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The use of flow cytometry to examine calcium signalling by TRPV1 in mixed cell populations

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ABSTRACT

Flow cytometric analysis of calcium mobilisation has been in use for many years in the study of specific receptor engagement or isolated cell:cell communication. However, calcium mobilisation/signaling is key to many cell functions including apoptosis, mobility and immune responses. Here we combine multiplex surface staining of whole spleen with Indo-1 AM to visualise calcium mobilisation and examine calcium signaling in a mixed immune cell culture over time. We demonstrate responses to a TRPV1 agonist in distinct cell subtypes without the need for cell separation. Multi parameter staining alongside Indo-1 AM to demonstrate calcium mobilization allows the study of real time calcium signaling in a complex environment.

Introduction

The ability to investigate calcium influx in response to known calcium inducers is of increasing importance [1]. The use of Indo-1 acetoxymethylester (Indo-1 AM) emission in flow cytometry has been known for many years [2] in the study of cell specific calcium channels on isolated single cell populations [3]. Most frequently, flow cytometric analysis is combined with microscopy or cuvette based techniques to detect calcium signaling in distinct populations [4]. However, the ability to compare cells of different types simultaneously would be beneficial where particular calcium channels are not uniquely expressed on specific cell types, or where the initiation of calcium signaling needs to be studied in mixed populations undergoing stimulation, for instance in whole blood or lymphoid populations [5]. During our study of immune/nerve cell interaction, we wanted to determine Transient Receptor Potential Vanilloid-1 (TRPV1) expression and function in a splenic population. Classically known as a vital neural receptor and calcium channel, TRPV1 plays a critical role in the release of calcitonin gene related peptide (CGRP) [6]. TRPV1 shapes the thermal regulating system in the body and is found on peripheral nerves and immune cells [7], although comparative expression in lymphoid tissue has never been shown. Given the mechanosensitive and thermal responsive nature of TRPV1, we needed to study calcium signaling with as little cell manipulation as possible. Furthermore we were interested in signaling within distinct immune cell populations in a co-culture environment. We chose to investigate calcium influx using Indo-1 AM (Fluorescence Ex/Em 335/405 and 475 nm) which provided the opportunity to maximise additional surface staining on remaining channels using an LSRII. To maximize surface receptor detection, we used a dilution staining gradient approach as described previously [8]. This staining method was initially described for monitoring immune cell populations of mouse blood and has since been developed for 10 channel flow cytometric analysis [9]. Here we combine five fluorescence parameter staining across four fluorochromes with Indo-1 AM to demonstrate the potential for calcium signaling as part of a multiplex analysis with minimum intervention.

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Materials and methods

Mice

Male C57BL/6Ola mice (6–8weeks old, Harlan Olac) were housed in the University of Manchester Biological Services Unit (BSU) in ventilated cages under specific pathogen free conditions at 21 °C with a 12 h light/dark cycle and free access to food and water. TRPV1 deficient mice (B6.129X1-*Trpv1tm1Jul*/J) were sourced from The Jackson laboratory and bred at the University of Manchester. Procedures were carried out under the Home Office Scientific Procedures Act 1986 (revised January 2013). For euthanasia, Mice were culled via CO_2 inhalation.

Ethics, consent and permission

All experiments were approved by the University of Manchester's ethics committee (http://www.manchester.ac.uk/research/environment/governance/ethics/), and the UK Home Office in accordance with Home Office Scientific Procedures Act 1986 (revised January 2013), adhering to the basic principles of replacement, reduction and refinement (3Rs). Dr Assas holds a personal license from the UK Home Office.

Splenocyte islolation

Isolated spleens were disaggregated through 100 μm sieves (BD Phamingen, Oxford, UK). Cells were centrifuged (400 g, 5 min, room temp), and re-suspended in complete media (RPMI-1640, supplemented with 50 μg/ml FCS, 100 μg/ml penicillin/streptomycin and 1 mM L-glutamine 0.42 mM calcium (Sigma Aldrich, UK)) at 5×10^7 cells/ml.

Intracellular Indo-1 AM splenocyte staining

This protocol is modified from Flow cytometry core facility, Camelia Botner Laboratories (https://www.ucl.ac.uk/ich/services/lab-services/FCCF/protocols/calcium_flux). 1×10^7 splenocytes were stained with Indo-1 AM at 4 μM for 30 min at room temperature (diluted in either complete media or calcium free media as detailed in the text). Cells were centrifuged (400 g, 5 min), washed and placed in a 96 well plate (5×10^5 cells per well). Cells were centrifuged (400 g, 2 min) and resuspended (1 mM calcium, 1 mM magnesium and 0.5% FBS (Sigma Aldrich, UK)) then rested (15 min) in preparation for surface antibody staining.

Cell surface staining

This protocol was modified from Frischmann *et al* (2006). Selected splenocytes were first incubated with anti-CD16/32 (2.5 μg/ml BDbiosciences, San Jose, USA) to block non-specific binding, washed and then labelled with the following antibody cocktail (20min, ice): anti-CD4-PE (0.1 μg/ml, eBioscience, San Diego, CA, USA), anti-CD8-PE (1 μg/ml, eBioscience, San Diego, CA, USA), anti-CD19-Alexa fluor 700 (0.25 μg/ml, eBioscience, San Diego, CA, USA), anti-F480-APC (0.1 μg/ml, eBioscience, San Diego, CA, USA), anti-CD11c-APC-Cy7 (0.5 μg/ml, eBioscience, San Diego, CA, USA). Cells were washed and re-suspended in complete media (400 μl of RPMI-1640, supplemented with 50 μg/ml FCS, 100 μg/ml penicillin/streptomycin and 1 mM L-glutamine 0.42 mM calcium (Sigma Aldrich, UK) or calcium free media for acquisition.

Instrument details (LSRII), configuration and settings

Instrument Model BD LSRII Flow Cytometer, Serial Number H08800001 (BD Biosciences, Oxford Science Park, Oxford). The instrument flow cell (fixed-alignment quartz cuvette), fluidics, light source, excitation optics or optical detectors/paths were not altered. There is a four-laser configuration as follows: 488-nm Coherent® SapphireTM air-cooled argon-ion laser, 50 mW; 640-nmCoherent® Cube air-cooled laser, 40 mW; 405-nm Coherent® Radius air-cooled laser, 50 mW; 355 nm Coherent Genesis 355-20 Air cooled laser, 20 mW. Detector arrays consist of BD octagons (561 and 488 nm laser lines) and BD Trigons (405 and 640 nm laser lines). The entire PMT arrangements are illustrated in the supplementary Table S1.

LSRII instrument setup

This protocol was modified from Flow cytometry core facility, Camelia Botner Laboratories (https://www.ucl.ac.uk/ich/services/lab-services/FCCF/protocols/calcium_flux) and the BD LSRII user's guide (http://www.gla.ac.uk/media/media_231654_en.pdf). The filters were configured as follows: 530/30 PMT A (Indo-1 blue) and 405/20 PMT B (Indo-1 violet) on the UV trigon and close violet laser shutter. This prevented the violet laser from interfering with the Indo-1 violet emission. Indo emission and excitation were monitored on a linear scale, and viewed on a time versus ratio (Indo-1 violet (numerator), Indo-1 blue (denominator)) plot. Parameters were set using Indo-1 single stain and surface only stained samples. Compensation was achieved using single stained beads (eBioscience, UK).

Sample acquisition

All samples were rested for 15 min at 30 °C prior to acquisition. Each sample was checked for clear Indo-1 blue staining (i.e. no calcium signaling) prior to acquisition and addition of agonists. Indo-1 blue staining was set up at approximately a 70° angle to allow movement to Indo-1 violet gate (Supplementary Fig. S1). Time ratio plots were monitored during acquisition. Events were acquired at 200 cells/second for all samples. Firstly, each sample was recorded for 10 s to establish a baseline (Fig.S1 B-D). With continuous recording, samples were treated with an agonist (capsaicin (100 μl in media, 100 μM, Sigma Aldrich, UK) or ionomycin (positive control, 100 μl in media, 2 μM, Sigma Aldrich, UK)) or vehicle (100 μl, bringing the total volume to 500 μl), vortexed and returned to the LSRII to record. Each sample was acquired for 360 s in total ($2–3x10^5$ events). The acquisition rod was cleaned between each sample, and distilled water was run for 5 min between each sample type (control, tests and ionomycin) to ensure no agonist was carried over.

Analysis

The data was compensated and analysed using FlowJo_V10.Ink (Treestar, Ashland USA). Labelled beads were used as compensation controls (eBioscience, San Diego, CA, USA) and analyzed as follows: splenocytes were gated on FSC-A/FSC-H to remove aggregates, and debris was viewed on FSC-A/SSC-A. T and B cells were gated on $CD4^+$ and $CD8^+$ (PE) and $CD19^+$ (Alexa flour 700) channels, with non-T non-B cells remaining in the unstained gate. CD11c + cells were analyzed on the APC-Cy7 channel, and F480 + cells in the non-T non-B cell gate on APC/APC-Cy7 channels. F480 + CD11c+ were also gated for interest (data not shown). Negative controls (media treated) were used to define the gates. The Indo-1 blue vs Indo-1 violet plot was then examined in each cell population. Data were plotted as number of cells over time migrating from Indo-blue (450 LP mirror) to Indo-violet (405/20 filter). Analysis was performed on FlowJo_V10.Ink (Treestar, Ashland USA).

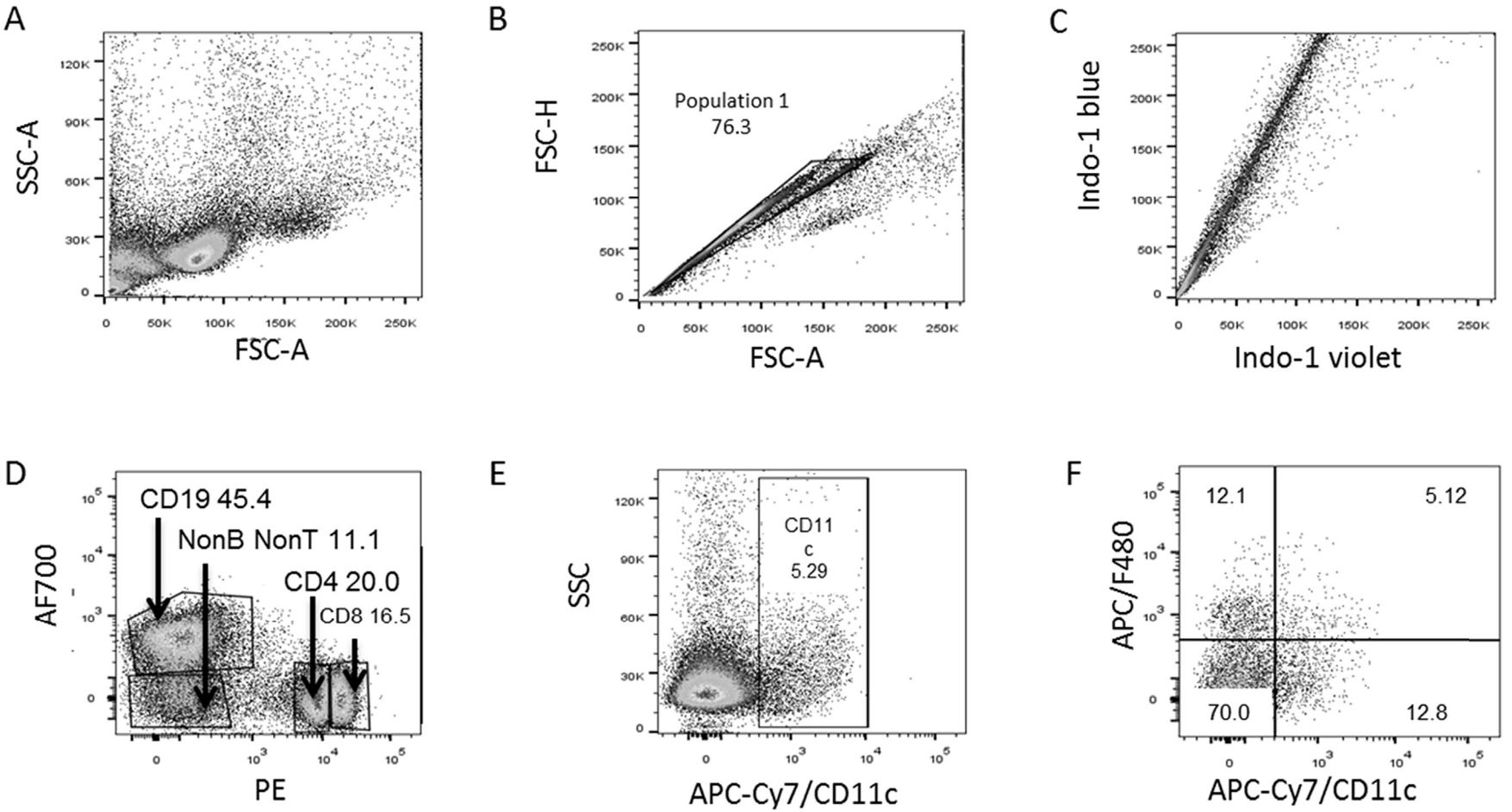

Fig. 1. Cell surface and Indo-1 AM staining demonstrates calcium signaling in distinct splenic populations. Splenoytes were gated according to surface staining and targeted for calcium signaling analysis. A&B: splenocytes were selected and aggregates were outgated on FSC-A/FSC-H. C: Indo-1 blue vs Indo-1 violet for unstimulated whole spleen. D: Splenocyte populations were gated according to their fluorochrome. E&F: CD11c + cells were gated on APC-Cy7 (E). F480 + CD11c-cells were gated on APC/APC-Cy7 (F).

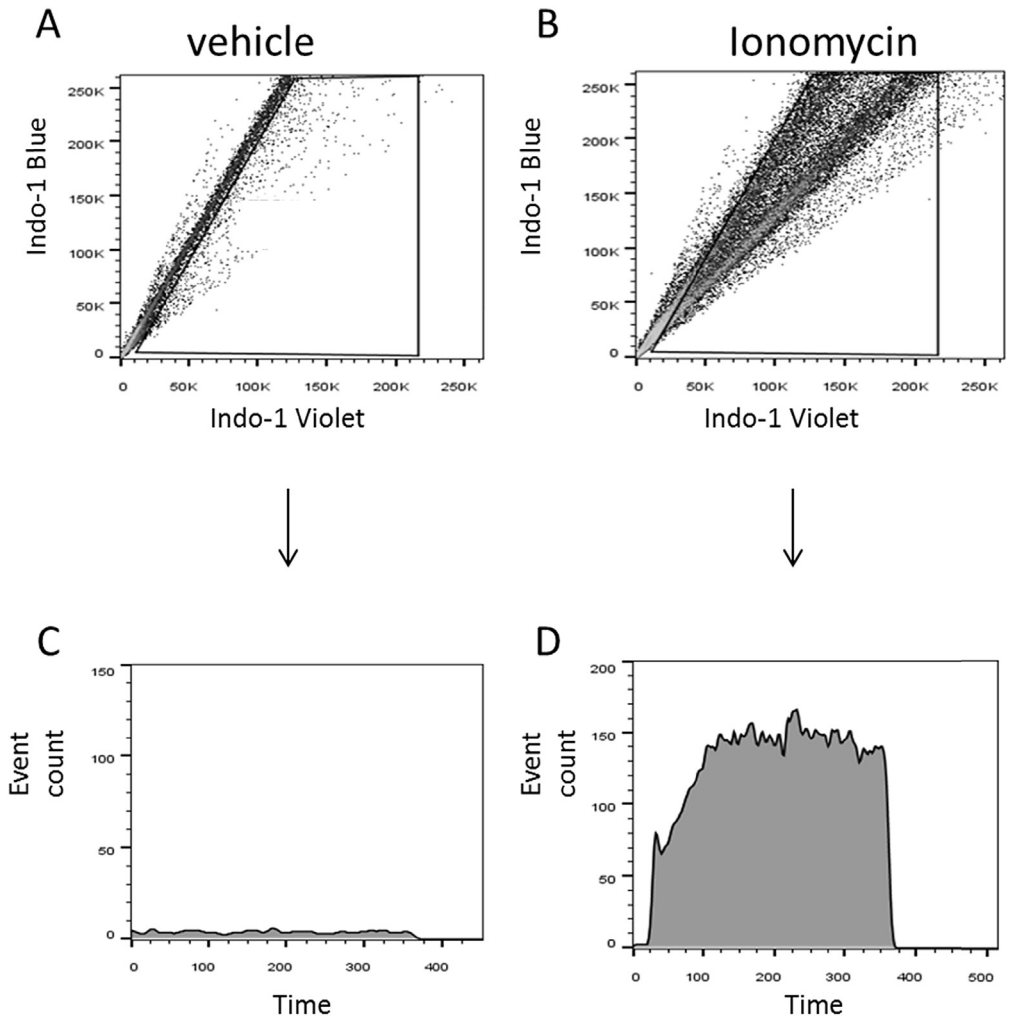

Fig. 2. Ionomycin was used as a positive control for calcium signalling. Ionomycin was used to determine the migration of cells from Indo-1 blue to Indo-1 violet. A: Gate shown applied to vehicle treated samples for negative control. B: Same gate was applied to ionomycin positive control to ensure the inclusion of all cells which had migrated towards Indo-1 violet from Indo-1 blue. C: low calcium levels with no increase pattern over time confirms the validity of the vehicle control and set a clear threshold. D: The high calcium signaling recording confirms the gating for positive control sample treated with Ionomycin. Ionomycin was used at (2 μM). Data is representative of 3 separate experiments, n = 3 in each group.

Theory

Here we applied a similar cell surface staining technique as in Frischmann et al. [8] but in combination with real-time calcium signaling identified with Indo-1 AM. Our achieved objectives were as follows:

1 Rigorous calcium sensing over time in response to specific agonist
2 A more economical protocol with reduced cell manipulation and intervention
3 A clearer understanding of TRPV1 signaling in mixed cell populations

Results

Splenoytes were gated according to stained populations (Table S2) and analyzed for calcium signaling. After out gating of cell aggregates (Fig. 1A and B), overall calcium signaling was viewed on Indo-1 blue vs Indo-1 violet (Fig. 1C). Samples were then gated using AF700 vs PE identifying $CD19^+$, non T non B cells, $CD4^+$ and $CD8^+$ (Fig. 1D). CD11c + cells were identified on APC-Cy7 (Fig. 1E), and gated using APC vs APC-Cy7 identifying F480 + CD11c- (Fig. 1F). Identified populations were then plotted using Indo-1 blue vs Indo-1 violet for calcium analysis.

Positive and negative controls displayed expected influx patterns of calcium-bound Indo-1 violet cells when migration was plotted against time (Fig. 2). Ionomycin (positive control) and vehicle (negative control) were used to set the gates for migration to Indo-1 violet (Fig. 2A and B). Indo-1 violet + cells were gated over time identifying the accumulation of intracellular calcium (Fig. 2C).

After stimulation, a gate was then drawn over the migrating cells moving from Indo-1 blue (calcium-free) towards indo-1 violet (calcium-bound) in response to TRPV1 agonist capsaicin (Fig. 3A). Gated data were represented over time to show the accumulation of indo-1 violet cells (360 s, Fig. 3B). Each defined cell population was analysed in this way (Fig. 3).

Lastly to demonstrate the specificity of capsaicin stimulation, calcium signaling was measured in TRPV1 deficient mice (Fig. 4). When TRPV1 deficient whole spleen was incubated with capsaicin,

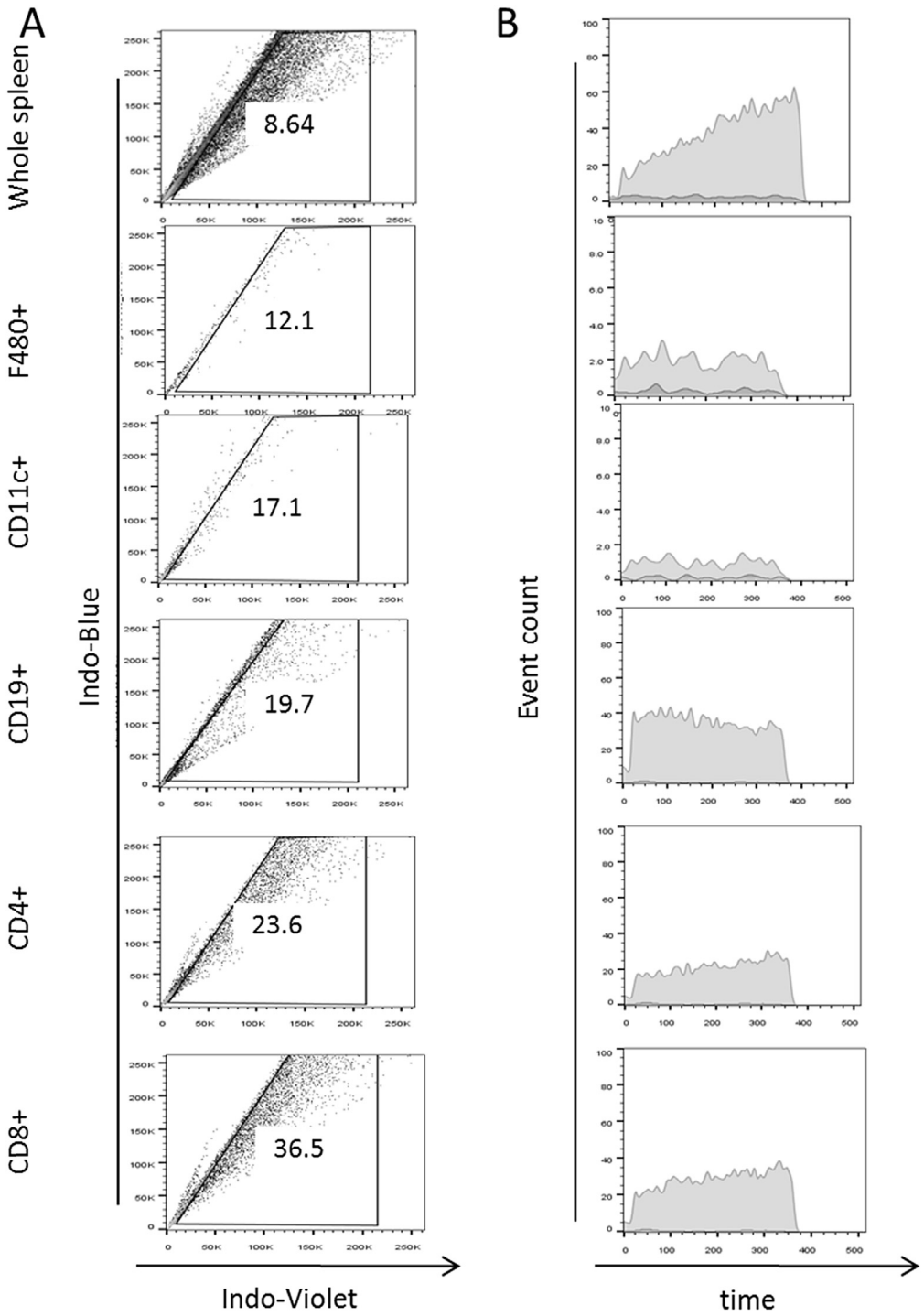

Fig. 3. Distinct cell populations were identified for cell specific response to capsaicin. A: Gated splenocytes were plotted on Indo-1 blue vs Indo-1 violet according to the positive controls. B: The ratio of Indo-1 blue/Indo-1 violet cells (light grey) were plotted against time (360 s) and compared to vehicle (dark grey). Capsaicin was at (100 μM). Data is representative of 3 separate experiments, n = 3 in each group.

no calcium signaling was recorded (Fig. 4A–H) in contrast to WT (Fig. 4I–P).

No calcium signaling was observed in C57BL/6 whole spleen in calcium free media indicating that calcium mobilization in response to capsaicin is dependent on calcium from the extracellular environment (Supplementary Fig. S3). Experimental repeats are shown in supplementary Figs S4 and S5.

Discussion

Indo-1 AM has been used to study intracellular calcium mobilization for many years. Given the essential role that Ca2+ plays in cell function including cell-cell signaling, transcription and apoptosis, methods to maximize the use of intracellular calcium indicators with other immunological parameters are needed. Although microscopy is widely used to study Ca2+mobilization (eg

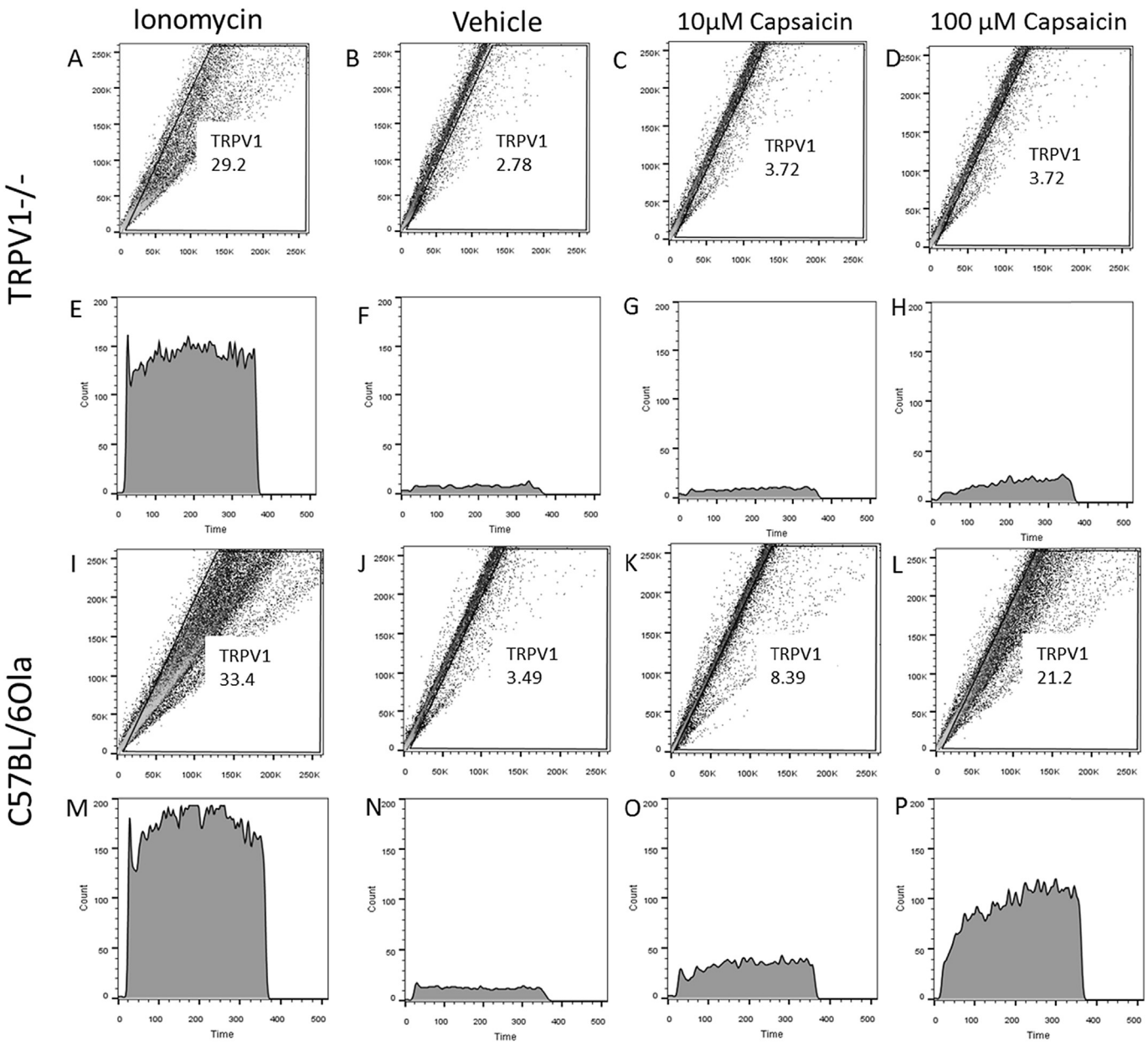

Fig. 4. TRPV1 deficient mice do not respond to exogenous capsiacin. TRPV1 functionality was tested by the treatment of whole spleen cell populations with TRPV1 agonist capsaicin. A-D& I–L: Representative flow cytometry plots showing cell migration from indo-blue (calcium free) to indo-violet (calcium bound) in response to positive control Ionomycin, media and capsaicin in TRPV1 deficient (A–D) and C57BL/6J (I-L)spleen cells. E-H & M–P: Calcium influx induced by capsaicin is dose dependent. Representative plots of cell migration from Indo-1 blue to indo-1 violet gate over time (360sec) in TRPV1 deficient ((E–H) and C57BL/6J (M–P) whole spleen. Media controls shown in dark grey. Data is representative of 3 separate experiments, n = 3 in each group.

Fura-2 Ex/Em 340/510 nm), it is most often used separately from flow cytometry methods [3,10]. Here we demonstrate that combining multiplex surface staining with Indo-1 AM provides an efficient method for Ca2+signaling in mixed cell populations. Combining multiplex surface staining with Indo-1 AM allows the study of relatively low numbers of cells in situ, where specific responses of distinct but rare cell subsets are needed. It is worth noting that the Fluo4 (Ex/Em 494/516 nm)/Fura red (Ex/Em, 436/637) combination has also been used as a calcium indicator in flow cytometry [16], and could potentially substitute Indo-1 AM here. However, whilst using Fluo-4/Fura Red does not require a specialist UV laser, they are detected on the green/far red channels, precluding them from use in a surface staining panel. Indo-1 AM has also been used in flow cytometry to study the mobilization of intracellular calcium stores in response to cell-cell signaling [12], and receptor mediated signaling [13]. Given the importance of calcium mobilization in receptor engagement processes during an immune response [14], the ability to study cell specific responses to cytokines would be beneficial in a multi-cellular environment. Furthermore, combination of this approach with the use of spatiotemporal calcium mobilization could allow the distinction to be drawn between intracellular mobilization and influx of extracellular calcium [15]. Finally the dynamic nature of Indo-1 migration from Indo-1 blue to Indo-1 violet in a multicellular culture gives a quantitative and kinetic representation of calcium influx over time, which coupled with multiplex staining could provide

valuable information on the comparative responses and speed of signaling in neighbouring cells. We have demonstrated recently how this can lead to mechanistic insight [11].

Conclusion

In conclusion, this approach demonstrates the potential for combining Indo-1 AM excitation with multi-parameter staining. We believe this approach could be valuable in the study of cell interactions in mixed population assays.

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Conflict of interest

The authors state no conflict of interest.

Availability of data

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.03.025.

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