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Transient receptor potential vanilloid 1 expression and function in splenic dendritic cells: a potential role in immune homeostasis

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Summary

Neuro-immune interactions, particularly those driven by neuropeptides, are increasingly implicated in immune responses. For instance, triggering calcium-channel transient receptor potential vanilloid 1 (TRPV1) on sensory nerves induces the release of calcitonin-gene-related peptide (CGRP), a neuropeptide known to moderate dendritic cell activation and T helper cell type 1 polarization. Despite observations that CGRP is not confined to the nervous system, few studies have addressed the possibility that immune cells can respond to well-documented 'neural' ligands independently of peripheral nerves. Here we have identified functionally relevant TRPV1 on primary antigen-presenting cells of the spleen and have demonstrated both calcium influx and CGRP release in three separate strains of mice using natural agonists. Furthermore, we have shown down-regulation of activation markers CD80/86 on dendritic cells, and up-regulation of interleukin-6 and interleukin-10 in response to CGRP treatment. We suggest that dendritic cell responses to neural ligands can amplify neuropeptide release, but more importantly that variability in CGRP release across individuals may have important implications for immune cell homeostasis.

Keywords: calcitonin-gene-related peptide; dendritic cell; lymph node; transient receptor potential vanilloid 1.

Introduction

The involvement of the nervous system in immune responses has gained momentum recently[1] and there is evidence implicating neurally related variations in disease.[2,3] Neural components such as sensory receptors, neuropeptides and neurotransmitters have been associated with profound immune functions.[4–6] Calcitonin-gene-related peptide (CGRP) in particular has been shown to have significant effects on T-cell polarization and is thought to influence primary immune responses. Although many studies have addressed the effect of neuropeptides on immune cells, their source is generally assumed to be neural. Immune cells have been shown in close proximity to sensory nerves in the periphery[7–9] and are able to respond to neuropeptides. However, immune cells have also been shown to produce neuropeptides independently of nerve–cell interaction,[10,11] implying cell-specific release. The presence and functionality of previously recognized 'neural' receptors on important immune cells has been unclear,[12] even though many studies have linked their function to immunological phenomena.[13] Whether neural components can function and are secreted independently of the nervous system is key to understanding their role in homeostasis of immune responses. It is likely that neural receptors and protein mediators are expressed far more ubiquitously than is currently understood.

We are particularly interested in transient receptor potential vanilloid 1 (TRPV1; also known as VR1) which is expressed along the entire length of the vanilloid-sensitive sensory neurons, from the periphery to the somata in

Abbreviations: APC, allophycocyanin; CGRP, calcitonin-gene-related peptide; IFN-γ, interferon-γ; IL-17, interleukin-17; LPS, lipopolysaccharide; PE, phycoerythrin; Th2, T helper type 2; TNF-α, tumour necrosis factor-α; TRPV1, transient receptor potential vanilloid 1

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the central nervous system.[14] TRPV1 is a ligand-gated non-selective cation channel, and mediates the release of CGRP in peripheral nerves. Studies have demonstrated that CGRP and other neuropeptides including substance P can influence both pro-inflammatory and anti-inflammatory immune responses, depending on concentration. In particular, CGRP has the potential to promote a T helper type 2 (Th2) phenotype through down-regulation of interferon-γ (IFN-γ) and interleukin-17 (IL-17) potentially through up-regulation of IL-10.[15,16] Recently, a homeostatic TPRV1–CGRP–Toll-like receptor 4 axis has been suggested,[17] with several studies demonstrating that Toll-like receptor 4 signalling can enhance the anti-inflammatory rather than pro-inflammatory effect of TRPV1 activation, through the release of CGRP. These studies suggest a critical role for TRPV1 expression and CGRP release in early immune cell polarization.

When sensory TRPV1-positive nerve terminals are activated an influx of calcium is induced that leads to the release of peptide mediators, including CGRP. Impulses are transmitted to the central nervous system via central fibres where they are perceived as pain, burn or itch. For a long time the distribution of TRPV1 was thought to be restricted to sensory peripheral neuronal fibres and tissue. However, the presence of TRPV1 on non-neuronal tissue is increasingly being shown, and includes keratinocytes,[18,19] gastric epithelial cells,[20] fibroblasts[21] and vascular endothelium,[22] as well as immune cells.[23,24] Recent studies on the role of TRPV1 in T cells revealed that the receptor functions as a calcium channel in immune cells in the same way as nerves,[12] although no corresponding release of neuropeptides was shown. TRPV1 activation in non-lymphocytes has been shown to induce other immune mediators. For instance, activation of TRPV1 in keratinocytes was reported to induce prostaglandin E_2 and IL-8 release as well as to increase cyclo-oxygenase 2 and matrix metalloproteinase 1 expression.[19,25,26]

Here we investigate for the first time the comparative expression of TRPV1 on primary splenic immune cells focusing on antigen-presenting cells. We show that TRPV1 can function as a calcium channel and induce the subsequent release of CGRP. Additionally, we have assessed the immune effects of CGRP across genetically unrelated strains of mice, to understand the role of CGRP in homeostasis.

Materials and methods

Mice

Male C57BL/6J mice (6–8 weeks old) were bred in the University of Manchester Biological Services Unit. Age-matched male AKR and BALB/c mice (6–8 weeks old) were purchased from Harlan Laboratories (Bicester, UK). TRPV1-deficient mice (B6.129X1-*Trpv1*^{tm1Jul}/J) were sourced from The Jackson Laboratory (Bar Harbor, USA) and bred at the University of Manchester. Mice were housed in the Biological Services Unit of the Faculty of Life Sciences in a temperature-controlled room at 24° with a 12-hr light/dark cycle and free access to food and water. All procedures were sanctioned by the local ethical review committee and by the Home Office Animal Procedures Inspectorate. Procedures were carried out under the Home Office Scientific Procedures Act 1986 (revised January 2013). For strain comparison experiments cell isolations were done in parallel from mice housed next to each other in the same room. All mice were housed in specific pathogen-free conditions in ventilated cages.

Spleen preparation

Spleens were disaggregated through 100-μm sieves (BD Pharmingen, Oxford, UK). Red blood cells were lysed, then cells were centrifuged (400 ***g***, 5 min, room temperature) and re-suspended in media (RPMI-1640, supplemented with 50 μg/ml fetal calf serum, 100 μg/ml penicillin/streptomycin and 1 mM L-glutamine) at 6×10^7 cells/ml.

Dendritic cell and CD4$^+$ T-cell isolation from spleen

A pan dendritic cell Isolation kit (Miltenyi Biotec, Bisley, UK) was used to isolate all dendritic cell subsets from splenic populations (purity 80·5%). This included plasmacytoid dendritic cells, and three conventional dendritic cell subsets (CD11b$^+$ CD4$^+$ CD8$^-$, CD11b$^+$ CD4$^-$ CD8$^-$, and CD11b$^-$ CD8$^+$ CD4$^-$) along with all dendritic cells commonly characterized by the expression of the dendritic cell marker CD11c. In parallel, a CD4$^+$ T-lymphocyte isolating kit (Miltenyi Biotec) was used to Isolate CD4$^+$ T cells from the same spleen population (purity 94·3%). In two separate procedures, mouse dendritic cells and CD4$^+$ T cells were isolated by depletion of non-target cells (negative selection) labelled with a cocktail of biotin-conjugated monoclonal antibodies against antigens that are not expressed by dendritic cells or CD4$^+$ T cells, provided by the manufacturer. In a second step, cells were labelled with anti-Biotin microBeads. The magnetically labelled non-target cells were depleted by retention within a MACS LS column.

Flow cytometry

Ten parameter identification by flow cytometry. This protocol was a modification from Frischmann and Muller.[27] Splenocytes were first incubated with an anti-Fc receptor antibody anti-CD16/32 (BD Biosciences, San Jose, CA) to block non-specific binding, and then labelled with the appropriate monoclonal antibody cocktail (20 min, ice): phycoerythrin-conjugated anti-CD4 (anti-CD4-PE;

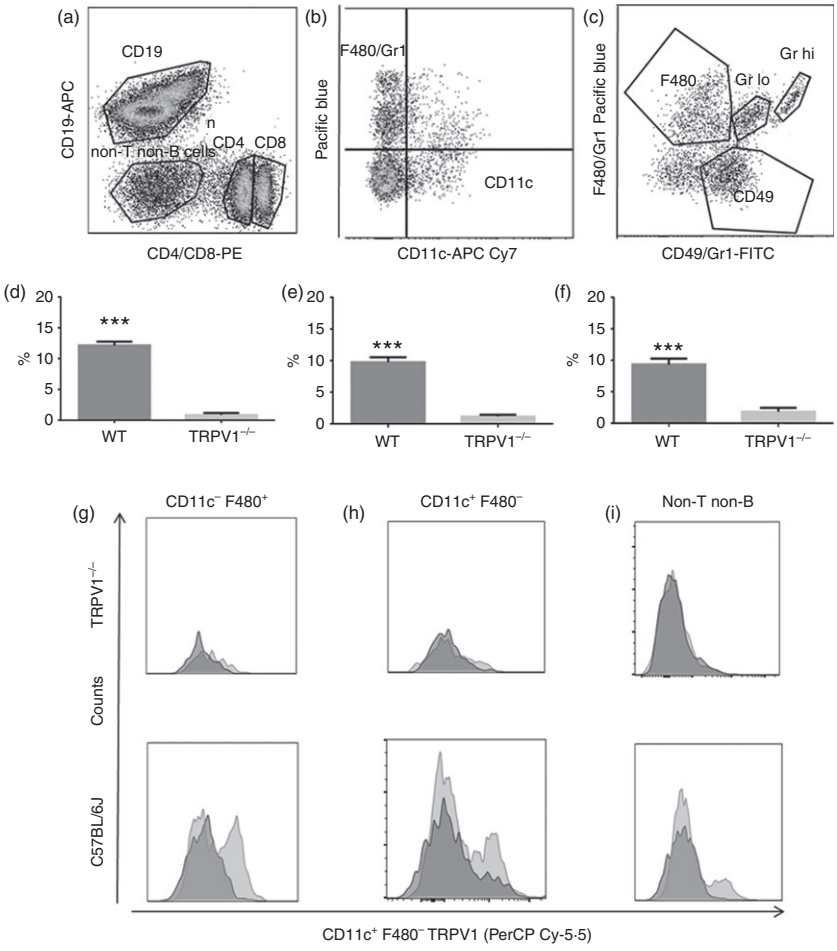

Figure 1. Transient receptor potential vanilloid 1 (TRPV1) is expressed on antigen-presenting cells in the spleen. TRPV1 expression was investigated in C57BL/6J whole spleen populations using flow cytometry. (a–c) Representative dot plots illustrate 10-cell gating strategy used to identify distinct immune cell populations.[27] (d–f) TRPV1 expression in Lin^- $CD11c^-$ $F480^+$ (d), $CD11c^+$ $F480^-$ (e) and non-T non-B (f) cells in C57BL/6J splenocytes. TRPV1-deficient splenocytes are shown as a control. (g–i) Histograms showing TRPV1 surface expression in control TRPV1-deficient and C57BL/6J mice by flow cytometry: Lin^- $CD11c^-$ $F480^+$ (g), lin^- $CD11c^+$ $F480^-$ (h) and non-T non-B (i) cell populations. F–1 controls are shown in dark grey. Data are representative of three experiments, $n = 3$ in each group. Significance shown by *t*-test (c–e). $***P < 0{\cdot}001$.

0·1 μg/ml), anti-CD8-PE (1 μg/ml), allophycocyanin-conjugated anti-CD19 (anti-CD19-APC; 0·25 μg/ml), anti-IgD-FITC (0·5 μg/ml), anti-IgM-Pacific blue (1 μg/ml), anti-Ly6g-Pacific blue (0·25 μg/ml), anti-Ly6g-FITC (0·5 μg/ml), anti-F480-FITC (0·1 μg/ml), anti-CD11c-APC-Cy7 (0·5 μg/ml) and CD49 (0·25 μg/ml). All antibodies were supplied by eBioscience (San Diego, CA). Ten-colour staining was supplemented with polyclonal anti-TRPV1-biotin where indicated (1·25 μg/ml; Bioss Antibodies, Woburn, MA). After washing (FACS buffer) cells were stained with Peridinin-chlorophyll protein-Cy5.5-conjugated streptavidin (1 μg/ml; eBioscience). F minus 1 controls[27] were unstained with streptavidin, alongside cell populations from TRPV1-deficient mice as negative controls (20 min on ice). Cells were once again washed and re-suspended (200 μl FACS buffer) and acquired on the LSRII (BD Biosciences). Using FlowJo software (V10.Ink; Tree Star, Ashland, OR), the data were compensated using labelled bead controls and analysed according to the following strategy (Fig. 1; see Supplementary material, Fig. S1): splenocytes were gated to FSC-A/FSC-H to remove aggregates, and debris was outgated on FSC/SSC. Splenocytes were then analysed on CD4 and CD8 (PE) and CD19 (APC), with non-B non-T cells remaining in the unstained gate. $CD19^+$ cells were gated on Pacific blue/FITC displaying distinctive populations for IgM, IgM/IgD and IgD. The non-B non-T-cell population was analysed on Pacific blue/FITC, showing $F480^+$, $CD49^+$, $Gr1^{++}$ (Ly6G hi and lo). Each distinctive population was gated on SSC/Peridinin-chlorophyll protein-Cy5.5 for TRPV1 identification. $CD11c^+$(APC-Cy7) dendritic cells were gated against $F480^+$(FITC). For cell surface expression of CD25 and CD80/86, target cells were washed, and surface expression was measured on APC (1·25 μg/ml; eBioscience) and FITC (1·25 μg/ml; eBioscience), respectively.

Intracellular interferon-γ detection by flow cytometry. Target cells were washed, fixed and permeabilized according to the manufacturer's instructions (FIX and PERM; eBioscience). Post permeabilization, cells were labelled with intracellular IFN-γ-Pacific blue (1·25 μg/ml; eBioscience). Cells were acquired on LSRII (BD Biosciences) and analysed using FlowJo software (V10; Tree Star).

Calcium signalling

Splenocytes (1×10^7) were stained with Indo-1 AM at 4 μM for 30 min at room temperature. Cells were pelleted

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(400 ***g***, 5 min), supernatants were removed and cells were re-suspended (complete media) and rested (15 min). Splenocytes were divided into aliquots of 5×10^5 cells, each in 400 μl of medium (1 mM calcium, 1 mM magnesium and 0·5% fetal bovine serum). Anandamide, capsaicin (100 μM) or ionomycin (positive control, 2 μM; Sigma Aldrich, Dorset, MO) were added. Samples were acquired with an LSRII flow cytometer (BD Biosciences, 355-nm UV laser, 360 seconds, low acquisition rate) according to the manufacturer's instructions: http://www.gla.ac.uk/media/media_231654_en.pdf. Using FlowJo software, the data were compensated and analysed. For analysis of specific immune cells, Indo-1 AM loaded cells were additionally stained with the 10-colour staining protocol (above, 20 min on ice), washed (400 ***g***, 4°) and re-suspended in 400 μl of medium (1 mM calcium, 1 mM magnesium and 25 μg/ml fetal bovine serum). Cells were then acquired as above. Data were plotted as number of events against time migrating from Indo-blue (450 LP mirror) to Indo-violet (405/20 filter).

Whole splenocyte in vitro polarization

A 48-well flat-bottomed plate (Sigma-Aldrich) was coated with 100 μl of anti-CD3 (3 μg/ml; eBioscience) overnight at 4°. After removing residual anti-CD3 antibody, spleen cells were re-suspended in the coated plate at 2×10^6 cells/ml (400 μl/well). All cells were stimulated with anti-CD28 antibodies (5 μg/ml; eBioscience) and divided into three separate conditions: Th0 polarization (no added reagents) or Th1 [50 ng/ml of recombinant IL-12, (PeproTech EC Limited, London, UK), 20 μg/ml neutralizing anti-IL4 (eBioscience)]. Cells were incubated (37°, 95% humidity/5% CO_2, 48 hr) and selected wells were treated with 2 μM of CGRP (Broome 200b; Bachem), capsaicin or anandamide (100 μM each; Sigma Aldrich). Supernatants were harvested at 72 hr and stored at − 80° until analysis.

In vitro stimulation of isolated dendritic cells and $CD4^+$ T lymphocytes

For $CD4^+$ T lymphocytes culture (1×10^6 cells/well), plates were coated with anti-CD3 as described above, and anti-CD28 was added. A single treatment at Day 0 of lipopolysaccharide (LPS; 5 μg/ml; Sigma Aldrich) was added to selected wells of isolated dendritic cells (1×10^5 cells/well). When required, CGRP (2 μM; Bachem, Weil am Rhein, Germany) was administered once at day zero. Where detailed in the text, capsaicin or media treatment was given as a single dose at day 0 of culture (100 μM; Sigma Aldrich). Cells were cultured for 72 hr; supernatants were collected at 24 hr for CGRP release, and 72 hr for cytokines. Cells were harvested at 72 hr.

CGRP ELISA

The CGRP-coated plates (Bertin Pharma, Montigny-le-Bretonneux France) were incubated with samples along with the secondary antibody according to the manufacturer's instructions. Standard range 2–500 pg/ml. Results were read on a Dynex MRX11 plate reader (Dynex Technologies, Worthing, UK) at 405 nm.

Cytometric bead array for IL-6, TNF-α and IL-10

Interleukin-6, tumour necrosis factor-α (TNF-α) and IL-10 from dendritic cell culture supernatants were measured using the manufacturer's instructions (BD Biosciences). Briefly, supernatants were incubated with the capture beads specific for IL-6, IL-10 and TNF-α (room temperature, 1 hr), followed by a 2-hr incubation for detection with streptavidin. Bound cytokines were then compared to known standards on MACSQuant (Miltenyi Biotec) using the FCAP array v3 software (BD Biosciences).

Statistics

All statistics were performed using Graphpad prism 6. Where stated, one-way analysis of variance was performed with *post hoc* Tukey test and in-group comparison. A *t*-test was performed where appropriate for direct comparison between two suitable groups (see text). Statistical significance was noted at $P < 0{\cdot}05$.

Results

Subpopulations of splenic dendritic cells and macrophages express TRPV1

Transient receptor potential vanilloid 1 expression has been shown previously on bone-marrow-derived dendritic cells[28] and T cells,[29] although this has never been studied comparatively in primary lymphoid tissue. TRPV1 expression in whole spleen of C57BL/6J mice was analysed using a panel of 10 immune cell markers[27] (Fig. 1a–f). Comparisons were made with unstained TRPV1 populations (F minus 1 controls[27,30]), and splenic populations from TRPV1-deficient ($TRPV1^{-/-}$) mice were stained as negative controls (Fig. 1d–i). There was no heterogeneity in mean fluorescence intensity across cell types (no hi or lo expression). $TRPV1^+$ cells made up approximately 10% of the total $CD11c^-$ $F480^+$, $CD11c^+$ $F480^-$, and non-T non-B-cell populations (Fig. 1d–f) in C57BL/6J mice. Representative flow cytometry histograms are shown for F–1 staining of $CD11c^-$ $F480^+$, $CD11c^+$ $F480^-$ and grouped non-lymphocyte populations in $TRPV1^{-/-}$ mice and C57BL/6J wild-type controls (Fig. 1g–i). The $CD11c^+$ $F480^-$

population contained both $CD4^+$ and $CD8^+$ subpopulations of dendritic cells (see Supplementary material, Fig. S1).

Transient receptor potential vanilloid 1 induces calcium influx in $TRPV1^+$ subpopulations

To determine the functional potential of TRPV1 in splenocytes, calcium influx was measured by flow cytometry (Indo-blue to Indo-violet on 355 UV laser) (Fig. 2a–c). Ionomycin was used as a positive control. TRPV1 agonists capsaicin and anandamide induced a dose-responsive intracellular calcium influx in whole spleen of C57BL/6J mice (Fig. 2d, and see Supplementary material, Fig. S2), which was not seen in calcium-free media (see Supplementary material, Fig. S3). The effect of each agonist was not cumulative: co-incubation with both agonists did not augment independent effects (see Supplementary material, Fig. S2B) suggesting competitive binding sites. Calcium influx in response to the chemical or physiological agonists for TRPV1 was identical in $TRPV1^+$ cell types; $TRPV1^+$ $CD11c^+$ $F480^-$ and $TRPV1^+$ $CD11c^-$

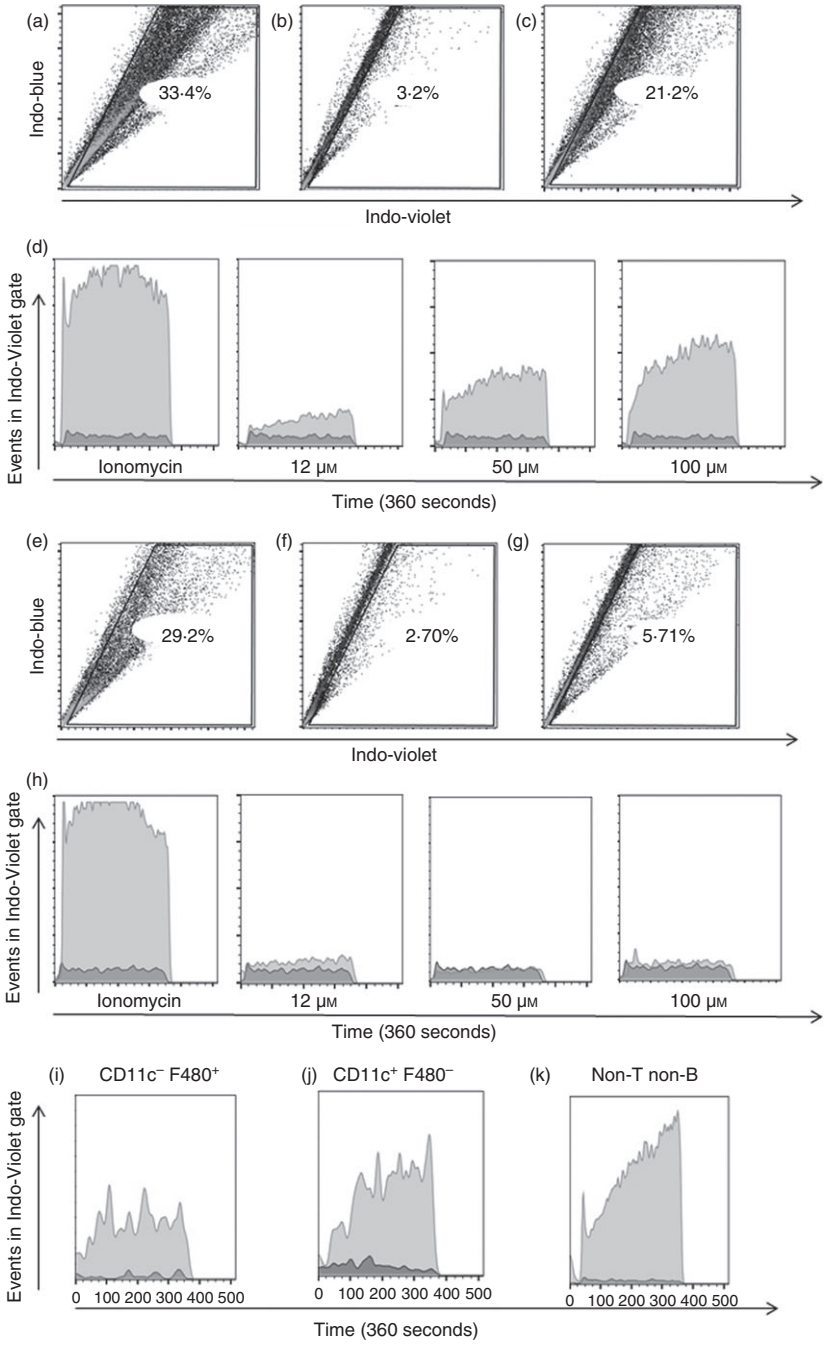

Figure 2. Transient receptor potential vanilloid 1 (TRPV1) is an active calcium channel in $TRPV1^+$ subpopulations. TRPV1 functionality was tested by the treatment of whole spleen cell populations with TRPV1 agonist capsaicin. (a–c) Representative flow cytometry plots showing cell migration from Indo-blue (calcium-free) to Indo-violet (calcium-bound) in response to positive control ionomycin (a), media (b) and capsaicin (c) in C57BL/6J whole spleen. (d) Calcium influx induced by capsaicin is dose dependent. Representative plots of events in Indo-violet gate over time (360 seconds) showing dose-dependent increase in calcium influx after capsaicin treatment (12–100 μM) in C57BL/6J. (e–g) Representative flow cytometry plots showing cell migration from Indo-blue (calcium-free) to Indo-violet (calcium-bound) in response to positive control ionomycin (e), media (f) and capsaicin (g) in TRPV1-deficient mice. (h) Calcium influx recordings post capsaicin in TRPV1-deficient mice. Time plots of events in Indo-violet gate over 360 seconds are shown for capsaicin treatment (12–100 μM). (i–k) $CD11c^-$ $F480^+$ (i), Lin^- $CD11c^+$ $F480^-$ (j) and non-T non-B (k) cells all respond with calcium influx during treatment with 100 μM capsaicin over 360 seconds. Media controls shown in dark grey. Data are representative of three separate experiments, $n = 3$ in each group.

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F480$^+$ and non-T non-B-cell responded to both capsaicin (Fig. 2i–k) and anandamide (see Supplementary material, Fig. S4). Importantly, TRPV1-deficient mice showed no calcium signalling in response to capsaicin (Fig. 2e–h) demonstrating the receptor specificity of the calcium response. TRPV1-deficient splenocytes did respond to ionomycin (Fig. 2e).

BALB/c mice have significantly larger subpopulations of TRPV1$^+$ CD11c$^+$ F480$^-$ dendritic cells and TRPV1$^+$ CD11c$^-$ F480$^+$ macrophages in the spleen compared with both C57BL/6J and AKR mice

We were particularly interested in the mouse strain variation of TRPV1 expression and function in the context of calcium mobilization. We therefore defined TRPV1 expression and functionality in BALB/c and AKR mice using flow cytometry as described above (Fig. 3a,b). Although BALB/c expressed twofold less TRPV1 than AKR overall ($P < 0{\cdot}01$, data not shown), expression was weighted towards antigen-presenting cells. BALB/c expressed significantly more TRPV1 on both CD11c$^-$ F480$^+$ macrophages (Fig. 3c), CD11c$^+$ F480$^-$ dendritic cells (Fig. 3d) and non-T non-B cells (Fig. 3e) compared with both AKR and C57BL/6J. Expression was not indicative of differences in cell number/immune cell distribution (data not shown). Again, there was no heterogeneity in per-cell expression across cell types. Representative histograms of surface TRPV1 staining are shown for CD11c$^-$ F480$^+$ macrophages, CD11c$^+$ F480$^-$ dendritic cells and non-T non-B-cells (Fig. 3f–h). Despite variation in cellular TRPV1 distribution, there were no significant differences in calcium influx between BALB/c and AKR in whole spleen populations, either in response to capsaicin (Fig. 4a–c), anandamide, or both (see Supplementary material, Fig. S4). In fact BALB/c and AKR demonstrated calcium influx similar to that seen for C57BL/6J (Fig. 2, and see Supplementary material, Fig. S2). This held true for individual TRPV1$^+$ populations; there was no significant difference between calcium

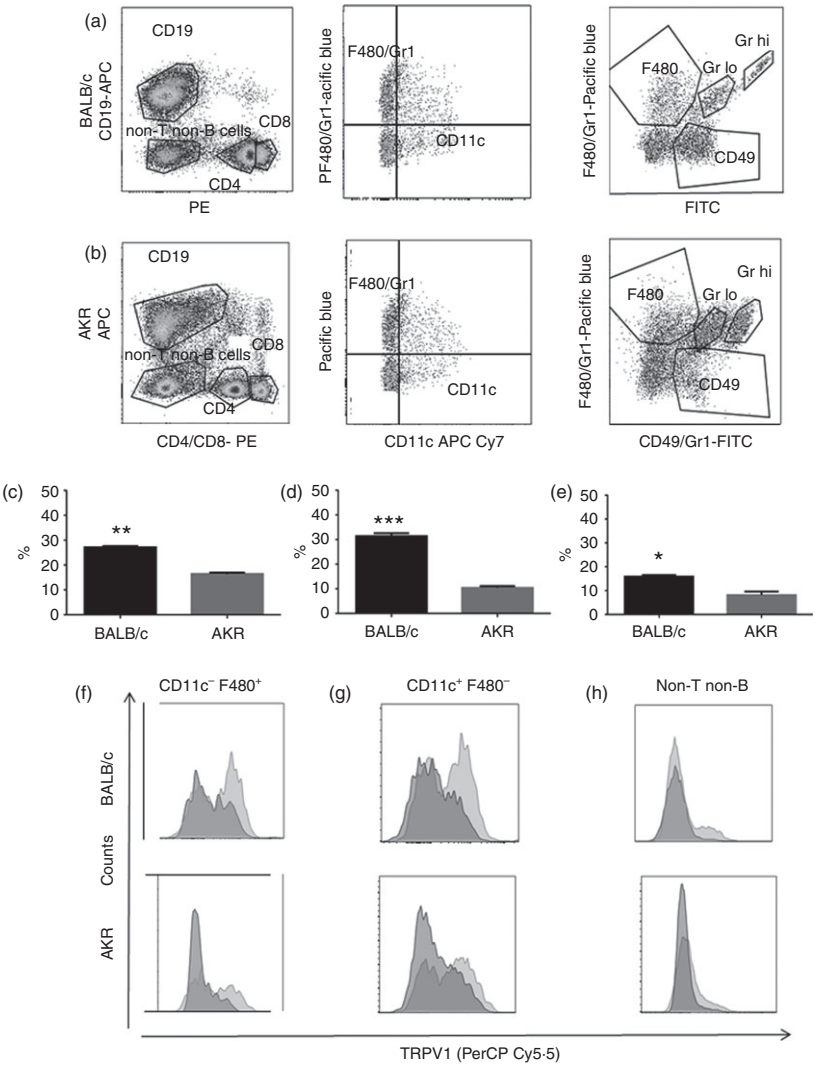

Figure 3. BALB/c have significantly more transient receptor potential vanilloid 1-positive (TRPV1$^+$) antigen-presenting cells compared with AKR. TRPV1 expression was investigated in BALB/c and AKR whole spleen populations using flow cytometry. (a, b) Representative dot plots illustrate 10-cell strategy used to identify distinct immune cell populations in the spleen of BALB/c (a) and AKR (b). (c–e) TRPV1 expression in CD11c$^-$ F480$^+$ (c), CD11c$^+$ F480 (d) and non-T non-B (e) cells in BALB/c (black bars) and AKR (grey bars) splenocytes. (f–h) Histograms showing TRPV1 expression by flow cytometry in CD11c$^-$ F480$^+$ (f), CD11c$^+$ F480$^-$ (g) and non-T non-B (h) splenocytes in BALB/c and AKR. F–1 controls are shown in dark grey and TRPV1 staining in light grey. Data are representative of three experiments, $n = 3$ in each group. Significance shown using *t*-test (c–e).*$P < 0{\cdot}05$, ***$P < 0{\cdot}001$.

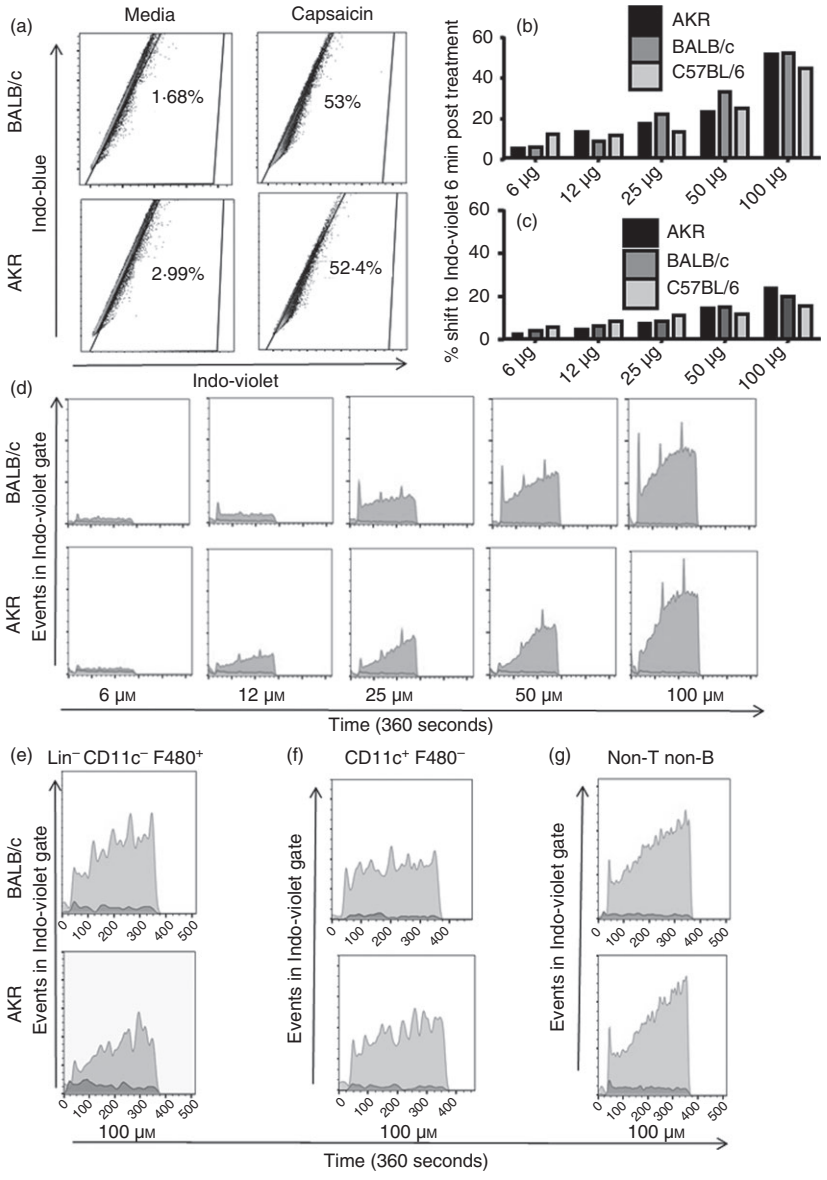

Figure 4. BALB/c and AKR splenocytes respond to transient receptor potential vanilloid 1 (TRPV1) agonist capsaicin. TRPV1 functionality was tested by treating whole spleen cell populations with TRPV1 agonist capsaicin. (a) Representative flow cytometry plots showing cell migration from Indo-blue (calcium-free) to Indo-violet (calcium-bound) in BALB/c and AKR splenocytes in response to media and capsaicin. (b, c) Quantitative comparison of dose-dependent increase in calcium influx in AKR, BALB/c and C57BL/6J splenocytes in response to capsaicin (b) and anandamide (c). (d) Representative time plots of events in Indo-violet gate over time (360 seconds) showing dose-dependent increase in calcium influx (6–100 µM) in BALB/c and AKR splenocytes. (e–g) $CD11c^-$ $F480^+$ (e), $CD11c^+$ $F480^-$ (f) and non-T non-B (g) cells in BALB/c and AKR respond to treatment with 100 µM capsaicin. Media controls are shown in dark grey. Data are representative of three experiments, $n = 3$ in each group.

influx in either $CD11c^+$ $F480^-$, Lin^- $CD11c^-$ $F480^+$ or non-T non-B-cell populations in BALB/c and AKR in response to capsaicin (Fig. 4e–g) or anandamide (see Supplementary material, Fig. S5). Of note, $CD4^+$ and $CD8^+$ T-cell populations also responded to capsaicin as shown previously[12] (see Supplementary material, Fig. S6).

Capsaicin induced CGRP release from dendritic cells but not T cells in isolated culture

To determine whether TRPV1 activation by capsaicin could also induce neuropeptide release in $TRPV1^+$ cells, isolated $CD4^+$ T cells and dendritic cells were cultured separately with or without relevant stimulation (Fig. 5). Although $CD4^+$ T cells were able to release a small amount of CGRP, there was no significant difference with capsaicin treatment, and release was not enhanced with TCR activation (Fig. 5a). Conversely, dendritic cells demonstrated significantly enhanced release of CGRP in direct response to capsaicin treatment after LPS activation; this was not seen in TRPV1-deficient dendritic cells (Fig. 5b).

Both dendritic cells and T cells respond to CGRP *in vitro*

To determine the effect of released CGRP on isolated $CD4^+$ T lymphocytes and dendritic cells, each population was cultured separately with exogenous CGRP (2 µM) for 72 hr. CGRP concentration was optimized according to previous data[31] (see Supplementary material, Fig. S7). Our observations are in agreement with published data showing that at lower concentrations CGRP exerts an anti-inflammatory effect.[32] For each cell type, CGRP induced significant down-regulation of both intracellular

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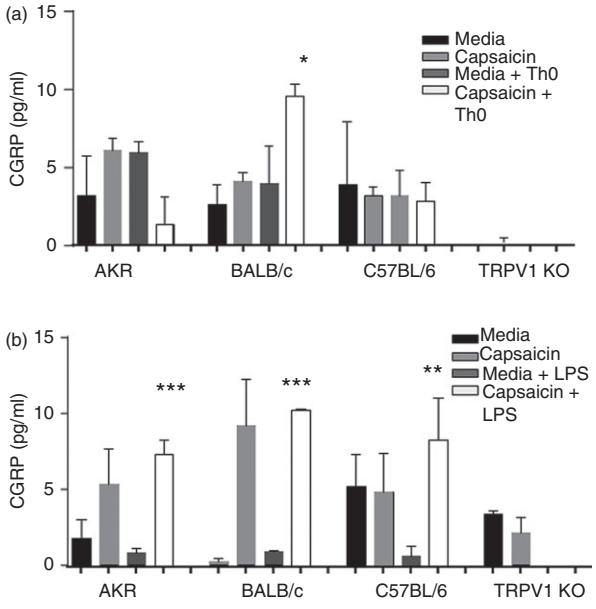

Figure 5. Transient receptor potential vanilloid 1 (TRPV1) agonist capsaicin induced calcitonin-gene-related peptide (CGRP) release from dendritic cells but not $CD4^+$ T cells in isolated culture. (a) Isolated splenic $CD4^+$ T cells do not release CGRP in response to TRPV1 agonist capsaicin, either in resting state or after CD3/CD28 stimulation. CGRP release from isolated T-cell culture was measured by ELISA after 24 hr of incubation. (b) Capsaicin (100 μM) stimulated the release of CGRP from isolated dendritic cells with lipopolysaccharide (LPS) stimulation. Data show released CGRP by ELISA after 24 hr of culture. TRPV1-deficient dendritic cells or T cells did not release CGRP in response to capsaicin under any conditions. Data representative of three experiments, $n = 5$ in each group. Significance shown by one-way analysis of variance followed by Tukey post hoc within each strain. $^*P < 0{\cdot}05$, $^{***}P < 0{\cdot}001$.

IFN-γ and surface expression of activation markers CD25 and CD80/86, respectively (Fig. 6a,e). In both cases, this was only seen in the presence of relative activating factors anti-CD3/anti-CD28 or LPS (Fig. 6b,f). These effects were not due to cytotoxicity as shown by flow cytometry plots of cell size and granularity (see Supplementary material, Fig. S8) but were due to the direct effects of CGRP as CGRP inhibition abrogated the observed effects (see Supplementary material, Fig. S8a,c). Of key significance, culture of isolated dendritic cells with exogenous CGRP in the presence of LPS stimulation induced up-regulation in IL-6 and IL-10 release, while significantly inhibiting TNF-α release (Fig. 6i–k). The up-regulation of IL-6 in response to CGRP has been shown previously in mice,[33] and for keratinocytes[34] and hepatocytes.[35]

Capsaicin significantly down-regulated IFN-γ in whole spleen cell cultures during Th1 polarization

To determine whether TRPV1-mediated CGRP release could have a significant effect on T-cell polarization, whole spleen cell populations were cultured with capsaicin during Th0 or Th1 stimulating conditions. CGRP has been reported to down-regulate IFN-γ production *in vitro*,[36] but this has not been linked to TRPV1 activation in immune cells. Under Th0 conditions, AKR and C57BL/6J splenocytes released substantial CGRP (50 pg/ml, SEM ± 2·16) (Fig. 7a). Capsaicin treatment significantly inhibited this release to below levels of detection under Th0 conditions. This was not due to cell cytotoxicity (data now shown). Conversely, capsaicin induced CGRP release from BALB/c splenocytes under the same conditions (95 pg/ml SEM ± 9·6 pg/ml). As expected, capsaicin had no effect on negligible IFN-γ release under Th0 conditions (Fig. 7c). Under Th1-driving conditions, capsaicin-induced CGRP release in BALB/c splenocytes; there was no significant effect on supernatant CGRP concentration in either AKR or C57BL/6J cells (Fig. 7b). However, in all three strains, capsaicin significantly reduced IFN-γ expression as measured by intracellular flow cytometry (Fig. 7d–f). Across both polarization conditions BALB/c consistently released significant CGRP in response to capsaicin; there was no discriminating response to TRPV1 agonism. This was unique to BALB/c and did not occur in the absence of capsaicin. Importantly, the effect of capsaicin on IFN-γ was absent in TRPV1-deficient cells, supporting a specific role for TRPV1 and CGRP release during Th1 polarization (Fig. 8).

Discussion

In this study we have demonstrated constitutive TRPV1 expression on primary splenic immune cell populations across three genetically unrelated strains of mice. TRPV1 expression has been shown previously on cultured mouse and human dendritic cells,[28,37] and T cells;[29,38] however, this is the first time that comparative expression has been shown in a primary resting lymph population. Across all three mouse strains, distinct subsets of $TRPV1^+$ $CD11c^-$ $F480^+$ macrophages and $TRPV1^+$ $CD11c^+$ $F480^-$ dendritic cells could be detected. In peripheral nerves, TRPV1 functions as a calcium channel. Calcium channels are known to be important in T-lymphocyte activation and proliferation.[39,40] However, controversy surrounds functionally similar channels in antigen-presenting cells. In 2005, O'Connell *et al.*[41] used calcium imaging to conclude that TRPV1 agonists do not induce calcium influx in bone-marrow-derived dendritic cells. Conversely, in the same year Basu and Srivastava[28] demonstrated active TRPV1 signalling in similar cultured cells. Here we have shown definitively that all $TRPV1^+$ splenic immune cell populations can respond in a dose-dependent manner to natural TRPV1 ligands capsaicin and anandamide, and that this results in an influx of exogenous calcium. Although our study cannot settle the controversy sur-

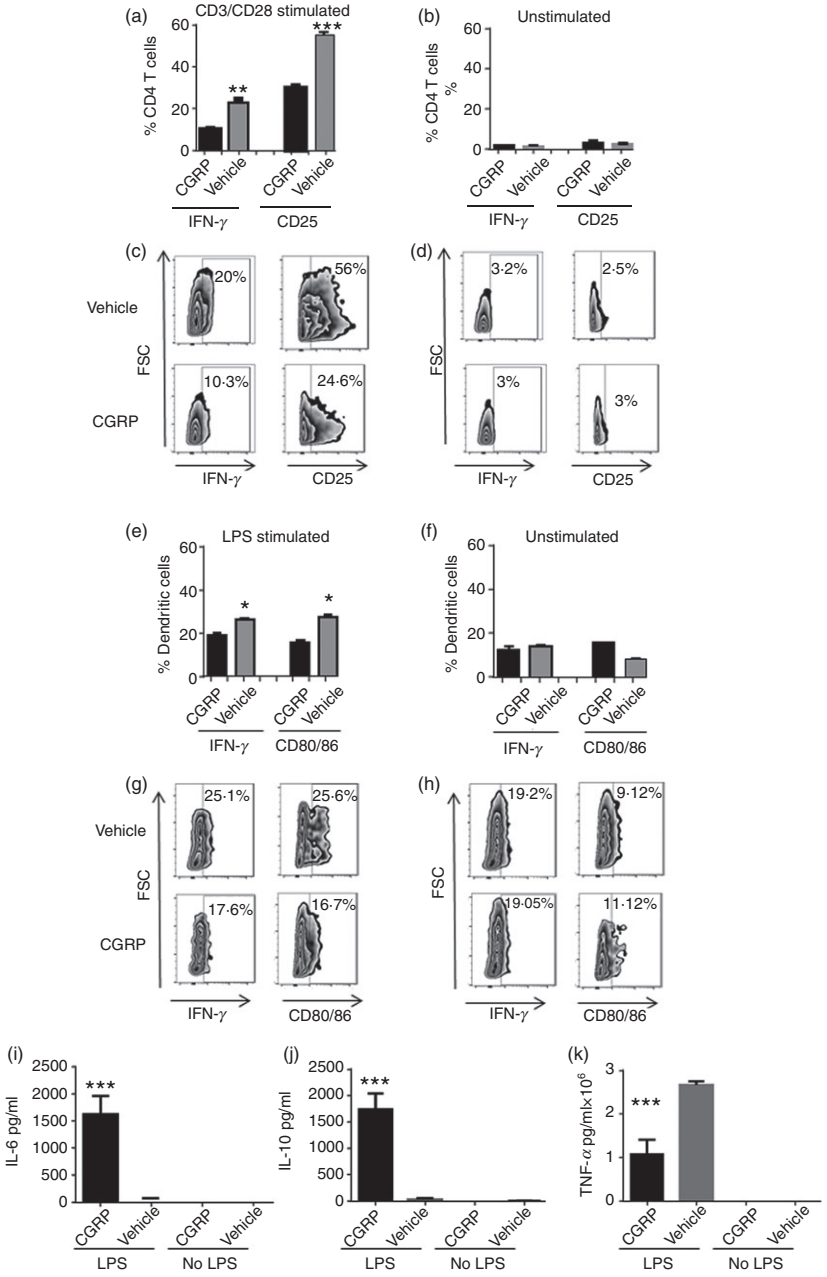

Figure 6. Both isolated dendritic cell and $CD4^+$ T-cell populations respond to calcitonin-gene-related peptide (CGRP) *in vitro*. (a, b) Isolated splenic C57BL/6J $CD4^+$ T cells respond to CGRP treatment *in vitro* only after CD3/CD28 stimulation. Quantitative representation of flow cytometry data for intracellular interferon-γ (IFN-γ) and cell surface CD25 in CD3/CD28 stimulated (a) or unstimulated (b) splenic $CD4^+$ T cells with (black bars) or without (grey bars) CGRP treatment. (c, d) Representative flow cytometry plots for stimulated (a) and unstimulated (d) $CD4^+$ T cells. (d) CGRP treatment had no effect on intracellular IFN-γ or CD80/86 expression in the absence of lipopolysaccharide (LPS) stimulation. (e, f) CGRP treatment of $CD11c^+$ dendritic cells with LPS induced down-regulation of intracellular IFN-γ and cell surface CD80/86 expression by flow cytometry. (g, h) Representative flow cytometry plots for stimulated (g) and unstimulated (h) $CD11c^+$ dendritic cells. (i–k) Cytokine levels released from $CD11c^+$ dendritic cells with or without LPS. Interleukin-6 (IL-6) (i) IL-10 (j) and tumour necrosis factor-α (TNFα) (k). These data are representative of three experiments, $n = 5$ for each group. Significance shown by one-way analysis of variance followed by Tukey post hoc test. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

rounding TRPV1 expression in bone-marrow-derived dendritic cells, we do make an important distinction with our data. First, by using primary uncultured splenocytes, the cell population has not undergone artificial maturation or activation. Second, a proportion of dendritic cells demonstrate $TRPV1^+$ surface expression and receptor functionality; these were found in $CD4^+$, $CD8^+$ and $CD4^-$ $CD8^-$ subsets. Our calcium signalling data are consistent with observations in $TRPV1^+$ T cells[12] and the known effect of TRPV1 ligands in peripheral nerves.

Although TRPV1-induced calcium influx has previously been shown in immune cells, neuropeptide release from immune cells as a result of TRPV1 engagement has not been shown to date, despite studies demonstrating separate anti-inflammatory effects of both TRPV1 agonism and neuropeptide CGRP. TRPV1 agonists have variously been shown to enhance IL-10-producing macrophages,[36,42] induce T-cell apoptosis[29,38] and inhibit dendritic cell activation and proliferation.[37] Similarly, the anti-inflammatory effects of TRPV1 agonists have been shown *in vivo*,[42–45] although none demonstrated the involvement of CGRP.

In peripheral nerves, one of the primary outcomes of TRPV1 activation is the release of CGRP, and its role in immune responses has been studied for more than a decade.[13] Several different systems have shown that

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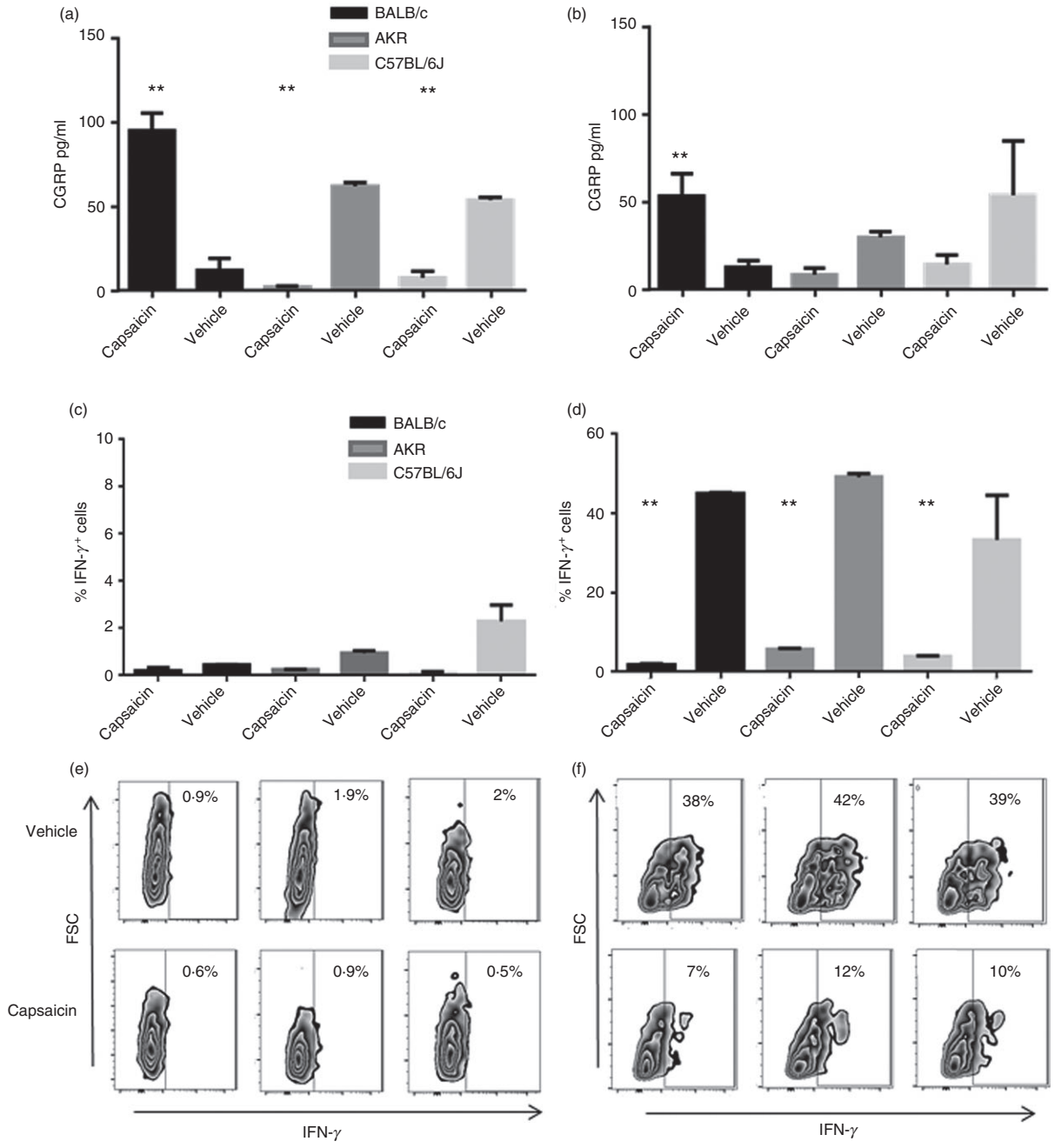

Figure 7. Capsaicin induced down-regulation of interferon-γ (IFN-γ) during T helper type 1 (Th1) polarization. calcitonin-gene-related peptide (CGRP) and IFN-γ expression were measured during Th0 and Th1 polarization in whole spleen cultures. (a) Capsaicin treatment (100 μM) down-regulated CGRP release from Th0 stimulated culture in AKR and C57BL/6 splenocytes, as measured by ELISA. Conversely, capsaicin induced CGRP release in BALB/c splenocytes under Th0 conditions. (b) Capsaicin (100 μM) had no effect on CGRP release from Th1 stimulated culture in AKR and C57BL/6 splenocytes, as measured by ELISA. Capsaicin significantly induced CGRP release in BALB/c splenocytes under Th1 conditions. (c) Capsaicin had no effect in any strain on negligible IFN-γ release during Th0 conditions, as measured by intracellular flow cytometry. (d) Capsaicin (100 μM) significantly inhibited IFN-γ expression in BALB/c, AKR and C57BL/6J splenocytes stimulated under Th1 conditions, as measured by intracellular flow cytometry. (e) Representative flow cytometry plots showing intracellular IFN-γ expression in Th0 (e) or Th1 (f) conditions. Data are representative of three experiments, $n = 5$ in each group. Significance shown by one-way analysis of variance followed by Tukey post hoc within each strain. $^{**}P < 0{\cdot}01$ BALB/c (black), AKR (grey) and C57BL/6J (light grey) are shown.

CGRP has a regulatory effect on both dendritic cell and T-cell functions,[4,37,46,47] down-regulating IFN-γ and promoting IL-10.[4,15,16,36] The over-riding opinion is one of immune control and regulation, under the premise that CGRP is released from TRPV1$^+$ sensory neurons.

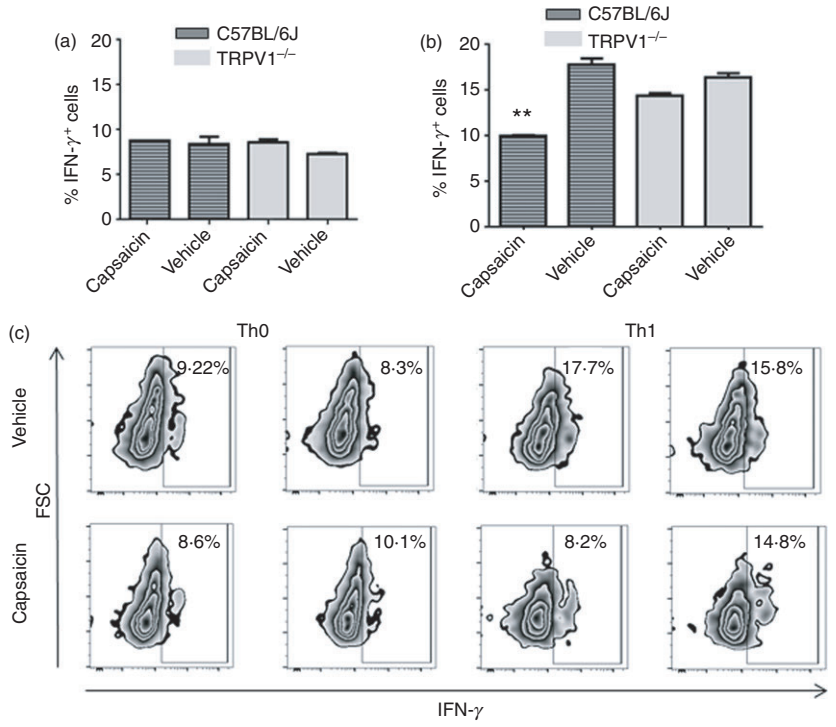

Figure 8. Capsaicin did not induce the down-regulation of interferon-γ (IFN-γ) during T helper type 1 (Th1) polarization in transient receptor potential vanilloid 1 (TRPV1) -deficient mice. IFN-γ expression was measured during Th0 and Th1 polarization in whole spleen cultures in C57BL/6J and TRPV1-deficient splenocytes. (a) Capsaicin treatment (100 μM) had no effect on negligible IFN-γ release during Th0 conditions for either strain, as measured by intracellular flow cytometry. (b) Capsaicin (100 μM) significantly inhibited IFN-γ expression in C57BL/6J splenocytes stimulated under Th1 conditions, as measured by intracellular flow cytometry. This was not seen in TRPV1-deficient splenocytes under the same conditions. Dark grey bars C57BL6; light grey bars TRPV1-deficient splenocytes. Data are representative of three experiments, $n = 3$ in each group. Significance shown by one-way analysis of variance followed by Tukey post hoc within each strain. $^{**}P < 0{\cdot}01$.

Our data both support and counter these observations. In our hands, $CD4^+$ T cells did not release CGRP in response to TRPV1 agonists, despite capsaicin-induced calcium signalling and clear responses to exogenous CGRP *in vitro*. $CD11c^+$ $F480^-$ dendritic cells on the other hand, responded to the TRPV1 agonist capsaicin with induction of calcium signalling and CGRP release. Although at first paradoxical, it has been suggested that the two processes of calcium influx and CGRP release are independent and may involve distinct mechanisms.[48] This is poorly understood. However, given that $TRPV1^{-/-}$ dendritic cells did not release CGRP in response to capsaicin, we are confident that the effects we have observed are TRPV1-specific. Dendritic cells are known to express the CGRP receptor CalcR, and respond to CGRP in an anti-inflammatory manner.[16,28] In our hands, CGRP treatment significantly reduced TNF-α release, while up-regulating IL-10 release in LPS-activated dendritic cells. These data suggest that TRPV1 activation of dendritic cells plays a role in their homeostasis and regulation in the spleen, through release of CGRP. It has been suggested that CGRP plays a role in the migration and inflammatory responses of dermal cells during contact hypersensitivity.[42] Langerhans cells in particular sit in close proximity to $CGRP^+$ c fibres[7,9] ideally situated to respond to released CGRP after TRPV1 activation. Our data suggest that splenic dendritic cells are additionally able to respond to 'neural' ligands and themselves amplify CGRP release through the same receptor as that found on nerves.

Finally, we have shown that capsaicin can inhibit the release of IFN-γ in Th1 polarized splenic cell culture across three strains of mice, demonstrating the link between immune cell expression of TRPV1 and polarization. Although release of CGRP under these conditions into the supernatant did not reflect that seen with stimulated isolated dendritic cells, the observations do not account for uptake of CGRP by activated T cells, which would drive the observed down-regulation of IFN-γ. Interestingly, BALB/c were unable to regulate CGRP release when stimulated through TRPV1. Given that BALB/c is the preferred strain for several disease models,[47–49] it is interesting to speculate that the unregulated release of CGRP could skew BALB/c T-cell polarization towards a Th2 immune response due to down-regulated IFN-γ. However, it is also possible that in whole spleen cell culture, additional TRPV1-independent mechanisms are at play;[8] this is the subject of further investigation.

In conclusion, TRPV1 expression may influence migration and activation of immune cells through its calcium channel functionality,[28] but may also contribute to immune regulation and homeostasis through induced release of CGRP and subsequent down-regulation of activation markers and cytokine release. The influence of TRPV1 on homeostasis and regulation in the spleen remains to be seen, but the fact that CGRP can be induced in immune cells using the same ligand : receptor process as that found in peripheral nerves suggests amplification of any nerve/immune cell cross-talk. It is becoming clear that many of the ligand/signalling processes that

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are well defined in the nervous system, may in fact be integral to the immune system, and vice versa.[17] We suggest that the true nature of host variability in response to the same pathogen may involve contributions from both nervous and immune systems.

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Disclosures

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative flow cytometry plots showing $CD11c^+$ cells in whole spleen. Representative flow cytometry plot showing heterogeneity of $CD11c^+$ dendritic cells with respect to CD4 and CD8 expression, from whole spleen.

Figure S2. Transient receptor potential vanilloid 1 (TRPV1) agonists anandamide and capsaicin induce calcium influx in C57BL/6J splenocytes. Known TRPV1 ligands capsaicin and anandamide were incubated separately and together with whole spleen cell populations, and calcium signalling was immediately recorded using flow cytometry (Indo-1 AM). Anadamide (a) and capsaicin and anadamide together (b) induced calcium influx in whole spleen population. This influx was dose responsive (6–100 μM of each agonist). Calcium influx was measured in 5×10^5 cells over 360 seconds. Media-only responses are shown in dark grey. Data shown are representative of three separate experiments. *y*-axis: count of cells responding to TRPV1 agonist. *x*-axis: time in seconds.

Figure S3. C57BL/6J splenocytes do not respond to capsaicin in calcium-free media. Indo-1 violet positive cells were gated over time identifying capsaicin-responsive cells. Known transient receptor potential vanilloid 1 (TRPV1) ligand capsaicin was incubated with whole spleen cell populations at 12, 50 and 100 μM, and calcium signalling was immediately recorded using flow cytometry (Indo1-AM). Ionomycin (2 μM) was used as a positive control while vehicle alone was used as a baseline negative control. Representative flow cytometry plots shown for Indo-blue to Indo-violet shift (left hand column), and events in Indo-violet gate over time (360 seconds) showing no change in calcium influx after capsaicin treatment (right hand column). Data are representative of two independent experiments, $n = 3$.

Figure S4. Transient receptor potential vanilloid 1 (TRPV1) agonists anandamide and capsaicin induced calcium influx in BALB/c and AKR whole spleen. Known TRPV1 ligands capsaicin and anandamide were incubated separately and together with whole spleen cell populations, and calcium signalling was immediately recorded using flow cytometry (Indo1-AM). A single treatment of anandamide (a, c) and both agonists together (b, d) induced calcium influx in whole spleen population. This influx correlated with increased concentrations of both agonists (6, 12, 25, 50 and 100 μM). Calcium influx was measured in 5×10^5 cells over 360 seconds. Data shown are representative of three separate experiments. *y*-axis: number of cells responding to TRPV1 agonist. *x*-axis: time in seconds.

Figure S5. Calcium influx in C57BL/6J, BALB/c and AKR primary immune cells in response to anandamide. Calcium influx in response to anandamide (100 μM) in $CD11c^-$ $F480^+$ (a, d, g), $CD11c^+$ $F480^-$ (b, e, h) and non-T non-B (c, f, i) cells, respectively. Media-only responses are shown in dark grey. Data are representative of three experiments, $n = 3$ in each group.

Figure S6. Capsaicin and anadamide induced calcium influx in $CD4^+$ and $CD8^+$ T lymphocytes. Known transient receptor potential vanilloid 1 (TRPV1) ligands capsaicin and anandamide were incubated separately with whole spleen cell population. Calcium influx was measured after capsaicin and anandamide treatment (100 μM each) in BALB/c, AKR and C57BL/6J. The influx correlated with a single dose of capsaicin and anadamide both at 100 μM. Calcium influx was measured in an estimated $1{\cdot}25 \times 10^5$ cells in $CD4^+$, $0{\cdot}5 \times 10^5$ cells in $CD8^+$ and $0{\cdot}5 \times 10^5$ cells in non-B non-T lymphocytes over 360 seconds. Media-only responses are shown in dark grey. Data shown are representative of three separate experiments. *y*-axis: number of cells responding to TRPV1 agonist. *x*-axis: time in seconds.

Figure S7. Isolated cell populations respond to calcitonin-gene-related peptide (CGRP) *in vitro*. (a, b) Isolated splenic C57BL/6J $CD11c^+$ cells respond to CGRP treatment *in vitro* only after lipopolysaccharide (LPS) stimulation. Titration of CGRP (2–20 μM) treatment of $CD11c^+$ dendritic cells with LPS, induced down-regulation of intracellular interferon-γ (IFN-γ) (a, b) and cell surface CD80/86 expression (c, d) by flow cytometry only at lower concentrations. Inhibition of CGRP with peptide hCGRP abrogated any effect on intracellular IFN-γ or CD80/86 expression with or without LPS stimulation. Significance shown by one-way analysis of variance followed by Tukey post hoc test within each strain. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

Figure S8. SSC versus FSC plots showing no variation in cell size or granularity after either vehicle or capsaicin treatment over 3 days. Splenocytes post vehicle (a) or capsaicin (b) treatment under T helper type 0 (Th0) condition. Splenocytes post vehicle (c) or capsaicin (d) treatment under Th1 conditions.

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