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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

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Sofie De Bondt

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
Prof. dr. Bieke BROUX

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Regulatory T cells to the rescue: Unravelling Piezo1 interactions during blood-brain barrier migration in multiple sclerosis *

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*Running title: *Piezo1 activation in BBB-transmigrating Tregs*

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ABSTRACT

With over 2.8 million people affected worldwide, multiple sclerosis (MS) remains one of the leading causes of neurologic disability in young adults. Though both the number and functionality of regulatory T cells (Tregs) are diminished in these patients, the therapeutic potential of Treg therapy remains limited. It is suggested that this is in part due to the pro-inflammatory phenotype switch these cells experience during migration through the inflamed blood-brain barrier (BBB). Indeed, our group recently showed that Tregs lose their immunosuppressive and regenerative functions, limiting their ability to block the local immune response and tissue damage. The resulting inflammation increases BBB leakiness, leading to immune cell infiltration and neurologic damage. Our previous study additionally revealed that Piezo1, a mechanosensitive ion channel, was upregulated in BBB-transmigrated Tregs. Therefore, we investigated Piezo1 activation in Tregs, exploring their involvement in this phenotype switch. Using flow cytometry and suppression assays, we demonstrate a loss of Treg phenotype and functionality upon Piezo1 activation, while qPCR data provides evidence implicating purinergic receptor signalling in mediating these changes. Next, calcium imaging and flow cytometry analyses of T cells migrating through an *in vitro* inflamed BBB model reveal calcium influxes and a pro-inflammatory phenotype switch in these cells. Nevertheless, we were unable to restore the T cell phenotype using a Piezo1 inhibitor, implying involvement of other Treg-BBB interactions that require further investigation. Thereby, we aim to further elucidate the mechanisms underlying the phenotype switch of BBB-transmigrated Tregs, intending to improve the safety and efficacy of Treg-based MS therapies.

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) that affects more than 2.8 million people worldwide (1, 2). The disease is characterized by autoreactive immune cells that infiltrate into the CNS, causing neuroinflammation and demyelination of axons (3). These autoreactive immune cells emerge from peripheral CD4+ T cells activated by foreign antigens that cross-react with myelin antigens, a process called 'molecular mimicry' (4). Once activated, T cells migrate to the CNS, where myelin antigens cause reactivation of the cells, initiating an inflammatory response that damages myelin sheaths, and indirectly, axons (4, 5). This progressive accumulation of neurologic damage blocks signal conduction, prompting clinical symptoms such as motor dysfunction, visual and sensory deficits, and cognitive impairment (1, 5, 6). MS onset occurs mostly between 20 and 40 years of age, twice as often in women than men, making it one of the leading causes of neurologic disability in young adults (1, 6). In early stages, MS typically presents as a relapsing-remitting disease, often transitioning to a progressive phase with irreversible accumulation of neurologic disability. MS is induced by a combination of genetic and environmental factors that trigger migration of myelin-reactive immune cells across the **blood-brain barrier (BBB)** into the CNS (6, 7).

Consisting of endothelial cells connected by tight junctions, pericytes, astrocyte endfeet, and a basal membrane, the BBB plays an essential role in maintaining cerebral homeostasis (1, 8). It forms the barrier between the systemic circulation and the CNS, regulating bidirectional exchange of nutrients and waste products. Furthermore, the BBB limits entry of toxic metabolites, xenobiotics, and immune cells into the CNS (1, 9). In MS however, the integrity of this barrier is disrupted, causing it to become leaky and allowing infiltration of myelin-reactive immune cells into the CNS (7). This infiltration is facilitated by both downregulation of tight junction proteins, such as zonula occludens-1 (ZO-1) and occludin, and upregulation of adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1), within the BBB (1, 7, 10, 11). These changes are exacerbated by the inflammatory environment created in the CNS, with the presence of inflammatory cytokines like interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (7). Finally, increased expression of chemokines by endothelial cells of the BBB further accelerates immune cell infiltration into the CNS (7).

Current disease modifying treatments for MS focus on immunomodulation, aiming to restore the balance between autoreactive and regulatory immune responses (7). Rituximab for example, a monoclonal anti-CD20 antibody, achieves this by inhibiting B cell activation, while IFN-β decreases pro- and increases anti-inflammatory cytokine production. Other methods focus on preventing immune infiltration into the CNS, such as the migration inhibiting monoclonal antibody Natalizumab (7). Recent research into therapeutic approaches for MS has proposed a novel treatment strategy, harnessing the immunomodulatory properties of **regulatory T cells (Tregs)** (12-14).

Tregs are CD4+CD25hiCD127lo T cells marked by expression of the regulatory transcription factor forkhead box P3 (FOXP3) (15-17). These cells exhibit immunosuppressive capabilities that play a crucial role in preventing autoimmunity (16, 17). By suppressing autoreactive immune responses and reducing neuroinflammation, the main function of Tregs is maintaining immune tolerance. This suppressive function is accomplished via a combination of cell-cell contact and the release of cytokines. Treg cell-cell interactions are achieved via expression of co-inhibitory molecules, such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4). To maintain normal immune responses, there is a balance between Tregs, activated through CTLA-4, and effector T cells (Teff), induced by CD28 (18). Via constitutive expression of CTLA-4, Tregs competitively inhibit CD28 signalling on antigen presenting cells, thereby reducing activation of Teff (19). Moreover, CTLA-4 synergizes with transforming growth factor beta (TGF-β) to promote the generation of Tregs via upregulation of FOXP3, which in turn upregulates CTLA-4 expression (20). Besides direct interactions, the protective effect of Tregs is also dependent on secretion of the anti-inflammatory cytokines interleukin 10 (IL-10) and TGF-β (17, 21). IL-10 has been linked to various immunosuppressive effects, including differentiation of CD4+ T cells into an IL-10 producing (Tr1) Treg subset and controlling expansion of other T cell populations (22, 23). Similarly, TGF-β suppresses T cell differentiation into T helper (Th)1 or Th2 cells and inhibits expression of IFN-γ by CD8+ T cells, while promoting the generation of Tregs (24). Moreover, via production of amphiregulin (AREG) and CCN3, Tregs have demonstrated regenerative capacities in the CNS (25, 26). More specifically, AREG derived from brain Tregs was shown to regulate astrogliosis and promote neurological recovery in mice (26). Similarly, murine brain Treg-derived CCN3 showed improved CNS regeneration by enhancing oligodendrocyte differentiation and myelination (25).

In the context of MS however, Tregs are known to be deficient in their number and function, in both the periphery and after BBB transmigration (17, 27, 28). This loss of function is marked by downregulated expression of FOXP3 (exFOXP3 T cells) (16, 28). These findings encouraged research into the development of Treg-based cell therapies for MS patients. Here, *ex vivo* expanded Tregs are administered into patients to boost their Treg numbers and functionality, thereby helping to suppress the autoreactive immune response (12, 21). While these Tregs can either be allogeneic or autologous, patient-derived Tregs are often dysfunctional, requiring *ex vivo* manipulations to improve their stability and suppressive capacity (21).

Interestingly, Tregs are known to exhibit functional plasticity (29). In autoimmunity, they

can convert into inflammatory Th1 and Th17-like cells, depending on the microenvironment (29). For example, culturing human Tregs in the presence of IL-12 results in a Th1-like Treg phenotype with reduced suppressive activity (30). Meanwhile, conversion of murine Tregs into Th17-like cells seems to be mediated by IL-6, as well as TGF-β, IL-1, and IL-21 (31, 32). Previous research within our group has established that in MS, this plasticity can be induced by interaction with the inflamed BBB (16). Here, transmigration of Tregs through inflamed BBB-endothelial cells evoked a pro-inflammatory phenotype switch, with reduced suppressive and regenerative capacities. Interfering in the pathways involved in this Treg phenotype switch, such as using the mTORC1 inhibitor rapamycin, could aid in restoring the Treg phenotype (16, 33). Therefore, to improve the safety and efficacy of Treg-based MS therapies, there is a need to understand the interactions between Tregs and the BBB. Moreover, identifying and blocking these interactions might allow us to restore the Treg phenotype, potentially improving the stability of Tregs in the inflammatory environment of MS. Interestingly, our group also discovered that **Piezo1**, a mechanosensitive ion channel, is upregulated on BBB-transmigrated Tregs.

Besides structural changes within the BBB, the inflammatory environment contributes to demyelination of the CNS, which can cause a reduction in brain stiffness (34). Brain stiffness describes the tendency of brain tissue to resist deformation in response to mechanical forces (34). Since myelin content was shown to increase brain stiffness, demyelination could alter mechanical stiffness of the brain (35). In MS patients for example, reductions in this stiffness, particularly in areas of inflammation, were correlated to higher clinical disease scores (34). During BBB transmigration, Tregs encounter these changes in stiffness, potentially facilitating their inflammatory phenotype switch. Key players in sensing these mechanical changes within the CNS are receptors for mechanical forces, such as Piezo1, also present on (murine) Tregs (34).

This cation channel serves as a mechanical sensor, converting (external) mechanical stimuli into biochemical responses within the cell (36). Various mechanical forces, such as membrane stretching, matrix stiffness, or osmotic pressure, activate Piezo1, converting the channel from a closed to an open state. This open state allows influx of calcium (Ca^{2+}), activating chemical and electrical signalling pathways that regulate a variety of cellular functions. Piezo1 also plays a vital role in converting mechanical stimuli into intracellular pro-inflammatory signals. This is demonstrated by the upregulated expression of this channel in various chronic inflammatory diseases (36). In the CNS for example, activation of Piezo1 on neurons was shown to negatively regulate myelination and prevent axon regeneration, while pharmacological inhibition of the channel attenuated demyelination (37, 38). Moreover, inhibition of Piezo1 showed reduced disruption of the BBB by protecting the integrity and permeability of the barrier (39). In T cells specifically, Piezo1 plays a role in mediating cell activation and antigen priming by driving the cytoskeletal rearrangements needed for optimal T cell receptor signalling (40, 41). In doing so, T cells can implement mechanical changes from the microenvironment to optimally bind antigen presenting cells. This process occurs via activation of **calpains** and modulation of the TGF-β/SMAD signalling pathway (40, 41).

Calpains are a family of Ca^{2+}-dependent proteases that can degrade various intracellular proteins, including signalling, membrane, and myelin proteins (42). These proteases are known to be implicated in various neurodegenerative diseases, with calpain inhibitors offering a novel therapeutic strategy (43). In MS for example, upregulated calpain expression was indicated to increase myelin degradation (42). Calpain was also shown to play a role in modulating Treg stability (44). Specifically, inhibition of calpain 1 improved Treg stability by increasing TGF-β production. This improved stability resulted in an expansion of FOXP3+ Tregs and Treg-associated cytokines (44). Similarly, in an experimental autoimmune encephalomyelitis (EAE) mouse model of MS, inhibition of Piezo1 expanded the Treg pool and ameliorated disease severity (45). These findings indicate that Piezo1 can restrain Treg activity by decreasing TGF-β production, likely modulated by calpain activation. Furthermore, this indicates the potential of Piezo1 inhibition in restoring Treg functionality and accordingly, as a novel therapeutic approach for MS.

Another downstream pathway related to Piezo1 activation utilizes adenosine triphosphate (ATP) release and subsequent activation of **purinergic**

P2X receptors. In red blood cells for example, ATP is released in response to shear stress, with Piezo1 playing an important role in mediating this release (46). Via activation of several P2 receptors, including P2X1 and P2X7, this ATP release then stimulates signalling pathways that allow regulation of cell volume. Moreover, in mesenchymal stem cells, several P2X and P2Y receptors are involved in ATP-mediated regulation of cell migration in response to mechanical stimulation (46). A third pathway shown to be involved in Piezo1 signalling is the **calcineurin/nuclear factor of activated T cells (NFAT)** pathway. This was demonstrated in murine cardiomyocytes, where mechanical stimulation of Piezo1 evoked Ca^{2+}-mediated activation of calpain and calcineurin, resulting in myocardial hypertrophy (47). These changes were reversed via cardiac-specific Piezo1 deletion, which suppressed cardiomyocyte hypertrophic growth, resulting in improved cardiac function (47). Moreover, in the context of osteoarthritis, Piezo1-mediated activation of the calcineurin/NFAT1 signalling pathway resulted in aggravation of the disease (48). However, inhibition of Piezo1, calcineurin, or NFAT1 showed alleviated disease progression, thereby posing as a potential therapeutic strategy for these patients (48). Still, since most of the research on Piezo1 is limited to murine models, how exactly the channel modulates Treg functionality, and how BBB transmigration affects Piezo1 signalling in humans, has yet to be elucidated.

In conclusion, it remains unclear whether Piezo1 is involved in the phenotype switch these Tregs experience during BBB transmigration. Hence, this research aims to study *in vitro* Piezo1 interactions on Tregs migrating through the BBB, to clarify the mechanisms underlying their loss of regulatory functions (**Figure 1**). Based on these interactions, this study also explores the potential of Piezo1 inhibition as a strategy to improve the efficacy of Treg-based MS therapies. Here, we

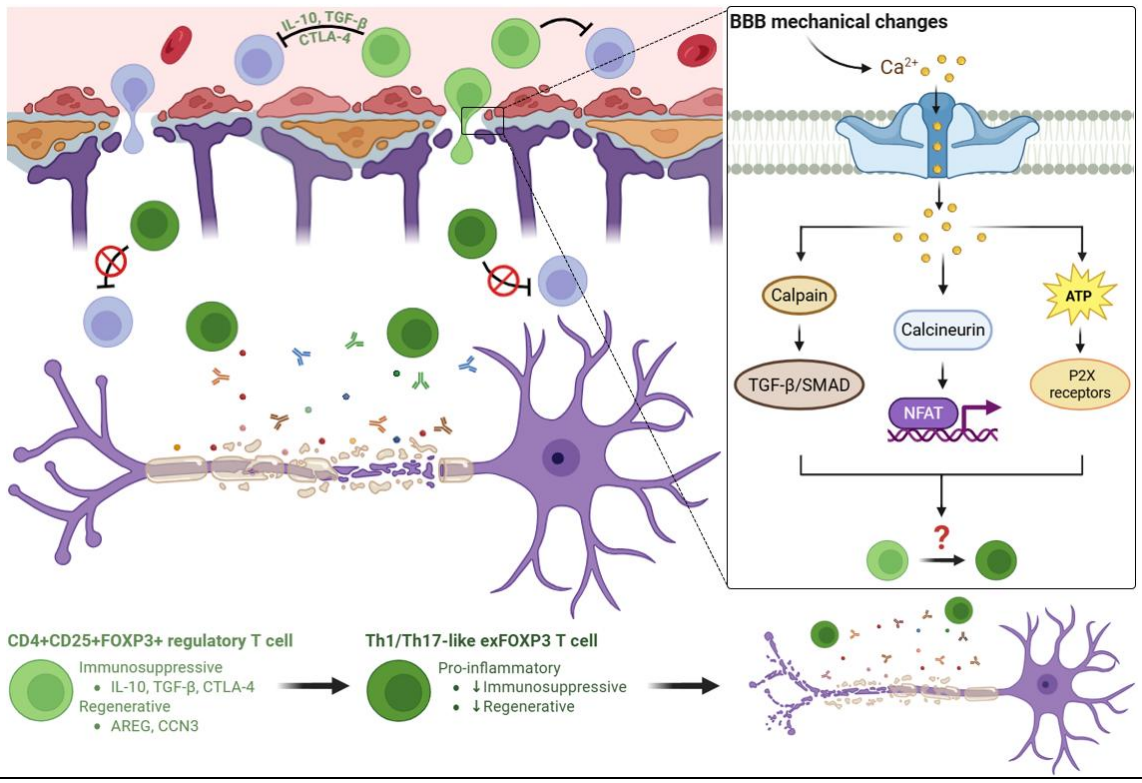

Figure 1 – BBB transmigration induces a pro-inflammatory Treg phenotype switch, potentially mediated by Piezo1. In the context of MS, the BBB becomes leaky, allowing infiltration of Teff (blue) and Tregs (green) into the central nervous system. Tregs interact with the inflamed BBB, causing a pro-inflammatory phenotype switch and worsening disease progression. Piezo1, a mechanosensitive ion channel known to restrain Treg functionality, might be involved in this process. Which downstream signalling pathway is involved in Piezo1 signalling within Tregs remains unclear. *ATP, adenosine triphosphate; AREG, amphiregulin; BBB, blood-brain barrier; CTLA-4, cytotoxic T lymphocyte-associated protein 4; FOXP3, forkhead box P3; IL, interleukin; NFAT, nuclear factor of activated T cells; Teff, effector T cell; TGF-β, transforming growth factor beta; Th, helper T cell; Treg, regulatory T cell.*

demonstrate that Piezo1 activation alters the phenotype and functionality of Tregs, potentially mediated by purinergic P2X receptor signalling. Secondly, we provide evidence of a pro-inflammatory phenotype switch together with calcium influxes in T cells migrating through an *in vitro* inflamed BBB model, potentially mediated by Piezo1 interactions. Finally, we show that inhibition of Piezo1 is not sufficient to prevent these phenotypic changes, suggesting involvement of other Treg-BBB interactions. By broadening our understanding of the mechanisms underlying the pro-inflammatory Treg phenotype switch in MS, these findings might enable us to further improve the safety and efficacy of MS therapies using Tregs.

EXPERIMENTAL PROCEDURES

Regulatory T cell isolation – Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples from healthy donors, using Ficoll density gradient centrifugation (Cedarlane lympholyte) for 20 minutes at 1800 rpm without brake, at room temperature. Further isolation of Tregs was achieved by negative selection of CD4 and positive selection of CD25 using magnetic beads (both Miltenyi Biotec), according to manufacturer instructions. Next, using CD4 APC-eFluor780, CD127 PE (both Thermo Fisher Scientific), and CD25 PE/Cy7 (BioLegend) antibodies, Tregs were sorted on the FACSAria Fusion (BD Biosciences). Tregs (CD4+CD25hiCD127lo) and Teff (CD4+CD25-) were used for further experiments.

hCMEC/D3 cell culture – The human brain endothelial cell line hCMEC/D3 was cultured in collagen I (rat tail, Sigma-Aldrich) coated culture flasks in growth medium (EGM-2MV medium [Lonza] with 2.5% fetal bovine serum [FBS, Gibco]). Cells were collected using trypsin and cultured in collagen I-coated µ-Slides ($5.5x10^4$ cells, Ibidi) for calcium imaging or in Thincerts ($8.25x10^3$ cells, 24-well, 3 µm, Greiner Bio-One) for migration assays. Three days before the experiment, cells were replenished with experimental medium (endothelial cell basal medium-2 [EBM-2, Lonza] with gentamicin [10 mg/ml], amphotericin B [1 mg/ml], fibroblast growth factor [FGF, 5 ng/ml], hydrocortisone [HC, 1.4 µM, all Lonza], and 2.5% FBS [Gibco]). 24 hours before the experiment, cells were treated with IFN-γ (10 ng/ml) and TNF-α (100 ng/ml, both Peprotech) in reduced medium (EBM-2 [Lonza] with gentamicin [10 mg/ml], amphotericin B [1 mg/ml], FGF [5 ng/ml, all Lonza], and 0.25% FBS [Gibco]).

Jurkat cell culture – Human leukemia cell line E6 Jurkat cells were cultured in growth medium (RPMI 1640 [Lonza] with 10% FBS [Gibco]) until used. For longer storage, cells were frozen in liquid nitrogen (10% DMSO [AppliChem] in FBS [Gibco]) and thawed when needed.

Jurkat cell IL-37 transfection – Cells were transfected with either an IL-37 overexpressing or an empty lentiviral vector (as described by Osborne et al.), while non-transfected Jurkat cells were used as a control (49). The IL-37 or empty vector was transfected into Jurkat cells (35 µg/$1x10^7$ cells) using electroporation (315 V, 10 ms, 1 pulse) with the ECM 830 electroporation system (BTX). After transfection, the amount of growth medium (RPMI 1640 [Lonza] with 10% FBS [Gibco]) was doubled daily until use.

Suppression assays – CD4+CD25- Teff were labeled with CellTrace Violet (5 µM, Invitrogen) before they were cultured alone ($2×10^4$ cells/well, representing a 1:0 ratio) or with different ratios of Tregs in a U-bottomed 96-well plate. Next, cells were stimulated with magnetic-activated cell sorting (MACS) Treg Suppressor Inspector beads (Miltenyi Biotec) in culture medium (XVIVO [Lonza] with 5% FBS [Gibco]) for five days at 37°C with 5% CO_2. Tregs isolated from blood were treated for 24 hours with either Yoda1 (10 µM in DMSO, Tocris Bioscience) or DMSO (vehicle, AppliChem) before being added to Teff. IL-37 transfected Jurkat cells were thawed and kept in culture medium (RPMI 1640 [Lonza] with 10% FBS [Gibco]) before being added to Teff. After five days, cell viability (fixable viability dye [FVD] eFluor 780, Invitrogen) and Teff proliferation (CellTrace Violet dilutions) were determined using the LSRFortessa and analyzed using FlowJo 10.10.0 (both BD Biosciences).

Quantitative polymerase chain reaction (qPCR) – To lyse cells, RLT lysis buffer (Qiagen) with β-mercaptoethanol (1%, Thermo Fisher Scientific) was used. Next, RNA was extracted using the RNeasy mini kit (Qiagen) according to manufacturer instructions, while mRNA concentration and purity were measured using a Nanodrop 2000/2000c spectrophotometer (Thermo Fisher Scientific). cDNA was generated

using qScript cDNA supermix (Quanta Biosciences) and qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) and the StepOnePlus real-time PCR detection system (Thermo Fisher Scientific). Data was normalized to the most stable housekeeping genes and relative gene expression was quantified using the comparative Ct method. Primer sequences are given in supplementary information (**Table S1**).

Calcium imaging – Jurkat cells ($7.5x10^5$ cells) were cultured for 24 hours with coated anti-CD3 (10 µg/ml, Invitrogen) and anti-CD28 (1 µg/ml, BD Biosciences). Next, they were treated for 35 minutes with Fluo 4-AM (5 µM, Invitrogen), after which they were added into µ-Slides with hCMEC/d3 cells ($5.5x10^4$ cells, Ibidi). Slides were imaged using the Zeiss Elyra PS.1 microscope and ZEN 3.11 software. Samples were imaged in 50% culture medium (RPMI [Lonza] with 10% FBS [Gibco]) and 50% Krebs solution (150 mM NaCl, 6 mM KCl, 10 mM HEPES, 10 mM glucose, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) at 37°C with 5% CO_2. Live cell images were obtained using a 10x objective and an additional 1.6x magnification with the OptoVar lens. Images were captured using structured illumination microscopy on the EMCCD camera (Ander iXon+ 897, 512x512) at one frame per second. Analysis of the images was performed using the ImageJ 1.54p software to acquire Fluo 4-AM intensity over time.

Boyden chamber migration assay – As described before, hCMEC/D3 cells were collected and cultured in collagen I-coated Thinserts ($8.25x10^3$ cells, 24-well, 3 µm, Greiner Bio-One). On days four and six, cells were replenished with experimental and reduced medium respectively. On day six, cells were treated with IFN-γ (10 ng/ml) and TNF-α (100 ng/ml, both Peprotech) On day seven, pre-treated Jurkat cells were added to the inserts ($5x10^5$ cells) and allowed to migrate for 24 hours, while Jurkat cells were treated with Yoda1 (10 µM in DMSO, Tocris Bioscience) for 24 hours without migration. Jurkat cells were pre-treated for 24 hours with either grammostola mechanotoxin 4 (GsMTx4, 5 µM in water, Abcam) or DMSO (vehicle, AppliChem). Jurkat cells were collected from the insert (non-migrated) and from the well (migrated) for flow cytometry.

Flow cytometry – For the phenotype check of Yoda1-treated Tregs, Tregs isolated from blood were treated for 24 hours with IL-2 (150 ng/ml, eBioscience) and either Yoda1 (1 or 10 µM in DMSO, Tocris Bioscience) or DMSO (vehicle, Applichem) before staining. For the phenotype check of Jurkat cells, control, empty, and IL-37 vector transfected cells were thawed and stimulated for 24 hours with coated anti-CD3 (10 µg/ml, Invitrogen), and anti-CD28 (1 µg/ml, BD Biosciences) before staining. For the Piezo1 staining, PBMCs from healthy donors were thawed and left to rest for two hours before staining. For the migration assay, Jurkat cells were collected from the insert and the well after 24 hours. First, all cells were stained using Zombie NIR live/dead staining (BioLegend). For phenotyping, Tregs and Jurkat cells were stained using CTLA-4 Brilliant Violet (BV) 785 and CD25 Kiravia Blue (both BioLegend). For the Piezo1 staining, PBMCs were stained with the following antibodies: CD3 Alexa Fluor 700, CD4 PerCP/Fire 806, CD8 BV510, CD14 Spark Blue 574, CD16 BV750, CD25 BV711, CD56 BV650, CXCR3 BV421, and CCR6 PerCP/Cyanine5.5 (all BioLegend). For the migration assay, Jurkat cells were stained using CD4 PerCP/Fire 806, CD25 BV711, CD69 BV421, and IL-17 BV785 (all BioLegend). Next, all cells were permeabilized using the FOXP3/transcription factor staining buffer kit (Thermo Fisher Scientific) according to manufacturer instructions. Tregs and Jurkat cells for phenotyping were further stained with FOXP3 BV421 (BioLegend), while PBMCs were stained with Piezo1 PE (Bio-Techne) and FOXP3 AF647 (BioLegend). Finally, for the migration assay, Jurkat cells were stained using IFNγ PerCP-Cy5.5, FOXP3 AF647 (both BioLegend), and Piezo1 PE (Bio-Techne). Data was acquired on the Aurora (Cytek) and analyzed using FlowJo 10.10.0 (BD Biosciences), to acquire percentages and mean fluorescence intensity (MFI).

Statistical analysis – GraphPad Prism version 10.4.1 (GraphPad Software) was used to perform all statistical analyses. Data are shown as mean ± SEM. To analyze data, a nonparametric Kruskall-Wallis test was performed, followed by a Dunn's test for multiple comparisons with a significance level of $p<0.05$.

RESULTS

IL-37 overexpression does not induce a Treg phenotype or functionality in Jurkat cells – In an attempt to establish a stable human Treg-like cell line to use for further experiments, E6 Jurkat cells

were transfected with an IL-37 overexpressing vector as described by Osborne et al. (49). First, qPCR was performed, measuring IL-37 expression, to validate that the transfection was successful. Here, cells treated with the IL-37 overexpressing vector showed increased expression of *IL37* compared to the empty vector or non-transfected Jurkat cells (**Figure 2a**). Secondly, acquirement of a Treg phenotype and functionality after transfection was examined. Using qPCR and flow cytometry, expression of typical Treg markers, including FOXP3, TGF-β, CD25, and CTLA-4, was evaluated. Here, the IL-37 transfected cells showed an increase in *TGFB*, but not *FOXP3*, mRNA expression (**Figure 2a**). Moreover, while there was a trend of increasing CD25 protein expression compared to both the empty vector and control cells, all groups displayed limited to no protein expression of FOXP3 or CTLA4 (**Figure 2b**). Finally, Treg functionality of the transfected cells was examined using a suppression assay, where both transfected and non-transfected Jurkat cells showed a similar decrease in Teff proliferation compared to the reference condition (1:0) (**Figure 2c**). Based on these results, we cannot validate that the IL-37 transfected Jurkat cells have acquired a sufficient Treg phenotype. For this reason, all further experiments were performed using primary Tregs or non-transfected Jurkat cells.

Piezo1 is expressed on all immune cell subsets, including high levels on Tregs – For Piezo1 activation to be a potential cause of the Treg phenotype switch, Piezo1 needs to be expressed on the surface of Tregs as they migrate through the BBB. To validate this, expression of the channel on different immune cell subsets was evaluated using flow cytometry. Here, PBMCs from healthy controls were stained for a variety of immune subset-specific markers, as well as Piezo1. All live cells, monocytes, natural killer (NK) cells, and T cells showed expression of the channel (**Figure 3a**). Within the different immune cell subsets, T cells displayed the highest PIEZO1 expression levels, with significant increases in Th17 cells compared to monocytes or NK cells (**Figure 3b**). Moreover, all Jurkat cells expressed PIEZO1, with a slight decrease in expression upon Yoda1 activation (**Figure 3c**). These results validate that Piezo1 is expressed on Tregs, implying that the channel might interact with the BBB during transmigration.

Activation of Piezo1 alters the phenotype and functionality of Tregs – Since Piezo1 was shown to negatively affect the Treg pool and EAE disease severity, we aimed to evaluate how exactly Piezo1 activation affects the phenotype and functionality of Tregs (45). First, the phenotype of Tregs treated with the Piezo1 agonist Yoda1 was investigated using flow cytometry. Here, Tregs treated with the highest concentration of Yoda1 (10 μM) showed a significant decrease in viability relative to the lowest concentration (1μM) but not the untreated cells (0 μM), demonstrating a small increase in viability in the lowest treatment group (**Figure 4a**). Although not significant, there is also a trend of decreasing CD25 expression with increasing concentrations of Yoda1 (**Figure 4b**). The expression of CTLA4 showed an increasing trend with higher concentrations of Yoda1, with a significant increase in the highest treatment group (**Figure 4c**). Moreover, while the percentage of FOXP3 positive cells remained unchanged, these cells showed a significantly reduced expression of FOXP3 with increasing concentrations of Yoda1 (**Figure 4d**). Next, the suppressive capacity of Tregs after Piezo1 activation was investigated using a suppression assay. Here, Tregs pre-treated with Yoda1 (10 μM) showed a reduction in their suppressive capabilities compared to a vehicle control (**Figure 4e**). Combined, these results imply that Piezo1 activation might alter the phenotype of Tregs by decreasing expression of Treg-associated markers, such as FOXP3, thereby reducing the suppressive capacity of these cells.

Piezo1 activation in Jurkat cells potentially mediates purinergic receptor signalling pathways – Next, since Piezo1 activation seems to affect the Treg phenotype and functionality, the intracellular pathways underlying these functional changes were investigated. For this, qPCR was performed on Yoda1-treated Jurkat cells (10 μM). First, the effect of Piezo1 activation on *PIEZO1* mRNA expression was examined, demonstrating a trend of increasing expression upon Yoda1 treatment (**Figure 5**). Next, since the calpain signalling pathway was shown to restrain Treg functionality by modulating TGF-β production, expression of both calpain-1 catalytic subunit 1 (*CAPN1*) and *SMAD6* were investigated, revealing no changes in expression (44). Thirdly, since ATP release was shown to be involved in Piezo1 signalling via activation of purinergic P2X receptors,

expression of the *P2RX7* gene was evaluated (41). Here, there was a trend of decreasing expression, suggesting that Piezo1 activation in Jurkat cells might modulate purinergic receptor signalling. Finally, since the calcineurin/NFAT pathway has also been linked to Piezo1, mRNA expression of phosphatase 3 catalytic subunit alpha (*PPP3CA*), the catalytic subunit of calcineurin, and NFAT cytoplasmic 1 (*NFATC1*), a component of the NFAT DNA binding transcription complex, were evaluated. Moreover, the mRNA expression of two modulators within this pathway, calcium/calmodulin-dependent protein kinase type IV (*CAMK4*) and CAMK2 inhibitor 1 (*CAMK2N1*), was also measured. Here, expression of *PPP3CA, NFATC1, CAMK4,* and *CAMK2N1* remained unaltered upon Piezo1 activation. Together, these results suggest that both the calpain and calcineurin/NFAT pathways are not involved in Piezo1 signalling within Jurkat cells. Moreover, while not significant, the trend of increasing *PIEZO1* and decreasing *P2RX7* expression does imply potential involvement of purinergic receptors.

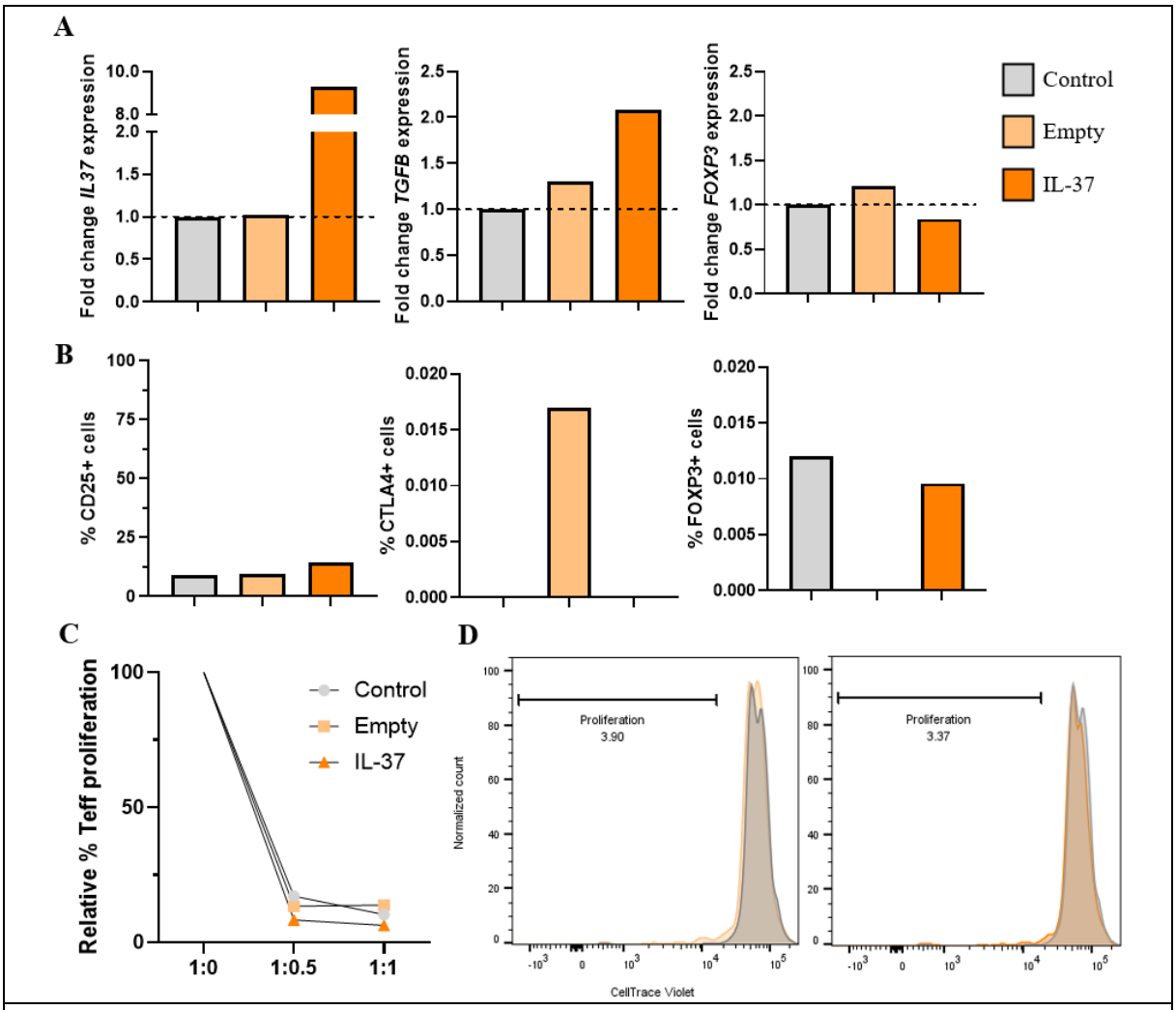

Figure 2 – IL-37 overexpression in Jurkat cells does not induce a Treg phenotype or functionality. Jurkat cells were transfected with an IL-37 overexpressing or empty lentiviral vector. **(a)** qPCR analysis of *FOXP3*, *TGFB*, and *IL37* gene expression (n=1). **(b)** Flow cytometry analysis of CD25, CTLA4, and FOXP3 protein expression in control, empty vector, or IL-37 vector transfected Jurkat cells (n=1). Gating strategy in **Figure S1**. **(c)** Suppression assay showing relative proliferation of Teff cultured for five days alone (1:0) or with different ratios (1:0.5, 1:1) of control, empty vector, or IL-37 vector transfected Jurkat cells (n=1). **(d)** Representative CellTrace Violet dilution plots of control and empty vector, or control and IL-37 vector transfected Jurkat cells (1:1). Relative percentage proliferation represents dilution of CellTrace Violet with different ratios of Jurkat cells, normalized to the 1:0 condition. Gating strategy in **Figure S2**. *CTLA4, cytotoxic T lymphocyte-associated protein 4; FOXP3, forkhead box P3; IL, interleukin; qPCR, quantitative polymerase chain reaction; Teff, effector T cell; TGFB, transforming growth factor beta; Treg, regulatory T cell.*

Migration through an in vitro inflamed BBB model induces calcium influxes and a pro-inflammatory phenotype in Jurkat cells – Finally, we aimed to elucidate whether migration through the inflamed BBB alters the phenotype of Tregs via activation of Piezo1. Therefore, we first examined whether migration through inflamed BBB endothelial cells causes Piezo1 activation in Jurkat cells. For this, calcium imaging was used, measuring calcium influxes in Jurkat cells migrating through inflamed hCMEC/D3 cells. Here, Jurkat cells showed increased calcium influxes, likely caused by interaction with or migration through the endothelial cells (**Figure 6a, b**). These results confirm that calcium influxes occur within Jurkat cells, possibly caused by migration through inflamed endothelial cells. Moreover, these calcium influxes are potentially regulated by the Piezo1 ion channel. Secondly, to examine changes in the phenotype of Jurkat cells after BBB transmigration, flow cytometry was performed on non-migrated and migrated Jurkat cells treated with the Piezo1 inhibitor GsMTx4 (5 µM) or a vehicle control (DMSO). These results were then compared to Yoda1-treated Jurkat cells (10 µM) that did not undergo migration. Here, inhibition of Piezo1 was found to reduce the migratory capacity of Jurkat cells compared to the control (**Figure 6c**). Secondly, migration through inflamed BBB endothelial cells reduced the viability of Jurkat cells, with the lowest viability in the GsMTx4-treated group (**Figure 6d**). The Yoda1-treated group showed a comparable decrease in viability. Similarly, the expression of PIEZO1 followed a decreasing trend after migration, but not Yoda1 treatment (**Figure 6e**). Thirdly, while expression of CD69 showed a slight increase, CD4 expression considerably decreased after migration (**Figure 6f, g**). While the Yoda-1 treated cells showed a similar CD69 expression to non-migrated cells, their expression of CD4 followed that of migrated cells. Next, expression of CD25 and the Treg- specific marker FOXP3 showed mixed results, with CD25 increasing and FOXP3 decreasing after migration, both with or without Piezo1 inhibition (**Figure 6h**).

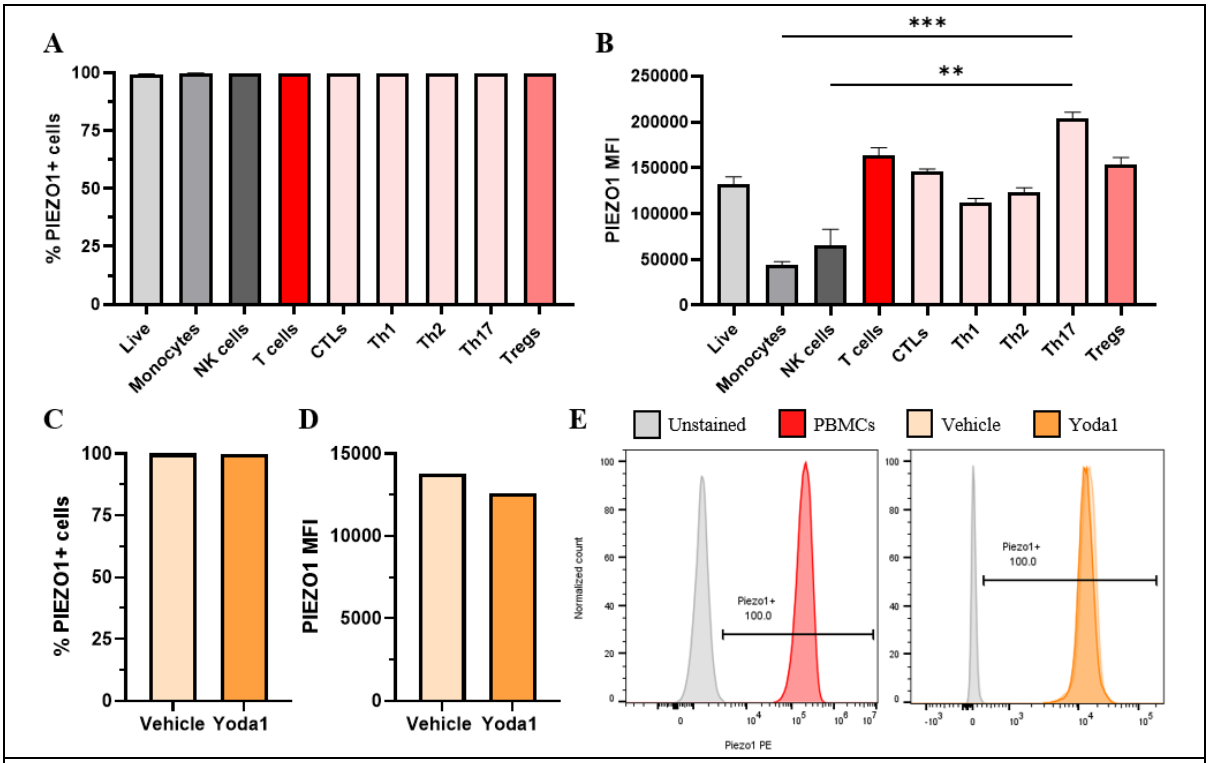

Figure 3 – Piezo1 is expressed on all immune cells, including Tregs and Jurkat cells. Validation of Piezo1 expression on various immune cell subsets, including Tregs and Jurkat cells. Flow cytometry analysis showing **(a, c)** percentage expression or **(b, d)** MFI of PIEZO1 on **(a-b)** live cells, monocytes, NK cells and T cell subsets (n=5), or **(c-d)** Jurkat cells treated with Yoda1 (10 µM) or a vehicle control (n=1). Data are depicted as mean ± SEM. **(e)** Representative histograms of PIEZO1+ cells within PBMCs or (Yoda1-treated) Jurkat cells. Gating strategy in **Figure S3**. Kruskall-Wallis test, Dunn's test for multiple comparisons. **p<0.01, ***p<0.001. *CTL, cytotoxic T lymphocyte; MFI, mean fluorescent intensity; NK, natural killer; PBMCs, peripheral blood mononuclear cells; Th, helper T cell; Treg, regulatory T cell.*

Interestingly, the Yoda1-treated cells showed opposite results, with a decrease in CD25 and an increase in FOXP3 expression. Finally, protein expression of the pro-inflammatory cytokines IL-17 and IFN-γ was increased in migrated compared to non-migrated Jurkat cells (**Figure 6i**). Again, Yoda1 treatment induced opposite effects, with decreased expression of both cytokines. Together, these results suggest that migration through an *in vitro* inflamed BBB model induces a pro-inflammatory phenotype switch in Jurkat cells by decreasing expression of the Treg-specific marker FOXP3 and increasing expression of pro-inflammatory cytokines. Furthermore, we found that Yoda1 treatment induces a phenotype similar to the non-migrated control, while GsMTx4-treated Jurkat cells share a phenotype with the migrated control. Altogether, these results show that Piezo1 inhibition cannot prevent a pro-inflammatory phenotype switch, implying that Piezo1 interactions alone are not sufficient in mimicking

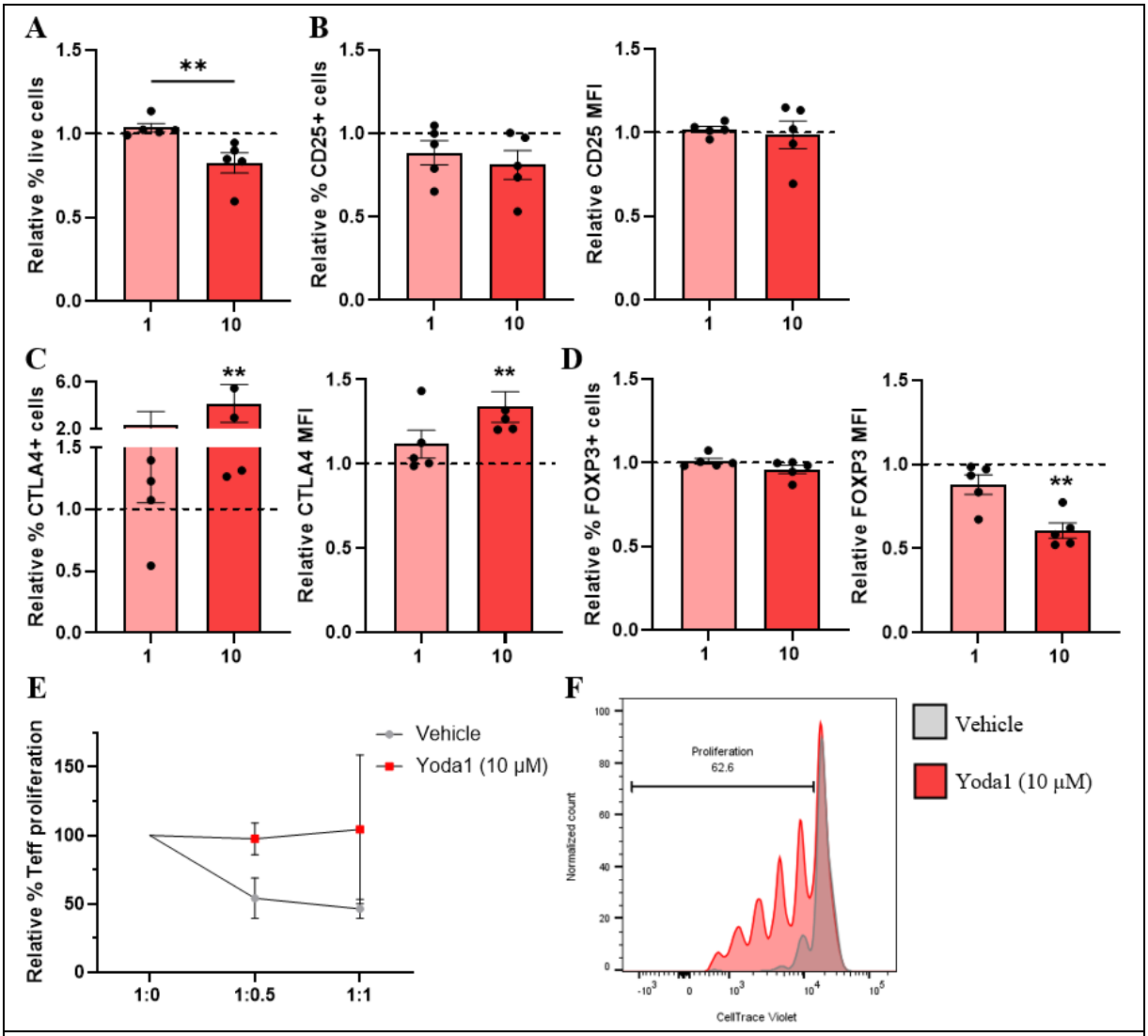

Figure 4 – Alterations in Treg phenotype and functionality following Piezo1 activation. The phenotype and suppressive capacity of Yoda1-treated Tregs was examined. Flow cytometry analysis shows **(a)** Treg viability and **(b-d)** relative percentage expression or MFI of **(b)** CD25, **(c)** CTLA4, and **(d)** FOXP3 on Yoda1-treated Tregs (1, 10 µM, n=5). Gating strategy in **Figure S1**. Kruskall-Wallis test, Dunn's test for multiple comparisons. $^{**}p<0.01$. **(e)** Suppression assay showing relative proliferation of Teff cultured alone (1:0) or with different ratios (1:0.5, 1:1) of Tregs treated with Yoda1 (10 µM) or a vehicle control (n=3). **(f)** Representative CellTrace Violet dilution plots of vehicle and Yoda1-treated Tregs (1:0.5). Relative percentage proliferation represents dilution of CellTrace Violet with different ratios of Tregs, normalized to the 1:0 condition. Gating strategy in **Figure S2**. All data are depicted as mean ± SEM. *CTLA4, cytotoxic T lymphocyte-associated protein 4; FOXP3, forkhead box P3; MFI, mean fluorescent intensity; Teff, effector T cell; Treg, regulatory T cell.*

the phenotypic alterations caused by migration through the inflamed BBB.

DISCUSSION

The phenotype switch Tregs experience during BBB transmigration in MS limits the efficacy of Treg-based cell therapies for these patients. However, the underlying mechanisms that cause these pro-inflammatory alterations in Tregs remain unclear. Here, we investigated the involvement of Piezo1, a mechanosensitive ion channel, in modulating these changes. We found that *in vitro* Piezo1 activation alters the phenotype and functionality of Tregs, potentially mediated by purinergic P2X receptor signalling. Moreover, we discovered that T cell interactions with *in vitro* inflamed BBB endothelial cells induce calcium influxes and a pro-inflammatory phenotype switch, possibly mediated by Piezo1. Nonetheless, inhibition of the channel was not able to prevent this phenotype switch, suggesting that Piezo1 activation alone is not the cause of these changes.

Since the number of Tregs that could be acquired from the blood of healthy controls was limited, we attempted to create a Treg-like cell line to use in further experiments. Interestingly, several studies had already demonstrated that IL-37 can enhance the suppressive capacity of Tregs by upregulating CTLA-4 and FOXP3 expression, as well as promoting production of TGF-β1 (49, 50). Based on these findings, we attempted to create a stable Treg-like Jurkat cell line by transfecting an IL-37 overexpressing vector into the E6 Jurkat cell line (49). Here, we showed an increase in *IL37* mRNA expression, validating that the transfection was successful. Nevertheless, there was no significant increase in expression of typical Treg markers, such as FOXP3 and CD25, or suppressive capacity of the IL-37 transfected cells. Although both non-transfected and IL-37 transfected Jurkat cells demonstrated the ability to suppress Teff in a suppression assay, this is more likely caused by uncontrolled overgrowth of the Jurkat cells, limiting proliferation of the Teff. Based on these results, Piezo1-induced alterations in phenotype and functionality were studied in Tregs, rather than Jurkat cells. Nevertheless, due to the high numbers of Tregs required to perform the other experiments, these were still carried out using CD4+ Jurkat T cells.

Therefore, the similarity in Piezo1 expression on these cells compared to Tregs was validated by examining expression of the channel on PBMCs and Jurkat cells using flow cytometry. These results showed that Piezo1 is expressed on all immune cell subsets, including NK cells, monocytes, and lymphocytes. Nevertheless, there were differences in how much Piezo1 is expressed on the different cell types. While T cells showed the highest expression levels, Th17 cells specifically showed a significant increase in Piezo1 expression compared to NK cells and monocytes.

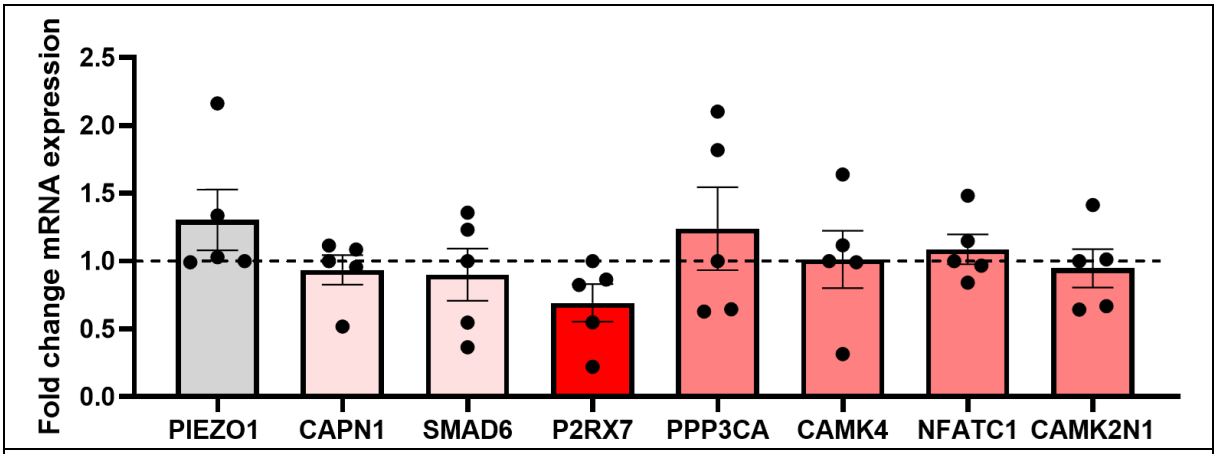

Figure 5 – Piezo1 signalling in Jurkat cells might be mediated by purinergic P2X receptors. Analysis of intracellular pathways potentially involved in Piezo1 signalling in Jurkat cells. qPCR data shows gene expression of various signalling pathway mediators in Yoda1-treated Jurkat cells (10 µM, n=5). Kruskall-Wallis test, Dunn's test for multiple comparisons. Data are depicted as mean ± SEM. *CAMK2N1, calcium/calmodulin-dependent protein kinase type II inhibitor 1; CAMK4, calcium/calmodulin-dependent protein kinase type IV; CAPN1, calpain 1; FOXP3, forkhead box P3; NFATC1, nuclear factor of activated T cells cytoplasmic 1; PPP3CA, phosphatase 3 catalytic subunit alpha; qPCR, quantitative polymerase chain reaction.*

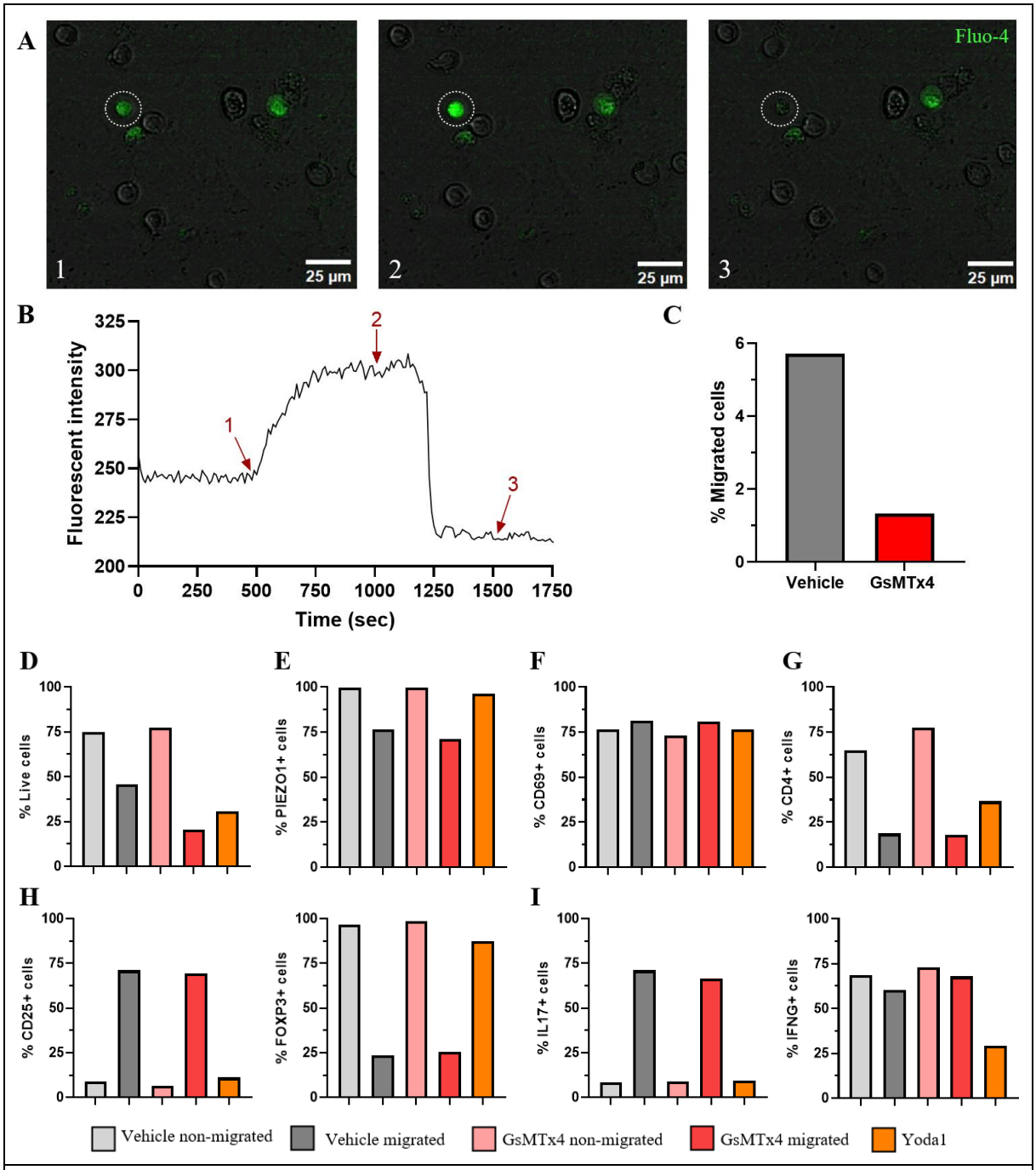

Figure 6 – Migration through an inflamed BBB model induces calcium influxes and a pro-inflammatory phenotype in Jurkat cells. Analysis of calcium influxes and phenotype of Jurkat cells migrated through an *in vitro* inflamed BBB model. **(a)** Fluo-4 staining shows calcium influxes (green) at different timepoints (500, 1000, 1500 sec) in Jurkat cells migrating through inflamed hCMEC/D3 cells. Dotted lines represent possible migrated cells based on Fluo-4 intensity. Images taken at 16x magnification. Scale bars represent 25 µm. **(b)** Fluo-4 fluorescent intensity within a Jurkat cell during possible migration through inflamed hCMEC/D3 cells. **(c-h)** Jurkat cells were treated with GsMTx4 (5 µM) or a vehicle before migration through inflamed hCMEC/D3 cells, or treated with Yoda1 (10 µM) without migration. Flow cytometry analysis showing **(c)** percentage of migrated cells, **(d)** viability of Jurkat cells, or percentage expression of **(e)** PIEZO1, **(f)** CD69, **(g)** CD4, **(h)** CD25, FOXP3, **(i)** IL17, and IFNG. Gating strategy in **Figure S4**. Data shown was acquired from a single measurement. *BBB, blood-brain barrier; FOXP3, forkhead box P3; GsMTx4, grammostola mechanotoxin 4; IFNG, interferon gamma; IL, interleukin; MFI, mean fluorescent intensity.*

This follows the idea that functional expression of Piezo1 on T cells is necessary for optimal T cell development and activation (40, 45). Moreover, Piezo1 expression on Jurkat and primary human CD4+ T cells is known to play a vital role in regulating migration of T cells. This was demonstrated in a transwell chemotaxis assay, where GsMTx4- or siRNA-mediated Piezo1 knockdown significantly reduced T cell migration (51). Combined, these results prove that Piezo1 is expressed on both Jurkat cells and Tregs, highlighting potential involvement of the channel in mediating the phenotype switch of BBB-transmigrated Tregs.

While migration through the inflamed BBB was already proven to induce a pro-inflammatory phenotype switch in Tregs, it remains unclear whether mechanical changes affect these cells in a similar way (16). Therefore, to examine how the phenotype and functionality of Tregs is affected by activation of mechanosensitive ion channels, Tregs were pre-treated with the Piezo1 agonist Yoda1. Here, Yoda1 treatment resulted in a significant decrease in the viability of Tregs. This is in line with results from various other cell types, where higher concentrations of Yoda1 significantly reduced cell viability. In human endothelial cells for example, Yoda1 concentrations above 10 µM were shown to cause a significant amount of cell death after four hours (52). This follows the idea that Piezo1 plays a role in mediating calcium-dependent cell death in response to abnormal mechanical stresses, particularly in cancer cells that express high levels of the channel (53). Contrary to this however, another study documented that the viability of Yoda1-treated (0.1-10 µM) human breast cancer cells increased in a dose-dependent manner (54). This somewhat aligns with the small increase in viability we observed in the treatment group with the lowest concentration of Yoda1 (1 µM). Nonetheless, Piezo1-mediated cytotoxicity also limits the therapeutic potential of Tregs. Secondly, based on expression of Treg-associated markers, Yoda1-treated Tregs displayed a loss of their regulatory phenotype. Notably, there was a significant decrease in FOXP3 expression. Such a decrease was previously shown to reduce functional stability and suppressive capacity of Tregs in the context of autoimmunity and inflammation (16, 28). Similarly, Yoda1-induced activation of Piezo1 evoked a trend of decreasing CD25 expression. This trend has also been demonstrated in Tregs migrating through an *in vitro* inflamed BBB model, where migrated Tregs exhibited an exFOXP3 phenotype with decreased expression of CD25 (16). Interestingly however, the Yoda1-treated Tregs showed a significant increase in CTLA4 expression. Similarly, CTLA4 expression was found to be amplified after *in vitro* mechanical compression of human glioma cells, which was shown to be regulated by the Piezo1 pathway (55). Yet, no studies in Tregs have directly reported a similar increase in CTLA4 with decreases in CD25 and FOXP3 expression. Nevertheless, these changes in Treg phenotype need to be validated upon mechanical activation of Piezo1, and more specifically, upon migration through the inflamed BBB. Next, a suppression assay performed with Yoda1 pre-treated Tregs unveiled a trend of reduced suppressive capacity compared to the vehicle control. Contrary to these results, in an EAE model with a T cell specific genetic deletion of Piezo1, the *in vitro* suppressive capacity of Piezo1$^{-/-}$ Tregs compared to those expressing the channel remained unaltered (45). Interestingly, this knockout did show diminished disease severity, as well as an increase of the Treg pool *in vivo* (45). Regardless of the increase in CTLA4 expression, a loss of suppressive capacity aligns with the altered phenotype that Tregs acquire upon Piezo1 activation. Combined with findings from another study, where CTLA-4 knockout Tregs retained their suppressive abilities *in vitro* but not *in vivo*, this suggests that CTLA-4 alone is not sufficiently able to maintain the suppressive capabilities of Tregs (56). These findings, together with the small sample size, large variation, and lack of significance in our results, warrant further investigation into the suppressive capacity of Tregs upon Piezo1 activation.

Next, to explain how Piezo1 activation causes these alterations in the Treg phenotype, we investigated intracellular pathways that might be involved. For this, mRNA expression of various signalling molecules within these pathways was evaluated in Yoda1 pre-treated Jurkat cells. First, since previous RNA sequencing data from our group revealed that *PIEZO1* mRNA expression was upregulated in BBB-transmigrated Tregs, we examined whether Yoda1 affects expression in a similar way. Here, there was a non-significant trend of increasing mRNA expression upon Yoda1 treatment. This aligns with flow cytometry data we acquired measuring PIEZO1 expression in these cells, which remained unchanged in both groups (data not shown). Moreover, this is similar

to the protein expression of PIEZO1 measured in erythroblasts treated with Yoda1, which also remained unaltered (57). One study suggests a model where Yoda1 changes the conformation of Piezo1, thereby lowering the mechanical threshold and activating the channel (58). This could explain increased Piezo1 activation without a need for increased expression of the channel. Secondly, expression of CAPN1 was examined based on the role calpain was found to play in regulating Treg stability upon Piezo1 activation (44). Since this regulation was linked to modulation of the TGF-β/SMAD pathway, expression of the TGF-β regulator SMAD6 was also evaluated (44). Here, no significant alterations in mRNA expression of *CAPN1* or *SMAD6* were found upon Piezo1 activation in Jurkat cells. One study focusing on the involvement of calpain in Jurkat chemotaxis showed that administering the calpain inhibitor calpeptin reduced their chemotaxis in a dose-dependent manner (59). Interestingly, while Jurkat cells showed basal levels of calpain expression, these levels did not change upon migration with or without the inhibitor. This was explained using the short timeframe of the migration resulting in changes in calpain activity, rather than protein expression (59). Therefore, examining activation of calpain, rather than mRNA expression, could provide more comprehensive results. Additionally, while SMAD6 does not seem to be involved in Piezo1 signalling in Jurkat cells, involvement of other SMADs should not be ruled out. One example are SMAD3 and 4, which were shown to play a vital role in regulating TGF-β1 induced expression of the IL-2 receptor (IL-2R) α chain (60). IL-2 signalling via the IL-2R is vital for the development and functionality of Tregs (61). Next, the release of ATP and consequent activation of purinergic P2X receptors has also been linked to Piezo1 activation (41). Therefore, we examined expression of the *P2XR7* gene, which showed a non-significant but decreasing trend in Yoda1-treated compared to control Jurkat cells. Purinergic receptors are known to modulate T cell functions, with silencing of these receptors resulting in impaired activation, proliferation, and migration of T cells (62, 63). Combined with our data, this suggests that reduced expression of the P2X7 receptor upon Piezo1 activation might affect the migratory capacity of Tregs. Moreover, silencing of the P2X7 receptor was shown to inhibit calcium influx, NFAT activation, and IL-2 expression, thereby suppressing T cell activation (63). However, it is interesting to note that Jurkat cells have a lower expression of this receptor compared to CD4+ T cells, contributing to their growth by protecting the cells from apoptosis (63). Thirdly, involvement of the calcineurin/NFAT pathway, as seen in the context of myocardial hypertrophy and osteoarthritis, was explored (47, 48). For this, mRNA expression of *PPP3CA*, *NFATC1, CAMK4,* and *CAMK2N1* were evaluated, where no significant changes were found. In T cells, activation of the T cell receptor is known to induce calcium increases, leading to calcineurin activation by calmodulin (64). Calcineurin then dephosphorylates NFAT, resulting in nuclear translocation and modulating expression of genes, such as IFN-γ (64). Therefore, to investigate alterations within this signalling pathway, examining phosphorylation rather than mRNA expression might yield more conclusive results.

One reason for the lack of significance in this experiment could be the low number of samples that were used, limiting the power of statistical analyses. Secondly, it is possible that 24 hours of Yoda1 exposure is not sufficient to induce large changes in gene expression within these pathways. Nevertheless, as Tregs are only submitted to Piezo1 activation during their short migration time through the BBB, longer exposure periods could limit the biologic relevance of the experiment. Next, due to the lack of starting material that could be obtained from Tregs, the experiment was performed using Jurkat cells. As one of the most used cell lines in T cells research, working with the immortalized CD4+ Jurkat T cell line offers a practical advantage compared to primary Tregs. However, there are important differences between Jurkat and primary CD4+ T cells that need to be kept in mind. Examples include differences in innate responses to bacteria, transcriptional modulation, and cytoskeletal organization (65-67). Hence, investigating the same signalling pathways within Tregs could yield different results. Furthermore, the signalling molecules explored in this experiment were limited to known pathways found in literature. Therefore, further research into the involvement of these pathways, as well as other pathways within Tregs should be carried out.

Although we proved that agonist-mediated activation of Piezo1 could alter the phenotype of Tregs, it remained unclear whether mechanical

changes within the BBB induce similar effects. The important role Piezo1 plays in cell migration has already been established in various cell types. In microglia for example, altered substrate stiffness was shown to modulate expression of Piezo1, thereby regulating production of pro-inflammatory cytokines and migration of these cells (68). Similarly, the migration rate of HEK cells was found to be increased in cells treated with a Piezo1 agonist, compared to a decrease in cells treated with an inhibitor (69). In T cells specifically, Piezo1 activation in response to mechanical cues was shown to be integral in regulating T cell motility (51). Here, increased membrane tension in migrating T cells resulted in a redistribution of Piezo1 towards the leading edge of these cells (51). In our study, we first examined Piezo1 activation by investigating calcium influxes into Jurkat cells migrating through an *in vitro* inflamed BBB model. This way, we were able to demonstrate that Jurkat cells allowed to migrate across inflamed endothelial cells experienced an increased influx of calcium. Combined with the knowledge that Piezo1 is expressed on the surface of Jurkat cells, this implies that the channel could be involved in mediating calcium influxes during migration through the inflamed BBB. It is important to note that migration of the Jurkat cells was determined based on increases in calcium influx. It is possible however that these influxes are caused by other calcium-related processes, but not migration through the endothelial cells. For example, adhesion of Jurkat cells to endothelial cells, mediated by very late antigen (VLA)-4 and ICAM-1 interactions, also results in calcium increases (70). Moreover, it was not validated that these influxes were caused by activation of Piezo1 specifically, suggesting that other ion channels could be involved in this process.

Hence, to validate the involvement of Piezo1, we examined the migratory capacity, Piezo1 expression, and phenotype of BBB-transmigrated Jurkat cells treated with or without a Piezo1 inhibitor. In doing so, we first found that inhibiting Piezo1 reduces the migratory capacity of Jurkat cells. This finding is comparable to results from another study, where Piezo1 siRNA transfected CD4+ T cells displayed significantly hindered integrin-dependent chemotactic migration (51). Secondly, GsMTx4 pre-treated Jurkat cells showed a decrease in PIEZO1 expression after migration, which was comparable to the vehicle control. Interestingly, since activation of Piezo1 was linked to conformational changes of the channel, it is possible that these changes prevent binding of the flow cytometry antibody (58, 71). As a result, this decreased expression would represent Piezo1 activation, rather than a decrease in expression. Nevertheless, since the Yoda1-treated group did not show the same trend, unless Piezo1 conformational changes differ between Yoda1- and migration-induced activation, this effect can be ruled out. Thirdly, we evaluated whether Piezo1 inhibition could prevent a phenotype switch in these cells. Interestingly, GsMTx4 pre-treatment was not able to prevent a pro-inflammatory phenotype switch in migrated Jurkat cells. This pro-inflammatory phenotype was characterized by reduced expression of FOXP3, together with increases in IL-17 and IFN-γ expression. These results are supported by similar findings in human Tregs migrating through the same *in vitro* model, as well as an *in vivo* EAE model (16). In these models, migrated Tregs displayed an exFOXP3 phenotype with increased expression of inflammatory cytokines, including IFN-γ (16). Notably however, expression of CD25 and CD69 were increased in migrated compared to unmigrated cells, in both control and GsMTx4-treated Jurkat cells. CD69 and CD25 are known early and late activation markers of lymphocytes, respectively, with CD25 expression remaining high and expression of CD69 dropping after several hours (72). Since our data shows a similar pattern, this increase could be explained as activation of the Jurkat cells in response to migration rather than phenotypic alterations of the cells (72). Altogether, these results suggest that Piezo1 inhibition alone is not sufficient in preventing a pro-inflammatory phenotype switch in BBB-transmigrating Tregs. Moreover, when comparing these results to Yoda1-treated Jurkat cells, there was a high phenotypic similarity to the non-migrated cells. Still, there were some differences, such as reduced viability and decreased expression of CD4 and IFN-γ. Interestingly, differences in CD4 expression are known to regulate Th1 or Th2 cell responses (73). Specifically, Th2 cells were found to have a twofold lower surface expression of CD4 when compared to Th1 cells (73). When combined with the decrease in the Th1-specifc cytokine IFN-γ, our results suggest a possible shift towards a Th2, rather than Th1, phenotype after migration.

While Piezo1 activation does alter the phenotype of Tregs, these effects still need to be validated in an *in vitro* BBB model. However, data acquired from Jurkat cells in this model suggests that these phenotypic changes might not be comparable to the pro-inflammatory phenotype acquired during migration through the inflamed BBB. This implies that, rather than Piezo1 alone, other Treg-BBB interactions are necessary to cause this phenotype switch. One example is the mTORC1 signalling pathway, which upon inhibition was able to restore Treg functionality after BBB transmigration (16). Nevertheless, since this experiment was performed using Jurkat cells, it is possible that Tregs interact differently with the BBB endothelial cells, potentially yielding different results. This is further highlighted by the lack of phenotype switch in Jurkat cells treated with Yoda1 compared to Tregs. Finally, the use of inflamed human endothelial cells as a pathological BBB model remains limited. As mentioned, the BBB is a structure consisting of a variety of cell types, including but not limited to endothelial cells (1). Furthermore, the static conditions of the migration assay used here are unable to mimic the physiological flow and shear stresses Tregs undergo *in vivo* (74). Therefore, research into the interactions between Tregs and other cells present within the BBB, as well as *in vivo* studies, should provide a more extensive overview of Treg-BBB interactions.

CONCLUSION

Altogether, these results reveal that Piezo1 activation causes a loss of Treg phenotype and functionality, potentially mediated by purinergic P2X receptor signalling. A similar pro-inflammatory phenotype switch was found in T cells migrated through an *in vitro* inflamed BBB model, verifying the physiological relevance of these changes. Furthermore, these changes in T cell phenotype were linked to calcium influxes within the cells, suggesting potential involvement of the Piezo1 ion channel. Nevertheless, Piezo1 inhibition on migrating T cells was not able to prevent a pro-inflammatory phenotype switch, suggesting involvement of other Treg-BBB interactions. Therefore, further research into elucidating the mechanisms underlying the pro-inflammatory phenotype switch of BBB-transmigrated Tregs is required. In doing so, the safety and efficacy of Treg-based therapies can be improved, ultimately ameliorating the quality of life of MS patients.

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Author contributions – SDB and JV conceived and designed the research. SDB and LK performed experiments and data analysis. SD provided assistance with calcium imaging. SDB wrote and JV carefully edited the manuscript.

SUPPLEMENTARY INFORMATION

Table S1 – Primer sequences for quantitative polymerase chain reaction.

Target gene	Primers (5' – 3')
FOXP3	F- GCACCAGCTCTCAACGGTGGATG R- GAAGACCCCAGTGGCGGTGG
PIEZO1	F- CGCGTCTTCCTTAGCCATTAC R- TCCTGCGGTGAAAGTCAATG
IL37	F- GCATTCATGACCAGGATCAC R- CAAAGAAGATCTCTGGGCGTA
TGFB	F- CACCTGGAGCTGTACCAGAA R- TGCAGTGTGTTATCCCTGCT
CAPN1	F- TCATTCCGAGACTTCATGCG R- AGTGTGGTGTTCCATTTGCG
SMAD6	F- GCCTTTCGTGCATGCAAATC R- AAGGCAGGCTTGTTGATACC
NFATC1	F- ATGGAACCTGCCTGAATTGC R- AGACACAAGAGACGCGATTG
PPP3CA	F- TGCAAAGCGCTACTGTTGAG R- GGCGGCATCCTCTCATTAATTC
CAMK4	F- AATTCAATGCCCGGCGTAAG R- TCGTTGCCATCTTGGATTGG
P2RX7	F- GGAAAGAGCCTGTCATCAGTTC R- AGACACTGTGCACCAACTTC
CAMK2N1	F- TGCAGGACACCAACAACTTC R- TCAGCACGTCATCAATCCTATC
TBP	F- TATAATCCCAAGCGGTTTGC R- GCTGGAAAACCCAACTTCTG
HMBS	F- GAATGAAGTGGACCTGGTTGT R- CTGGTTCCCACCACACTCTT
RPL	F- AAGTTGAAGTACCTGGCTTTCC R- GCCGTCAAACACCTTGAGAC
PGK1	F- CTGGGCAAGGATGTTCTGTT R- GCATCTTTTCCCTTCCCTTC

F, forward primer; R, reverse primer

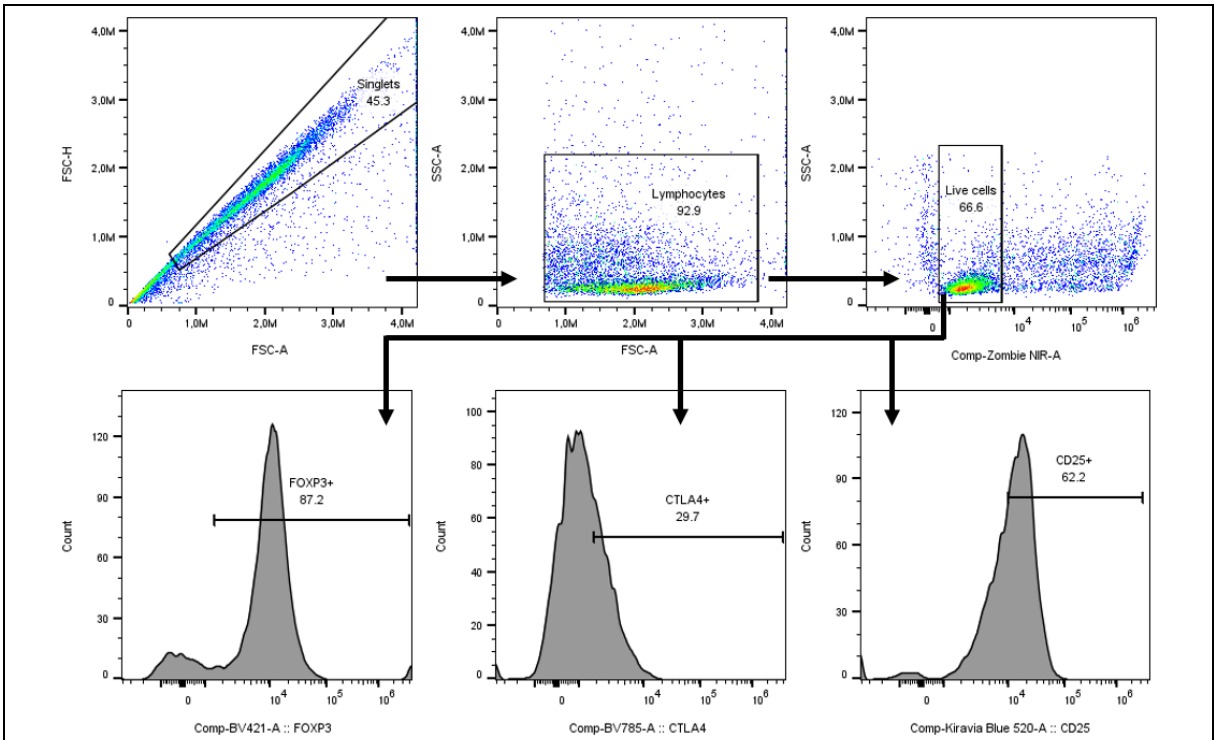

Figure S1 – Gating strategy for phenotype checks. Jurkat cells were transfected with an empty vector, IL-37 vector, or non-transfected, while Tregs were treated with increasing concentrations of Yoda1 (1, 10 µM) before staining. Treg phenotype was evaluated based on expression of Treg-associated markers. Gating strategy and representative plots for quantifying FOXP3, CTLA4, and CD25 protein expression. *CTLA4, cytotoxic T lymphocyte-associated protein 4; FOXP3, forkhead box P3; IL, interleukin; Treg, regulatory T cell.*

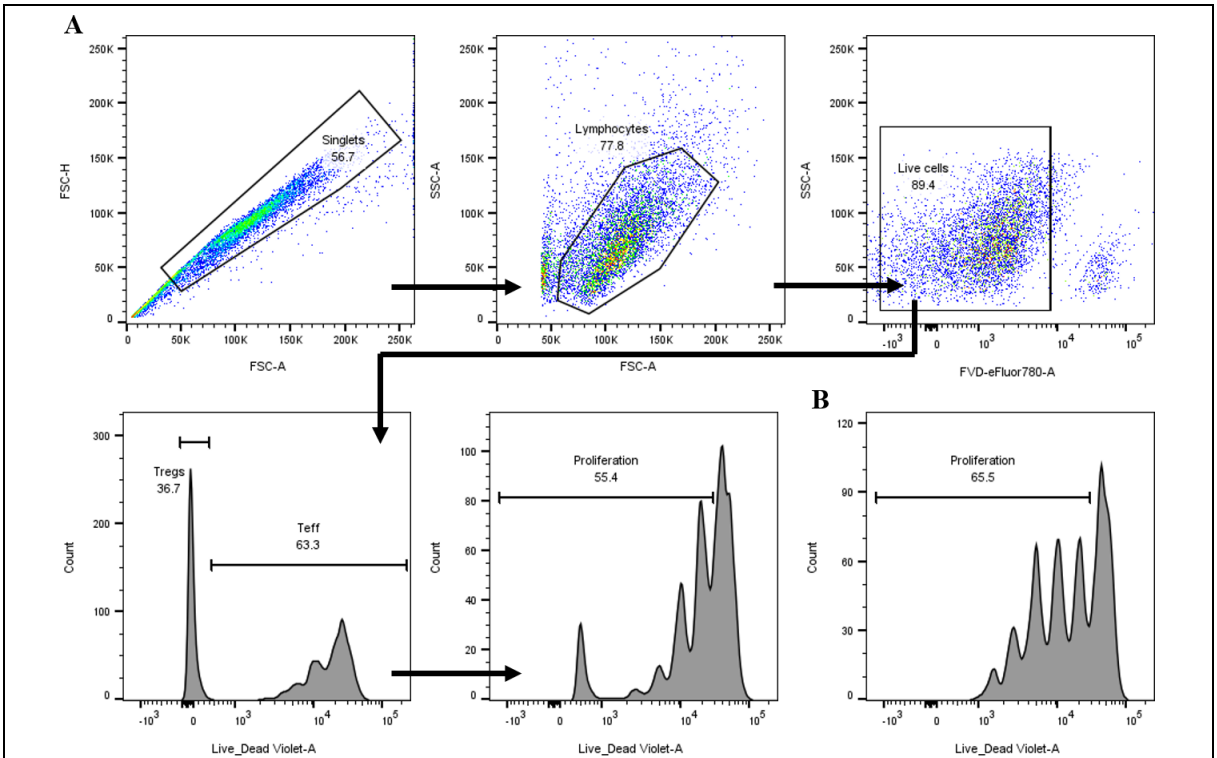

Figure S2 – Gating strategy for suppression assays. CellTrace-labeled Teff were cultured for five days with or without IL-37-induced or Yoda1-treated Tregs. CellTrace dilutions are used as a measure of proliferation. Gating strategy and representative plots quantifying proliferation of Teff cultured **(a)** with or **(b)** without (Jurkat) Tregs. *IL, interleukin; Teff, effector T cell; Treg, regulatory T cell.*

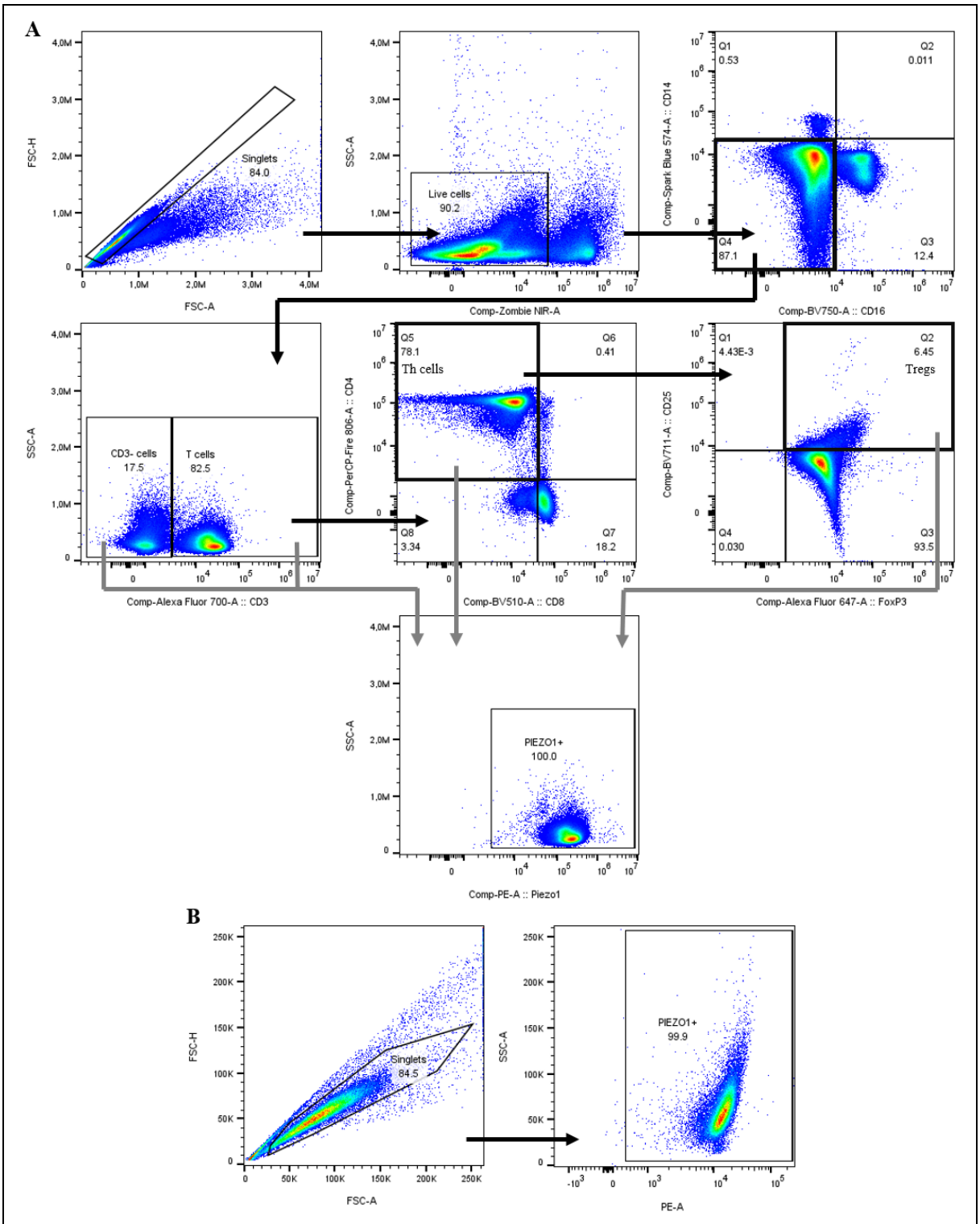

Figure S3 – Gating strategy for PIEZO1 expression on T cells. PBMCs were thawed, allowed to rest, and stained for T cell (subset) specific markers and Piezo1. Jurkat cells were treated with Yoda1 (10 µM) or a vehicle control and stained for Piezo1. Gating strategy and representative plots for quantifying PIEZO1 expression on **(a)** PBMCs and **(b)** Jurkat cells. *FOXP3, forkhead box P3; PBMCs, peripheral blood mononuclear cells; Th, helper T cell; Treg, regulatory T cell.*

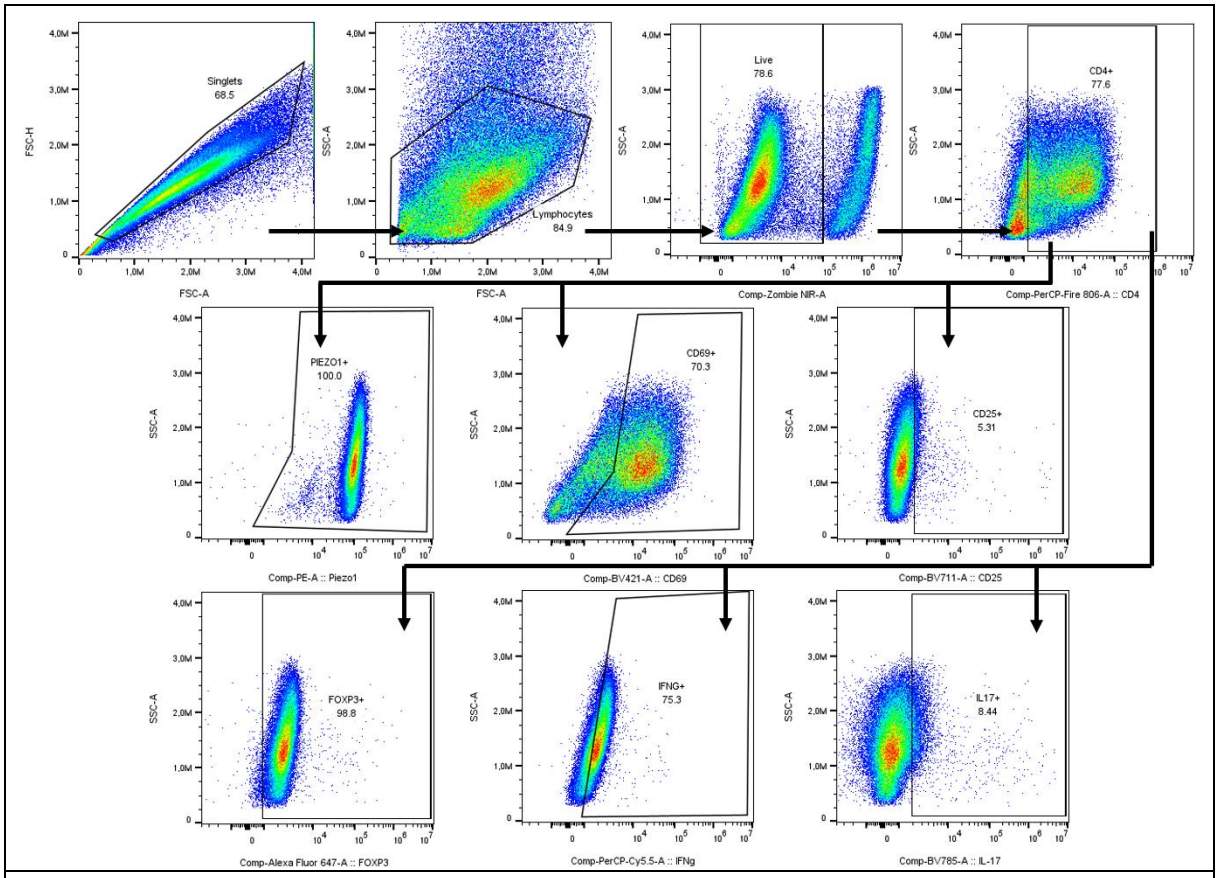

Figure S4 – Gating strategy for migration assays. Jurkat cells were treated with GsMTx4 (5 µM) or a vehicle control before migrating through inflamed hCMEC/D3 cells, or treated with Yoda1 (10 µM) without migration. Gating strategy and representative plots for quantifying expression of CD4, PIEZO1, CD69, CD25, FOXP3, IFNG, and IL17. *FOXP3, forkhead box P3; IFNG, interferon gamma; IL, interleukin.*