [10 nM IL-8 (R&D System, Cat. #208-IL/CF), C5a (R&D System, Cat. #2037-C5-025), or fMLP (Sigma-Aldrich, Cat. #F3506)] were placed, by triplicate, in the lower chamber, whereas 100,000 purified neutrophils in 50 μl Ca^{2+}-HBSS to the upper chamber. 1.26 mM Ca^{2+}-HBSS was used as a negative control. After 2 h incubation at 37 °C, the transmigrated cells were collected from the lower chamber, fixed with 2% paraformaldehyde, and counted by flow cytometry. For this, 20,000 of 20 μM size fluorescence beads (Polysciences, Inc., Warrington, PA) were added to each sample and counted along with the cells. The total number of transmigrated cells equals the number of counted neutrophils × the total number of beads/the number of beads counted [20]. Results are expressed as mean ± SEM of the chemotaxis index (CI) of triplicate wells. The CI represents the fold-change in the number of migrated cells responding to the chemoattractant divided by the spontaneous migration in the control (HBSS). Migrated cells were

counted in BD Accuri C6 flow cytometer and BD Accuri C6 Software version 1.0.264.21. Data were analyzed with FlowJo software version X.0.7 (Tree Star, Inc., Ashland, OR, USA), and GraphPad Prism version 6.0 (San Diego, CA, USA).

2.5. Calcium measurements by flow cytometry

Flow-cytometric calcium measurements were performed using BD Accuri™ C6 flow cytometer, as described by Vines A, et al., [21]. Briefly, 1×10^6 purified neutrophils/mL 1.26 mM Ca^{2+}-HBSS were loaded with 1 μM Fluo-4-acetoxymethyl ester (Fluo-4 AM; Invitrogen, Cat. #F14201) for 20 min at 37 °C. The final concentration of DMSO, the Fluo-4 AM diluent, was 0.05%. Next, the cell pellet was rinsed twice with 500 μl 1.26 mM Ca^{2+}-HBSS, and resuspended in 2 ml of the same solution (extracellular Ca^{2+} conditions) or zero Ca^{2+}-HBSS (0 Ca^{2+}-HBSS; Ca^{2+}-free conditions cells). Lastly, 3×10^5 cells (600 μl) were transferred to 1.5 ml Eppendorf tubes. The mean fluorescence of basal cytosolic Ca^{2+} was recorded for 60 s before the addition of the stimulus: 300 nM IL-8, 10 nM C5a, 100 nM or 1 μM fMLP, 25 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich, Cat. #L4391), or 2 μM thapsigargin (Invitrogen, Cat. #T7459), diluted in 1.26 mM Ca^{2+}- or 0 Ca^{2+}-HBSS, using a flexible long gel loading tip placed in the tube before the recording. Changes in cytosolic Ca^{2+} were monitored for up to 10 min by measuring the mean fluorescence emission of Fluo-4 AM on the FL-1 (494/506 nm) channel. Ca^{2+} signal patterns were defined as follows: Ca^{2+} release was defined as the transient increase in mean fluorescence of cytosolic Ca^{2+} in Ca^{2+}-free conditions; Ca^{2+} entry was defined as the slower rising wave-like phase present in 1.26 mM Ca^{2+}-HBSS and absent in 0 Ca^{2+}-HBSS. Data were analyzed with FlowJo software version X.0.7 using its kinetic function (Tree Star, Inc., Ashland, OR, USA). Ca^{2+} response was then normalized by converting the data to the F/F0 ratio, where F is fluorescence and F0 is the resting fluorescence value. F0 was defined as the average of the fluorescence in the first 60 s before the stimulus application. Therefore, F/F0 reflects changes in intracellular calcium. For statistical analysis, the AUC of the calcium transient and the maximum response time were calculated using the multipeak fitting 2 and curve fitting functions of Igor64 program version 8.04 (WaveMetrics).

2.6. Immunofluorescence

50,000 neutrophils in 100 μl 1.26 mM Ca^{2+} HBSS were attached to poly-L-Lysine pretreated-coverslips for 30 min at 37 °C. Cells were then fixed with 2% paraformaldehyde for 15 min at RT. Next, samples were permeabilized with 0.5% Triton X-100 for 5 min, rinsed with PBS, and blocked with 2% donkey serum for 90 min at RT. For TRPM2 subcellular detection, cells were incubated with 3.3 μg/ml rabbit anti-TRPM2 polyclonal antibody (Bethyl Laboratories, Cat. # A300-413A) or purified rabbit IgG antibody as a negative control, in combination with 20 μg/ml mouse anti-Rab5 (Sigma-Aldrich, Cat. #7904) or mouse anti-Rab7 monoclonal antibody (Sigma-Aldrich, Cat. #R8799), or 0.8 μg/ml purified mouse anti-human Lamp-1 (BD Transduction Laboratories, Cat. #611042) antibody, to detect early endosomes, late endosomes, and lysosomes, respectively, for 1 h at RT. For ORAI1, cells were incubated with a rabbit anti-ORAI-1 (NT) antibody (ProSci, Cat. # 4041–1003). Alexa Fluor 488 goat anti-rabbit (H + L) IgG (Invitrogen; Cat. #A11034) and Alexa Fluor 568 anti-mouse (H + L) IgG (Invitrogen, Cat. #A11031) were used as secondary antibodies for anti-channels' and anti-organelles' antibodies, respectively. Samples were incubated for 40 min at RT, rinsed with PBS, and mounted using ProLong Gold antifade reagent with DAPI (Invitrogen, Cat. #p36935). Images were acquired using a Leica TCS SP8x Confocal Microscope (Wetzlar, Germany) and Leica Microsystems LAS X software version 2.6.0 (Leica, Hudson, OH, USA). Image analysis was done with Image J (version 1.52 K; NIH) and GraphPad Prism version 6.0c (San Diego, CA, USA).

2.7. Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted from purified neutrophils using TRIzol LS reagent (Invitrogen, Cat. #10296010) according to manufacturer's protocol. First strand cDNA was prepared from 500 ng RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Cat. #205311). For qRT-PCR, 10 μl reactions were performed with 50 nM cDNA, 400 nM TRPM2 (68 bp; 5′-ACCTCCTCATCGCCATGTT-3′, 5′-CTTCCA AATCTGGTCCGTGT-3) [22] or 300 nM *ORAI1* (190 bp; 5′-ATGAGCCT CAACGAGCACT-3′, 5′-GTGGGTAGTCGTGGTCAG-3′) [23], and 300 nM β-actin (218 bp; 5′-CAGAGCAAGAGAGGCATCCT-3′, 5′-ACGTACATGGCTGGGGTG-3′) specific primers, using qPCR SYBR Master Hi-ROX (Green Jenna Bioscience, Cat. #PCR-374S). Amplification setting was: 95 °C 2 min; 45 cycles: 95 °C 15 s, 58 °C 45 s, 72 °C 30 s; 1 cycle: 95 °C 1 min, 55 °C 30 s, 95 °C 30 s. We compared the amount of target normalized to the endogenous reference using the $2^{-\Delta\Delta CT}$ method (CT = threshold cycle) for relative quantification. Data were acquired using the Applied Biosystems StepOne Real-Time PCR system and StepOne software version 2.2.2 (Life technologies). Analysis was performed using the instrument software, Microsoft Excel version 16.16.24, and GraphPad Prism version 6.0c (San Diego, CA, USA).

2.8. Statistical analysis

Data are represented as mean ± SEM values. Statistic difference was determined by unpaired two-sided Student's *t*-test and Welch's correction using GraphPad Prism version 6.0 (San Diego, CA, USA). For all analyses, $p < 0.05$ was considered to be statistically significant.

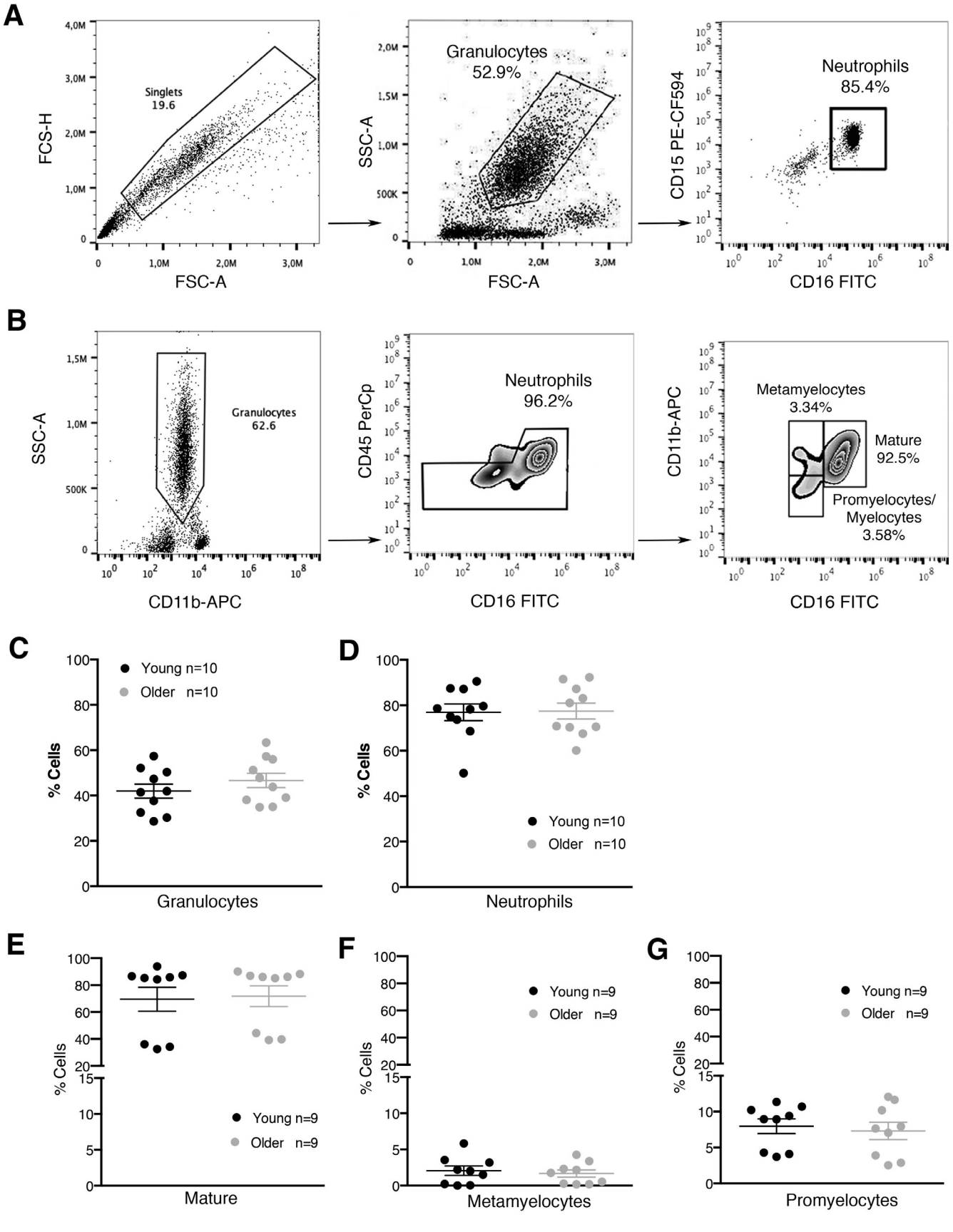
3. Results

3.1. Aging does not affect the proportion of circulating neutrophils and their precursors.

Characteristics of the study cohort are presented in Table 1. We assessed the percentage of granulocytes and neutrophils, and neutrophil subpopulations, in the whole blood of the participants by flow cytometry. Cellular aggregates and debris were excluded from single cells, and then granulocytes were identified by size and complexity. Last, neutrophils were differentiated based on CD15 and CD16 expression (Fig. 1A) [16]. There were no significant differences in the percentage of granulocytes (Fig. 1C) and neutrophils (Fig. 1D) between young and older adults. For circulating neutrophil subpopulations, granulocytes were identified by CD11b expression and complexity, and neutrophils were then selected based on CD45 and CD16 expression. Finally, mature neutrophils ($CD16^+/CD11b^+$), metamyelocytes ($CD16^-/CD11^+$), promyelocytes and myelocytes ($CD16^-/CD11b^-$), were distinguished according to the expression of CD11b and CD16 (Fig. 1B) [17]. No significant differences in the percentage of precursors were found between young and older adults (Fig. 1E-G).

3.2. Neutrophil chemotaxis to inflammatory stimuli decreases with aging

A decrease in neutrophil chemotaxis has been reported with aging; therefore, we examined whether this was evident in our population. Freshly purified neutrophils from young and older adults were used to test chemotaxis towards 10 nM IL-8, C5a, and fMLP, as described previously. Fig. 2 A-C shows that neutrophils from older adults migrated much less towards IL-8 ($p = 0.02$), C5a ($p = 0.04$), and fMLP ($p = 0.06$) in comparison to their counterparts in young adults. This migration was not because of differences in the expression of CXCR2, CD88, and FPLR1 between neutrophils of young and older adults (Fig. 2 D-F).


A
Singlets
19.6
FCS-H
FSC-A
Granulocytes
52.9%
SSC-A
FSC-A
Neutrophils
85.4%
CD15 PE-CF594
CD16 FITC
B
Granulocytes
62.6
SSC-A
CD11b-APC
Neutrophils
96.2%
CD45 PerCp
CD16 FITC
Metamyelocytes
3.34%
Mature
92.5%
Promyelocytes/
Myelocytes
3.58%
CD11b-APC
CD16 FITC
C
Young n=10
Older n=10
% Cells
Granulocytes
D
Young n=10
Older n=10
% Cells
Neutrophils
E
Young n=9
Older n=9
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Mature
F
Young n=9
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Promyelocytes

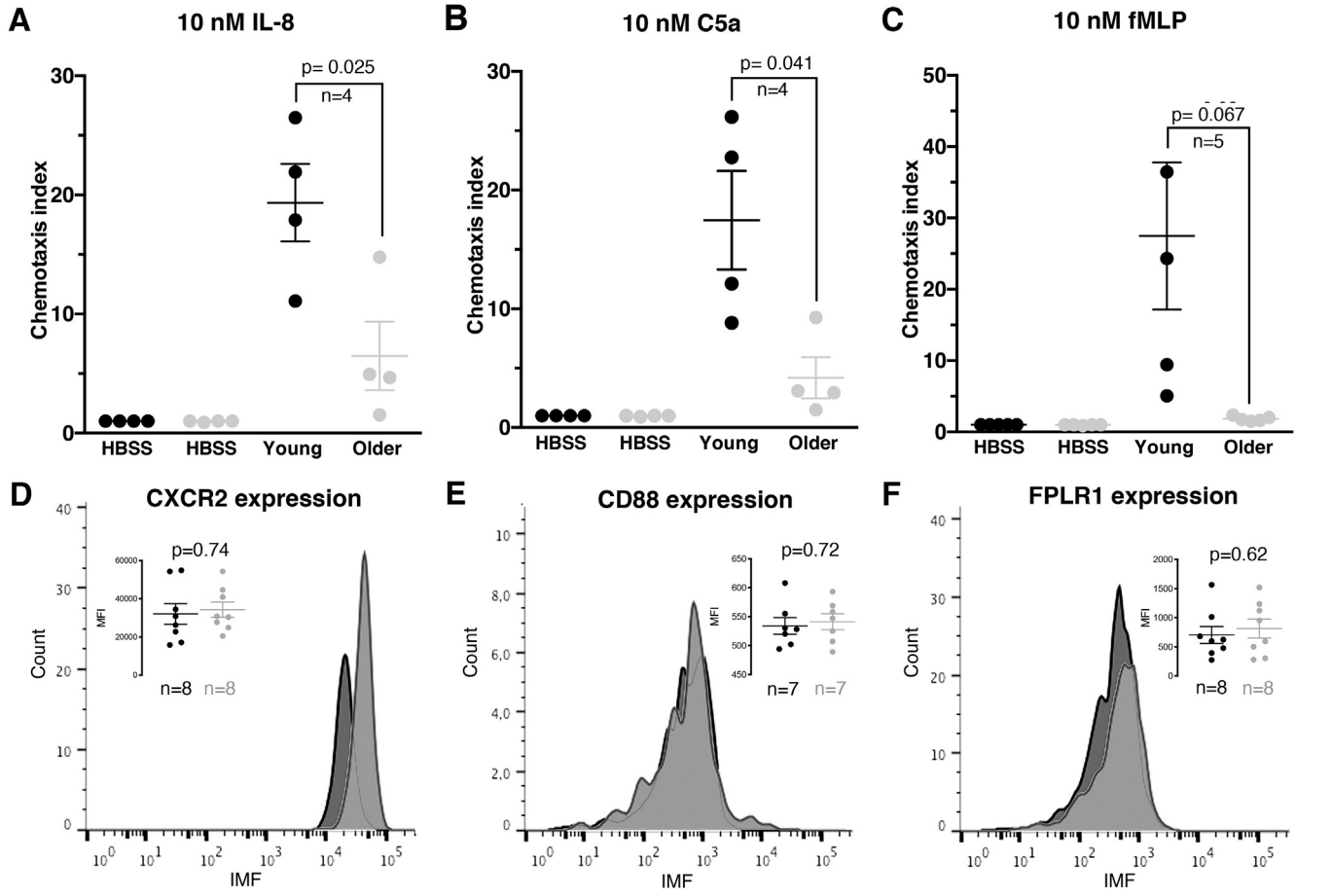


Fig. 2. Neutrophil chemotaxis to inflammatory stimuli decreases with aging. Neutrophils from young and older adults were analyzed in transwell chemotaxis assays using A) 10 nM IL-8, B) 10 nM C5a, or C) 10 nM fMLP, by triplicate per experimental condition. Transmigrated neutrophils were collected from the lower chamber after 3 h at 37 °C, fixed, and enumerated by flow cytometry. Results are expressed as the mean ± SEM of the chemotaxis index (CI) of 4–5 independent experiments. Expression of the chemoattractant receptors was examined by flow cytometry: D) CXCR2, E) CD88, and F) FPLR1, n represents the number of independent experiments made by duplicate. Statistical analysis was performed using unpaired two-sided Student's *t*-test and Welch's correction, differences were considered significant when p values were ≤ 0.05.

3.3. Ca^{2+} signals are altered in females' neutrophils with aging

Since neutrophil migration depends on Ca^{2+} signaling [3], we investigated whether chemotactic stimulus-induced Ca^{2+} mobilization is affected by aging. Fig. 3A shows that 300 nM IL-8 induced a rapid transient increase in cytosolic Ca^{2+} levels followed by Ca^{2+} entry in human neutrophils (black trace). The Ca^{2+} transient corresponded to Ca^{2+} release from intracellular stores (Fig. 3A, no difference between the gray and black traces) whereas the second wave to Ca^{2+} influx only (Fig. 3A, from ~ 150 s, the difference between the gray and black traces). No difference was observed in the Ca^{2+} kinet-icbetween neutrophils of young and older adult males (Fig. 3B, from 60 s to ~ 149 s). In contrast, the Ca^{2+} transient in neutrophils of older adult females was significantly higher (AUC = 126.53 ± 14.09) than in neutrophils of young adult females (AUC = 74.30 ± 10.12) (Fig. 3C, gray trace; p = 0.04). 10 nM C5a also induced similar Ca^{2+} kinetic in human neutrophils (Fig. 3D). However, no differences were observed in the Ca^{2+} kinetics between neutrophils of young adults and older adults of both sexes (Fig. 3E and 3F). Analogous to the other stimuli, 1 μM fMLP and 100 nM fMLP elicited a Ca^{2+} transient and Ca^{2+} influx in neutrophils (Fig. 3G and 3J); however, the Ca^{2+} transient induced by the lower concentration was almost entirely due to Ca^{2+} influx (Fig. 3J). No statistically significant difference was found in the Ca^{2+} response to 1 μM fMLP between neutrophils of young and older adults of males and females (Fig. 3H and 3I). Nor was there any difference in the Ca^{2+} response between both groups

Fig. 1. Aging does not affect the proportion of circulating neutrophils and their precursors. Analysis of percentage of granulocytes, neutrophils, and neutrophil precursors, in the blood of healthy young adults and older adults by flow cytometry. Representative graphs of the regions of A) singlet population, granulocyte population selected by size and complexity from the previous region, and the neutrophil population identified by CD16 FITC and C15PE-CF594 markers from the granulocyte region, B) granulocyte population selected by CD11b-APC and complexity from a singlet region (not shown), total neutrophil population selected by CD45 PerCP and CD16 FITC from the granulocyte region, and neutrophil precursors identified by CD11b APC and CD16 FITC from the previous region. Percentage of total C) granulocytes, and D) neutrophils, in young adults (black circle) and older adults (gray circles). Percentage of E) mature neutrophils, F) metamyelocytes, G) promyelocytes and myelocytes, in young adults (black circle) and older adults (gray circle). Statistical analysis was performed using unpaired two-sided Student's *t*-test and Welch's correction; differences were considered significant when p values were ≤ 0.05.

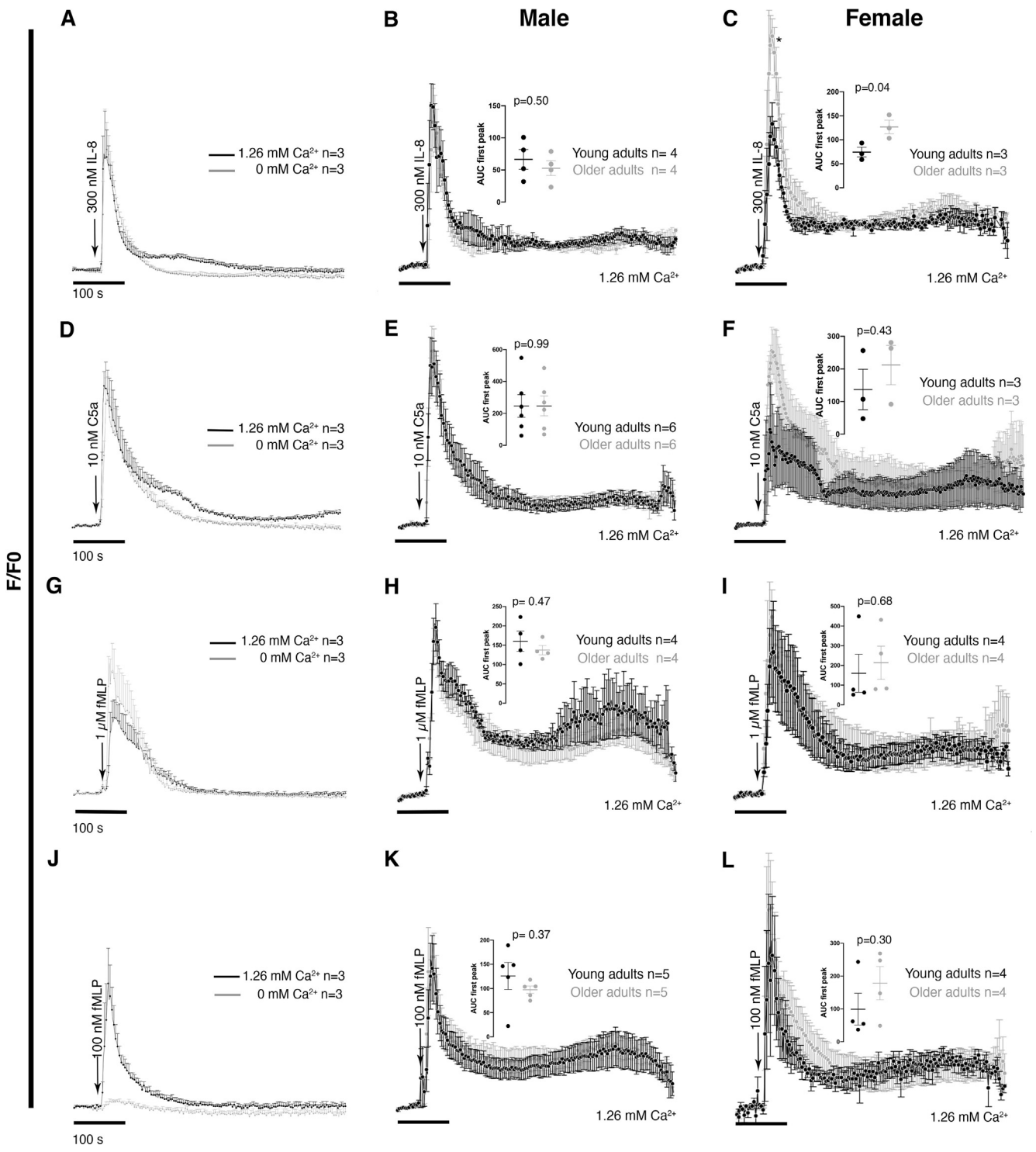


Fig. 3. Calcium response is altered in females' neutrophils with aging. Ca^{2+} response to inflammatory stimuli was analyzed by flow cytometry in purified neutrophils using 1 μM Fluo-4. Arrows indicate the timing of stimulus application (60 seconds). Average Ca^{2+} responses to A-C) 300 nM IL-8, D-F) 10 nM C5a, G-I) 1 μM fMLP, and J-L) 100 nM fMLP; A,D,G,J) with 1.26 mM external Ca^{2+} (black trace) or 0 Ca^{2+} (gray trace) to isolate Ca^{2+} release; in neutrophils of young adult (black trace) and older adult (gray trace) males (B,E,H,K) and females (C,F,I,L) in the presence of 1.26 mM external Ca^{2+}; n represents the number of independent experiments made by duplicate. AUC of the calcium transient (first peak) was calculated using the multipeak fitting 2 function of Igor64 program version 8.04 (WaveMetrics). Statistical analysis was performed using unpaired two-sided Student's *t*-test and Welch's correction; differences were considered significant when *p* values were ≤ 0.05.

with 100 nM fMLP (Fig. 3K and 3L). Next, we investigated whether the Ca^{2+} response to LPS, the main inflammatory stimulus from gram-negative bacteria, is altered with aging. In this case, 25 μg/ml LPS induced a slow increase of the cytosolic Ca^{2+} level with a maximum response at 444.69 ± 13.89 s, followed by a slow decline in neutrophils of young adult males (Fig. 4A, black trace). Unlike neutrophils in young adults, cells from older adult males showed a delay in their Ca^{2+} response, reaching a maximum at 519.09 ± 23.23 s that was fol-

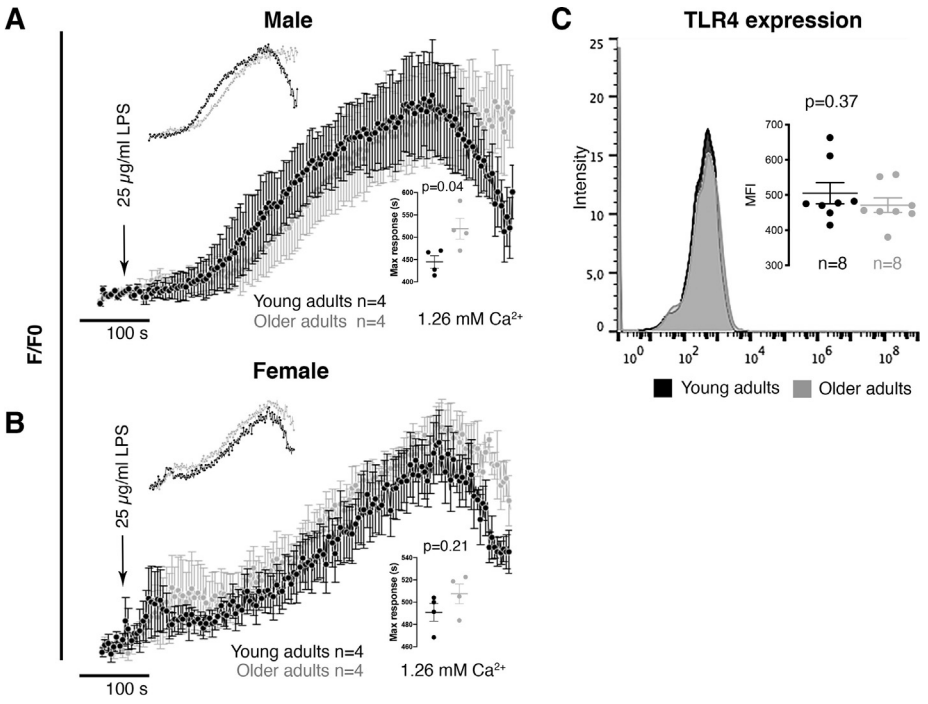


Fig. 4. Delayed Ca^{2+} response to LPS in older males. Ca^{2+} mobilization was analyzed as before. Arrows indicate the timing of the LPS application (60 s). Average Ca^{2+} response to 25 μg/ml LPS in young adult (black trace) and older adult (gray trace) A) males, and B) females; C) TLR4 (LPS receptor) expression was examined by flow cytometry. n represents the number of independent experiments made by duplicate. The maximum Ca^{2+} response was calculated using the curve fitting function of Igor64 program version 8.04 (WaveMetrics). Statistical analysis was performed using unpaired two-sided Student's *t*-test and Welch's correction; differences were considered significant when *p* values were ≤ 0.05.

lowed by a plateau (Fig. 4A, gray trace; p = 0.04). In turn, neutrophils of older adult females showed no difference in the Ca^{2+} response (490. 92 ± 7.95 s) compared to neutrophils of young females (479.54 ± 8. 78 s)(Fig. 4B). There was no difference in TLR4 (LPS receptor) expression between neutrophils of young adults and older adults (Fig. 4C).

3.4. Aging alters ORAI1 expression but not SOCE

Because alterations in calcium influx were observed in neutrophils of older adults, we investigated if SOCE was affected by aging. To activate ORAI1 channels, ER-calcium stores were passively depleted by inhibiting the Sarco-ER Ca^{2+}-ATPase (SERCA) with 2 μM thapsigargin (TG). Fig. 5 shows the characteristic sustained Ca^{2+} increase of SOCE activation; furthermore, in Fig. 5, we show there was no statistically significant difference in Ca^{2+} entry between neutrophils of older and young adults. Although, neutrophils of older females showed lower Ca^{2+} entry in the plateau phase than cells of young adults, it was not statistically significant (Fig. 5B). Confocal analysis revealed, unexpectedly, that ORAI1 channels were localized intracellularly in neutrophils (Fig. 5C). We also analyzed *ORAI1* mRNA expression by real-time qRT-PCR and the $2^{-\Delta\Delta CT}$ method. Data showed lower expression of this channel in neutrophils of older females ($2^{-\Delta\Delta CT}$ = 0.5216) than in neutrophils of older males ($2^{-\Delta\Delta CT}$ = 0.8819) compared to their corresponding young adults (Fig. 5D).

3.5. TRPM2 channel expression reduces in neutrophils with aging

TRPM2 channel has been detected in murine neutrophils and associated with their migration capacity [5–7]. Therefore, we investigated TRPM2 expression in neutrophils of young adults and older adults. Confocal microscopy analysis showed an intracellular dotted pattern of TRPM2 in human neutrophils (Fig. 6A). Based on the expression, two neutrophil populations were detected, $TRPM2^{low}$ neutrophils (Fig. 6A, middle panel) and $TRPM2^{high}$ neutrophils (Fig. 6A, right panel), the latter being predominant. These two populations were present in both young adults (48% and 52%, respectively) and older adults (40% and 60%, respectively) (Fig. 6A). No difference was detected in the CTCF between the $TRPM2^{low}$ neutrophil populations of young and older adults (Fig. 6B; p = 0.3095); however, it was lower in the $TRPM2^{high}$ neutrophil population of older adults in comparison to the same population in young adults (Fig. 6B; p = 0.0035). We next investigated whether the subcellular location of this channel was in lysosomes (Lamp-1), as previously reported [24,25], or in early/late endosomes (Rab5/Rab7). The analysis uncovered that 18.3% (Fig. 6C, white signal, upper left panel) of the total TRPM2 signal in the cell (Fig. 6C, green plus white signals, upper left panel) colocalized with Lamp-1 (lysosomes) in 32% (8 out of 25 cells) of neutrophils of young adults (Fig. 6C, upper left panel); no colocalization was observed in the remaining 68% of cells. Conversely, 30% of the signal was in lysosomes (Fig. 6C, white signal, lower left panel) in only 15% of neutrophils of older adults (3 out of 20 cells). On the other hand, only 4% of the TRPM2 signal colocalized with Rab5 (early endosomes) in 4% (3 out of 25 cells) neutrophils of young adults (Fig. 6C, upper middle panel), while 15% of the channel signal was in early endosomes in 20% (3 out of 20 cells) neutrophils of older adults (Fig. 6C, lower middle panel). Lastly, 53% of the TRPM2 signal colocalized with Rab7 in 28% (7 out of 25 cells) neutrophils of young adults (Fig. 6C, upper right panel); in contrast, only 14% of the channel signal was present in late endosomes of 5% (1 out of 20 cells) neutrophils of older adults (Fig. 6C, upper right panel). The data are summarized in a table and shown schematically in Fig. 6D. We also analyzed TRPM2 mRNA expression using real time qRT-PCR and $2^{-\Delta\Delta CT}$ method. Fig. 6E shows that the mRNA of this channel decreased in neutrophils of older adults of both sexes, with $2^{-\Delta\Delta CT}$ being 0.89 in neutrophils of older adult males and 0.53 in neutrophils of older adult females.

4. Discussion

Studies have reported that the number of circulating neutrophils remains unchanged with aging, but show a reduced chemotaxis capac-

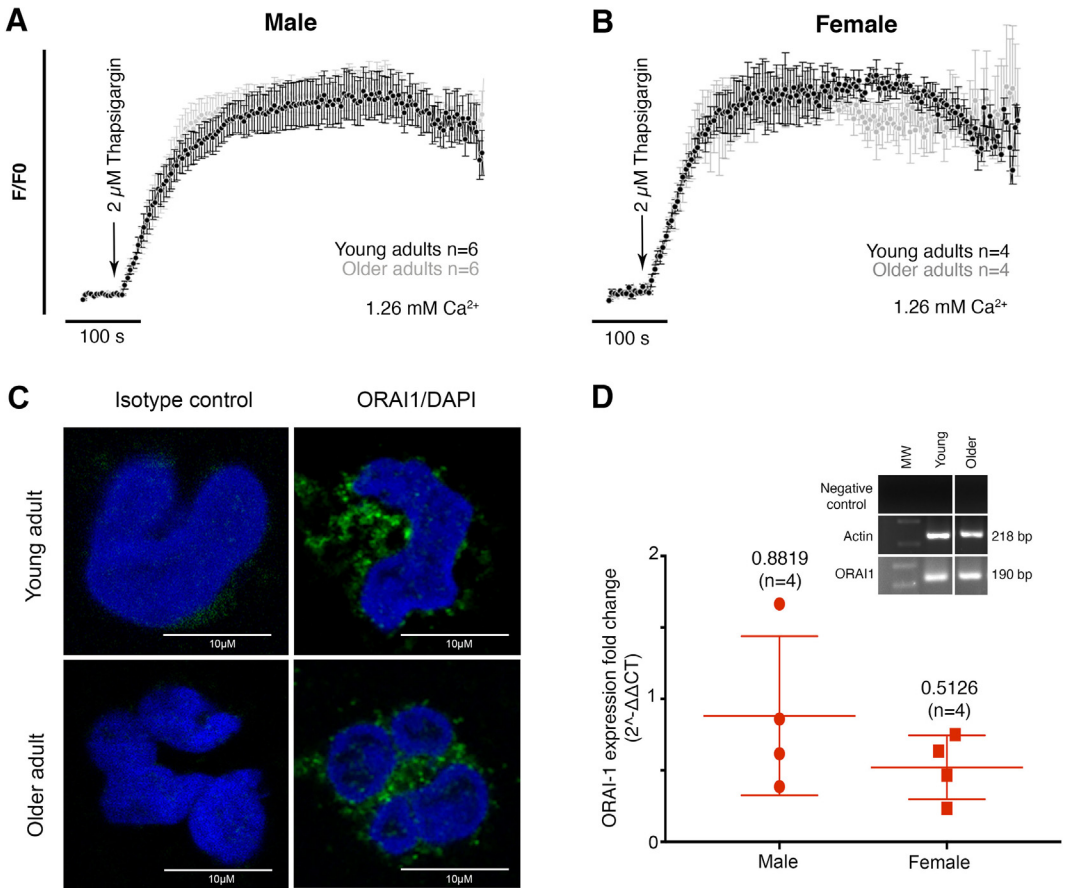


Fig. 5. Aging alters SOCE and reduces *ORAI1* expression in both sexes. Ca^{2+} mobilization was analyzed as described before. Arrows indicate the application time of 2 μM thapsigargin (60 seconds), a SERCA inhibitor. Average Ca^{2+} response in neutrophils of young adult (black trace) and older adult (red trace) A) males, and B) females; n represents the number of independent experiments made by duplicate; C) ORAI1 subcellular localization was assessed by immunofluorescence. Purified neutrophils were attached to poly-L-Lysine-pretreated coverslips and stained with rabbit anti-human ORAI1 antibody and Alexa Fluor 488 anti-rabbit IgG as a secondary antibody. Samples were analyzed by confocal microscopy. ORAI1 had a punctate intracellular expression in neutrophils of both young adults (upper right panel) and older adults (lower right panel). Images are representative of 20 and 40 images, respectively, from four independent experiments. Signals were not detected in the rabbit IgG isotype control. D) *ORAI1* mRNA expression was analyzed by qRT-PCR. *ORAI1* mRNA expression was lower in neutrophils of older males ($2^{-\Delta\Delta CT} = 0.8819$) and females ($2^{-\Delta\Delta CT} = 0.5216$) in comparison to cells of young adults. Graphs represent the mean of four independent experiments made in duplicate. Cropped gels of the qRT-PCR products are representative of the four independent experiments. Full-length gels are presented in Supplementary Fig. 1. Statistical analysis was performed using Student's *t*-test and Welch's correction, differences were considered significant when p values were ≤ 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

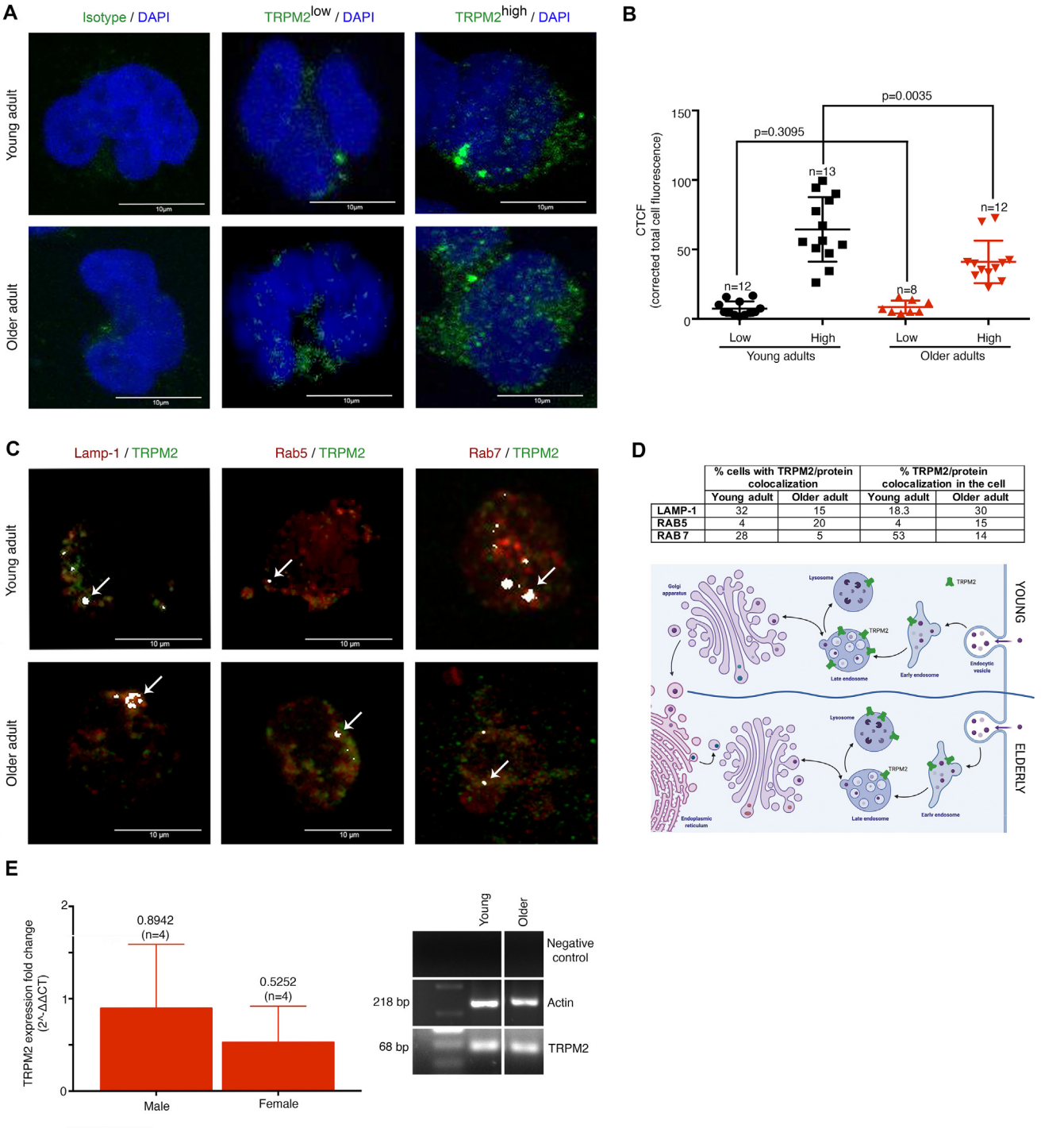
ity, phagocytosis, NET formation, ROS, and cytokine production [9,10]. We also found that the percentages of granulocytes, neutrophils, and neutrophil precursors, were comparable between young and older adults, and that aging reduced chemotaxis.

Typically, engagement of chemokine and formyl peptide receptors triggers a biphasic Ca^{2+}-signal; a Ca^{2+} phase mediated by phospholipase Cβ (PLCβ), which produces IP3 from plasma-membrane phosphatidylinositol 4,5 bisphosphate, IP3 binds and activates its receptor (IP3R), resulting in ER-stored Ca^{2+} release into the cytoplasm [3]. STIM1, an ER transmembrane protein, senses the drop in the Ca^{2+} level in the ER and activates CRAC channels (ORAI1), allowing extracellular Ca^{2+} influx into the cytoplasm (second Ca^{2+} phase) [3].

Accordingly, IL-8 induces a rapid Ca^{2+} increase in human neutrophils by activating PLC, and IP3 production [26]. Here we further show that IL-8 induces Ca^{2+} release followed by an extracellular Ca^{2+} entry. The initial Ca^{2+} transient response to IL-8 was higher in neutrophils of older adult females. Conversely, neutrophil chemotaxis to IL-8 was reduced in older individuals. These data suggest that lower migration of neutrophils with aging may not be caused by impaired Ca^{2+} mobilization. Nevertheless, IL-8-induced biological responses requiring calcium, such as respiratory burst or granule release [27], may be altered in women neutrophils by aging. Even though females appear to be less susceptible to oxidative stress by the antioxidant properties of estrogen [28], its concentration drops drastically after age ~ 60 [29].

In turn, cells from older adult males showed a prolonged Ca^{2+} response to bacterial LPS compared to the young adult males. Age and sex differences in the immune response to LPS in mice and humans have been previously described [30–32]. A study has shown that human neutrophils from males express more TLR4, release more TNF-α, and exhibit more robust activation of mitogen-activated protein kinases, and phosphatidylinositol 3-kinase, in response to LPS, than neutrophils of females [33]. As LPS primes neutrophils to release increased amounts of superoxide anion (O^{2-}), or can induce neutrophil extracellular traps, by increasing the intracellular Ca^{2+} concentration [34,35], is likely that neutrophils of older adult males produce more extended responses, with more significant damage occurring in this population during infections, as in sepsis.

Last, no significant difference in the Ca^{2+} response to C5a, and to the bacterial peptide fMLP, were observed in neutrophils, suggesting



	% cells with TRPM2/protein colocalization		% TRPM2/protein colocalization in the cell	
	Young adult	Older adult	Young adult	Older adult
LAMP-1	32	15	18.3	30
RAB5	4	20	4	15
RAB7	28	5	53	14

Fig. 6. TRPM2 channel expression is reduced in neutrophils with aging. Purified neutrophils were attached to poly-L-Lysine-pretreated coverslips and stained with rabbit anti-human TRPM2 antibody and Alexa Fluor 488 anti-rabbit IgG as a secondary antibody. Cells were also incubated with mouse anti-Rab5, mouse anti-Rab7 monoclonal antibody, or purified mouse anti-human Lamp-1 antibody, to detect subcellular organelles. Samples were analyzed by confocal microscopy. A) TRPM2 (green) showed a dotted intracellular pattern in neutrophils from young (upper panel) and older (lower panel) adults, and its expression level revealed two neutrophil populations: TRPM2^{low} (middle panel) and TRPM2^{high} (right panel). No significant signal was observed with the isotype control, rabbit IgG (left panel). B) CTCF analysis. There was no difference in the CTCF between the TRPM2^{low} neutrophil populations of young adults (black circle) and older adults (red triangle). In contrast, the TRPM2^{high} neutrophil population of older adults (red inverted triangle) had lower CTCF compared to TRPM2^{high} neutrophils of young adults (black squares) ($p = 0.0069$). C) Representative confocal images of neutrophils from young adults (upper panel) and older adults (lower panel) showing the colocalization (white) of TRPM2 (green) and Lamp-1 (red; left panel), Rab5 (red; middle panel), or Rab 7 (red; right panel). Images are representative of 25 cells from young adults and 20 cells from older adults, from six independent experiments. D) Summary of the confocal image analysis (table) and schematic representation of the findings. E) Quantification of TRPM2 mRNA by qRT-PCR. TRPM2 mRNA expression was lower in neutrophils of older males ($2^{-\Delta\Delta CT} = 0.8942$) and older females ($2^{-\Delta\Delta CT} = 0.5252$) than in cells of young adults. Graphs represent the mean of four independent experiments (n) made in duplicate. Cropped gels of the qRT-PCR products are representative of the four independent experiments. Full-length gels are presented in Supplementary Fig. 1. Statistical analyses were performed using Student's *t*-test, differences were considered significant when *p* values were ≤ 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that other mechanisms may be involved in the loss of migratory capacity of neutrophils of older adults toward these stimuli. The differences in the Ca^{2+} response alterations between women and men due to aging support the sexual dimorphism in the immune response that has been documented in humans [10,36]. However, further studies analyzing sex-dependent changes in neutrophils of older adults are necessary.

In neutrophils, CRAC channels have been implicated in the sustained Ca^{2+} influx response to IL-8, C5a, fMLP, or LPS [3,26,37,38]. *ORAI1* mRNA was found halved in neutrophils of older adult females but this was not directly reflected in the Ca^{2+} entry, suggesting that other ORAI isoforms may be present and build heteromeric CRAC channels with ORAI1, or that other channels could participate in SOCE (such as TRPC3 [39]), or compensatory mechanisms occur in human neutrophils; as proposed for neutrophils from patients with mutations in either ORAI1 or STIM1 that show only a modest reduction in SOCE [3]. Alternatively, ORAI1 mRNA could be more stable in neutrophils of older adults and, therefore, the expression of ORAI1 protein may not be altered. Cis-elements determine the stability of mRNAs, to which are bound *trans*-acting RNA-binding proteins to inhibit or enhance the mRNA decay rate [40]; this is regulated by factors in the cellular microenvironment, such as hypoxia, hormones, and cytokines [40], which are modified with aging.

Nevertheless, changes in the mRNA need to be correlated with channel activity (functional channels, altered properties or conductance) by electrophysiological analysis. In addition, ORAI1 was found intracellularly in freshly separated neutrophils, forming clusters or a dotted distribution, contrasting with the plasma membrane localization that has been observed in murine neutrophils [3,37,41] and human neutrophil cell lines [3,41], similar to what has been observed for this channel after cell activation [42]. Additional studies are necessary to characterize ORAI1 subcellular localization in primary human neutrophils and to identify the mechanisms that rule the organization of this channel in specialized structures or microdomains.

TRPM2 channels have also been detected in the plasma membrane of murine and human neutrophils by electrophysiology [43,44]. In contrast, we observed TRPM2 intracellularly in the human neutrophils by confocal analysis, similar to in mouse dendritic cells [24]; moreover, TRPM2 expression was found in two distinct cell populations: $TRPM2^{low}$ and $TRPM2^{high}$ neutrophils. The CTCF in the $TRPM2^{high}$ population of older adults was lower than the same population in young adults, suggesting a loss in expression, which was corroborated by the real-time qRT-PCR and $2^{-\Delta\Delta CT}$ method.

Interestingly, ~30% of neutrophils showed TRPM2 channels in lysosomes and late endosomes, mainly in the latter, in young adults; little signal of TRPM2 was observed in early endosomes. Meanwhile, 15% of neutrophils of older adults exhibited TRPM2 in lysosomes and 5% in late endosomes; in these cells, TRPM2 was observed mainly in lysosomes. In addition, 20% of neutrophils of older adults presented a higher signal of TRPM2 channels in early endosomes than cells of young adult.

Early endosomes receive extracellular molecules (nutrients), and plasma membrane components (receptors, ion channels, etc) to sort them into recycling (recycling endosomes) and degradative compartments (late endosomes and lysosomes) [45,46]. Our data might suggest a shift in the channel trafficking with aging. Possibly, due the decreased mRNA expression of TRPM2 by aging more TRPM2 channels are being recycled in the neutrophils of older adults. In contrast, in neutrophils from young adults TRPM2 channels are directed to be degraded.

In neutrophils, a central function of lysosomal enzymes is the digestion of bacteria engulfed via phagocytosis [47]. Since TRPM2 can permeate H^+, it can contribute to the lysosomal acidification for the optimal activity of hydrolases [47,48]. Lysosomes may also provide Ca^{2+} for membrane fusion between them and late endosomes, and the plasma membrane, and for regulation of distinct steps of lysosomal trafficking [48]. Further work is needed to understand the TRPM2 function, and the mechanisms that regulate TRPM2 trafficking in human neutrophils, and how remodeling of this channel localization affects the neutrophil function in aging.

In summary, despite alterations in CRAC and TRPM2 expression by aging, the Ca^{2+} response to inflammatory and chemotactic stimuli was only slightly affected, indicating that other mechanisms are involved in the loss of migratory capacity of neutrophils of older adults.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2022.05.002.

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