Spi1 -14 Kb upstream regulatory element (URE) is not required for maintenance of PU.1 expression in macrophages [version 1; peer review: 1 approved]

Ruth Jones1, Matthew Hill1, Philip Taylor1,2

1UK Dementia Research Institute at Cardiff, Cardiff University, Cardiff, CF24 4HQ, UK
2Systems Immunity Research Institute, Cardiff University, Cardiff, CF14 4XN, UK

Abstract

Background: Previous work suggested an upstream regulatory element (URE) of Spi1 was required to maintain constant expression of the PU.1 transcription factor in bone marrow and foetal liver cells. PU.1, encoded by Spi1, is essential for development and maintenance of myeloid and B-lymphocyte populations in mice. Deletion of this (-14 Kb) URE potentially reduces expression of PU.1 and therefore provides a way to investigate its role in myeloid populations in development and disease. This study aimed to examine the impact of removal of the -14 Kb Spi1 URE in Cx3cr1+ cells on the myeloid lineage formation and maintenance.

Methods: B6;129-Spi1tm1.2Dgt/J mice, whose -14 Kb Spi1 URE mice is flanked by LoxP sites ('floxed'), were bred to a strain with constitutively active Cre expressed under the Cx3cr1 promoter (B6J.B6N(Cg)-Cx3cr1tm1.1(cre)Jung/J) to delete the Spi1 URE in myeloid cells. The floxed mice were also bred to mice with a tamoxifen-inducible Cre expressed under the Cx3cr1 promoter (B6.129P2(C)-Cx3cr1tm2.1(cre/ERT2)Jung/J) to be used as URE intact controls and to permit temporally-controlled deletion of the URE if required. PU.1 protein expression was measured in the peritoneal macrophages and microglia by flow cytometry. Additionally, a Cre-encoding lentiviral vector was used to assess the impact on PU.1 expression in bone-marrow derived macrophages from these mice in vitro.

Results: Expression of the PU.1 transcription factor was not significantly altered in the peritoneal macrophages or microglia in mice lacking the -14 Kb Spi1 URE. Moreover, initial experiments utilising Cre encoding lentivirus did not reduce PU.1 protein in bone-marrow derived macrophages differentiated from the -14 Kb Spi1 URE floxed mice.

Conclusions: These observations suggest that the -14 Kb URE does not play a major role in PU.1 protein expression in either mature peritoneal macrophages or microglia.
Keywords
PU.1, Spi1, Macrophage, Microglia, URE
Introduction

Spi1 encodes the transcription factor PU.1 which has been shown to be critical for the development and maintenance of myeloid cells and B-lymphocytes[1–3]. Previous work has suggested that PU.1 regulates transcription of genes in a dose-dependent manner in macrophages (MØ) [4–5]. In hematopoietic development, low PU.1 in precursor cells commits them to a B-lymphocyte lineage whereas high PU.1 commits them to a MØ lineage[6,7]. Following development, PU.1 helps maintain MØ populations, especially tissue resident MØ where co-binding of tissue specific transcription factors alongside PU.1 results in a highly specialised phenotype[8,9].

Additionally, CSF1/M-CSF (colony stimulating factor 1/macrophase colony stimulating factor) acts through the CSF1-receptor (CSF1R) stimulating PU.1 expression resulting in increased CSF1R expression and further promoting the proliferation and survival of MØ populations[10–12]. CSF1R inhibitors have been successfully used to restrict microgliosis and deplete microglia in models of Alzheimer’s disease (AD)[13–15]. Preventing microgliosis with CSF1R inhibitors, which are thought to affect PU.1 expression, has been shown to be beneficial and improve performance on behavioural tests in several models of AD[16–20]. Therefore, PU.1 is thought to be both critical to the development of MØ populations and to contribute to microglial-dependent pathology in AD.

Recent reports have shown that lower levels of SPI1 expression are associated with reduced risk of AD and delayed onset of this neurodegenerative condition[21]. In the brain, Spi1/PU.1 is predominantly expressed by microglia[22]. Given these apparent links between a low Spi1/PU.1 expression levels and delay in AD onset there have been several studies looking at how the amount of Spi1/PU.1 present in microglia impacts gene expression[23,24]. However, these studies relied on utilising siRNAs and in vitro models of microglia[25] which likely fail to represent the complexities of in vivo microglia[26,27]. Therefore, there is a need to study the impact of a reduced Spi1/PU.1 dose on the transcriptome of microglia freshly extracted from transgenic mice with reduced levels of PU.1.

PU.1 expression is tightly regulated by itself and multiple other feedback loops including GATA1, GATA2 and RUNX1[28–30]. A key upstream regulatory element (URE) site located 14 Kb upstream of the Spi1 promoter (-14 Kb Spi1 URE) is thought to regulate PU.1 levels during MØ development[31,32]. Runx1, C/EBPα and Ikaros are transcription factors that bind at the -14 Kb URE, and other nearby Spi1 regulatory sites to maintain a high PU.1 expression in MØ[33,34]. When the -14 Kb Spi1 URE was ubiquitously removed in mice, PU.1 protein expression was reduced by 80% in total bone marrow. This reduction was associated with development of an acute myeloid leukaemia (AML)-like pathology at 3 months of age[35–37].

Thus the -14 Kb Spi1 URE floxed mouse model has the potential to determine effects of low PU.1 expression on the microglial transcriptome. We aimed to investigate the impact of removing the -14 Kb Spi1 URE on PU.1 protein expression specifically in MØ (peritoneal MØ (pMØ) and microglia) in adult mice. The approach taken in this work was to use two transgenic mouse lines where the Cre recombinase enzyme was expressed either constitutively (“Spi1-ARE[38]”) or after induction with tamoxifen (“Spi1-ARE[ERT2]”) under the control of the Cx3cr1 promoter. The URE should be deleted in the mice with constitutively active Cre in all cells where Cx3cr1 is normally expressed, or where it was previously expressed by precursor cells, but remain intact in those cells expressing tamoxifen-inducible Cre expressing mice, in the absence of tamoxifen.

Methods

Ethical considerations

All experiments were conducted in accordance with UK Home Office Guidelines and Animal [Scientific Procedures] Act 1986, which included approval by the local ethical review board (Animal Welfare and Ethical Review Body, AWERB, of the Biological Standards Committee) and the granting of a UK Home Office Project Licence (P05D6A456). The study was conducted in compliance with the ARRIVE guidelines[39].

URE assessment in publicly available ChiP-Seq datasets

Assay for Transposase-Accessible Chromatin-seq (ATAC-Seq) and Histone 3 lysine 27 acetylation sequencing (H3K27ac ChIP-Seq) datasets were accessed through the Cistrome database[40]. The datasets used in this study are detailed in Table 1. The -14 K b Spi1 URE sites of the selected datasets were compared using the UCSC genome browser (RRID:SCR_005780)[41].

Mice

All transgenic mice were purchased through Jackson Laboratories and maintained in the Animal Housing Unit. Tissue was collected from mice culled via asphyxiation with a slow rising level of CO2. Confirmation of death was provided via cervical dislocation. This killing method is described and authorised in Schedule 1 (humane killing of animals code of practice) of the Animal [Scientific Procedures] Act 1986. Mice matched for age and gender were processed in parallel, as indicated alongside the data. All mice were housed in conventional open top cages separated by genotype and gender, with between 1–7 cage companions, kept on a 12-hour light/dark cycle, provided water and chow ad libitum and with environmental enrichment. No additional procedures were conducted on these animals for this study and to ensure no additional suffering the welfare of the animals was regularly monitored.

B6;129-Spi1[12Ds1/J (Spi1-ARE[ERT2]; stock number 006099) mice were previously described in 31. To ensure the -14 K b URE was selectively excised in Cx3cr1 expressing cells, Spi1-ARE[ERT2] mice were crossed to constitutively expressing an active Cre recombinase under the Cx3cr1 promoter (B6.B6N(Cg)-Cx3cr1[12Ds1/J; stock number 025524 henceforth referred to as Cx3cr1[ERT2]), which is predominantly expressed by myeloid cells[38]. Additionally, Spi1-ARE[ERT2] mice were bred to mice expressing a tamoxifen-inducible Cre recombinase in Cx3cr1 positive cells (B6.129P2(C)-Cx3cr1[ERT2]; stock number 020940),
and in the absence of tamoxifen treatments served as a negative control for the study. These strains were termed Spi1-URE<sup>CONS</sup> and Spi1-URE<sup>EXT</sup> to indicate Spi1-URE<sup>CONS</sup> mice with constitutive Cre or tamoxifen-inducible Cre, respectively. A total of 57 mice were used in this study, as summarised in Table 2, the age and gender for each mouse/experimental unit are indicated in the appropriate figure legend. No animals were excluded during the experiment or from the analysis, though four microglia samples were removed from study due to an error in sample processing. There were no other criteria to exclude animals from the experimental analysis, to minimise impact of other cofounders equal numbers of Spi1-URE<sup>CONS</sup> and Spi1-URE<sup>EXT</sup> mice were processed in each independent experiment.

**Recombined allele polymerase chain reaction (PCR)**

The removal of the -14 Kb URE was assessed through genomic PCR. Firstly, genomic DNA was isolated from Spi1-URE<sup>CONS</sup> and Spi1-URE<sup>EXT</sup> peritoneal lavage cells using the GenElute<sup>TM</sup> Mammalian Genomic DNA Miniprep Kit (Sigma, cat# G1N70) to provide template DNA as per manufacturer’s instructions. DNA was also isolated from the Spi1-URE<sup>EXT</sup> MØP cell line (see below). PCR amplification was performed using GoTaq® G2 Flexi kit (Promega) using three primers designed by Jackson Laboratories to detect removal of the -14 Kb Spi1 URE (Spi1<sup>[m1.3D/F]</sup>/J primer 1: GCCAACCTCAGCTCAGGCA; Spi1<sup>[m1.3D/F]</sup>/J primer 2: CTGGATACGACCAATTGT; Spi1<sup>[m1.3D/F]</sup>/J primer 3: ACTGAGTGCCACGGGT CATC; all primer sequences written as 5’ to 3’). The PCR was performed in a Mastercycler<sup>®</sup> Nexus Gradient (Eppendorf) PCR machine utilising the following touchdown protocol adapted from the Jackson Laboratory. Samples were heated to 95°C for 2 minutes, before 10 touchdown cycles of 30 seconds at 95°C, 30 seconds at 70°C (decreasing by -1°C per cycle), and an extension at 72°C for 1 minute. Following this samples underwent an additional 20 cycles using the same cycling parameters as the touchdown, with an annealing temperature of 60°C. After a final extension step at 72°C for 5 minutes samples were held at 4°C.

As a positive control the B6;129-Spi1<sup>[m1.2Dgt]</sup>/J genotyping PCR was used. Here a Wild-Type or a mutant floxed Spi1 URE band of 250 bp or 450 bp were produced using the GoTaq<sup>®</sup> G2 Flexi kit as above, with the following primers (5’ to 3’) GGCC TCA GTTTC T CACCTG and CCTTCCA GGTGTTAG AAT.

**Cell isolation**

pMØ were extracted as a mixed peritoneal population from mice by lavage of the peritoneal cavity with 5 mL of ice-cold lavage solution (5 mM EDTA in DPBS). Briefly, mice were sacrificed, and the abdominal skin was retracted exposing the abdominal wall. The animal’s lower right abdominal quadrant was injected with the lavage solution using a 21-gauge hypodermic needle (BD Biosciences). The abdominal cavity was massaged and then the lavage fluid withdrawn slowly into the same syringe. Flow cytometric analysis of MØ expressed markers CD11b and CD45 indicated the mean proportion of pMØ to be 51 ± 17 (±SD, n=50) of all lavage cells (Table 2).

For microglia isolation, each mouse brain was removed and stored in 1 mL Hanks’ Balanced Salt Solution (HBSS) (without Ca<sup>2+</sup> or Mg<sup>2+</sup>) on ice. Brains were digested in a C-tube (Miltenyi Biotec) using the Neural Tissue Digest Kit with papain (Miltenyi Biotec, cat# 130-092-628) and then the lavage fluid withdrawn slowly into the same syringe. Flow cytometric analysis of MØ expressed markers CD11b and CD45 indicated the mean proportion of pMØ to be 51 ± 17 (±SD, n=50) of all lavage cells (Table 2).

**Assessing URE in vitro**

The Spi1 floxed URE model was also assessed in bone-marrow derived macrophages (BMDM) differentiated from the bone-marrow of Spi1-URE<sup>CONS</sup> and WT (C57BL/6J) control.

---

Table 1. Datasets selected from the Cistrome database<sup>26</sup> to investigate the state of the chromatin/DNA at the -14 Kb URE site upstream of the Spi1 promoter.

| Cell Type     | Data Type             | GEO Reference | Additional Information     |
|---------------|-----------------------|---------------|---------------------------|
| Microglia     | ATAC Sequencing       | GSM2692293    | C57BL/6 mice published<sup>10</sup> |
| pMØ           | ATAC Sequencing       | GSM2692320    |                           |
| Microglia     | H3K27ac ChiP-Sequencing | GSM1545963    | C57BL/6 mice published<sup>10</sup> |
| pMØ           | H3K27ac ChiP-Sequencing | GSM1545985    |                           |
| Microglia     | H3K4me1 ChiP-Sequencing | GSM1545960    |                           |
| pMØ           | H3K4me1 ChiP-Sequencing | GSM1545982    |                           |
|               |                       | GSM1545987    |                           |

---

Wellcome Open Research 2022, 7:154 Last updated: 30 MAY 2022
mice with BMDM media (DMEM supplemented with 4.5 g/L D-glucose, 4mM L-Glutamine 10% (v/v) Heat-Inactivated fetal bovine serum (FBS), 100 units/mL (v/v) Penicillin, 100 μg/mL (w/v), Streptomycin (all Gibco) and 20 ng/mL of M-CSF (Peprotech), as described in 40. After 7 days BMDM were detached from plates using AccuMax™ and re-plated at 1x10³ cells per well in BMDM medium in a 6-well plate. Cells were transduced with 200 μL/well of a lentiviral vector encoding Cre-recombinase (see 41 for plasmid design) and one of the expression marker, either truncated form of rat CD2 (trCD2) or an enhanced GFP (henceforth termed GFP) sequence. Non-infected cells and cells infected with control vectors, which expressed either a trCD2 or GFP as a reporter of infection, were utilised to control for changes caused by lentiviral infection. Lentiviruses were produced using the Effectene® Transfection reagent (Qiagen, cat# 301427) in HEK293T cells as previously described by 41 and purified by sucrose gradient per41. The infected BMDM media was replenished after 3–4 days in culture, before cells were harvested at 7 days using AccuMax™ per manufacturers direction (Thermofisher) and PU.1 expression was measured by flow cytometry.

Similar experiments were performed using M-CSF differentiated Spil-UREΔMOP cells. The Hoxb8 conditionally-immortalised macrophage precursor (MOP) cell line was derived from CD117⁺ bone-marrow cells pooled from three 10-week-old female Spil-UREΔMOP mice, using previously published methods41. Once differentiated with M-CSF in the absence of estrogen MOP cells are phenotypically similar to BMDM13,44. Conditionally-immortalised MOP cells were differentiated into MØ-like cells following the withdrawal of β-estradiol from the culture media (Roswell Park Memorial Institute (RPMI) 1640 with 4mM L-Glutamine 10% (v/v), Heat-Inactivated FBS, 100 units/mL (v/v) Penicillin, 100 μg/mL (w/v) Streptomycin (all Gibco)) and the addition of 20 ng/mL M-CSF for 4 days, per41,44. Following 3 days differentiation these cells were harvested with 8 mg/mL Lidocaine and 5 mM EDTA in DPBS, which was diluted with cytokine-free culture media before centrifugation at 350 x g for 5 minutes. M-CSF MØPs were re-plated at a 4x10³ cells well per well in a 24 well plate and infected with 250 μL lentivirus a Cre-expressing plasmid with a trCD2 reporter, as previously mentioned41, or a control vector virus. After 3–4 days cells the media was removed and cells were harvested using Lidocaine-EDTA (8 μg/mL (w/v) Lidocaine, 5 mM (v/v) EDTA in PBS) was washed by centrifugation at 350 x g for 5 minutes in PBS before PU.1 was assessed via flow cytometry as described in the following section.

Flow cytometric analysis of PU.1

Isolated peritoneal and brain cells were counted using the Muse® Cell Analyzer (Merck Millipore) per manufacturer’s instruction. Up to 4 × 10⁶ cells per sample were fixed in an equal volume of 8 % formaldehyde (1:1 volume to give a final concentration of 4 % formaldehyde). The samples were washed by centrifugation at 350 x g for 5 minutes before the cells were permeabilised in 90 % ice-cold methanol for 30 minutes on ice. The cells were washed again via centrifugation (350 x g for 5 minutes) before being re-suspended in 100 μL of block solution (4 μg/mL rat anti-mouse FcγRII/III (Clone 2.4G2, produced in-house), 5 % (v/v) filtered rabbit serum, 0.5 % (w/v) BSA, 5 mM EDTA and 2 mM NaN₃ in DPBS) and incubated on ice for 20 minutes. Samples were then divided into two, and stained with 2 μg/mL Anti-CD11b PerCP-Cy5.5 (Clone M1/70, BD Biosciences cat# 301247) in HEK293T cells as previously described by 41 and purified by sucrose gradient per41. The infected BMDM media was replenished after 3–4 days in culture, before cells were harvested at 7 days using AccuMax™ per manufacturers direction (Thermofisher) and PU.1 expression was measured by flow cytometry.

| Genotype          | % Female | Age in Weeks (± SD, n) | % pMØ (± SD, n) | % Microglia (± SD, n) |
|-------------------|----------|------------------------|-----------------|------------------------|
| Spi1-ΔURECONST    | 59.1     | 9.0 (± 2.7, 22)        | 44.2 (± 17.1, 22) | 86.1 (± 8.8, 20)       |
| Spi1-ΔUREERT      | 59.1     | 9.4 (± 2.2, 22)        | 56.6 (± 16.7, 22) | 87.8 (± 7.7, 20)       |
| Cx3cr1TCONST      | 33.3     | 9.4 (± 3.6, 6)         | 55.3 (± 8.6, 6)  | 87.8 (± 4.7, 6)        |
| All genotypes     | 50.5     | 9.2 (± 2.5, 50)        | 51.0 (± 17, 50)  | 87.0 (± 7.8, 46)       |

Table 2. Summary of mice used in this paper by genotype. *n=2 microglia samples were excluded due to an error in sample processing.
either 5 μg/mL of Anti-PU.1 Alexa Fluor™ 647, Rat IgG2a, Alexa Fluor™ 647 isotype control or 120 ng/ml Anti-Cre-recombinase (Clone D3U7F, Cell Signalling Technologies, cat# 12830) with a polyclonal Donkey Anti-Rabbit APC secondary antibody (2 μg/mL dilution, Jackson ImmunoResearch, cat# 711-136-152). M-CSF MØ cell experiments were assessed on a 9-colour Cyan™ ADP analyser (Beckman Coulter) and the BMDM experiment on a BD LSR Fortessa™ (BD Biosciences).

Antibody-specific staining was measured as median fluorescent intensity of the isotype-matched control antibodies subtracted from the receptor-specific reagents. Normalised protein expression was calculated by dividing the antibody specific staining by the control sample, which is indicated in the graph axes by an asterisk (*) where appropriate.

**Imaging cytometric analysis**

Samples were stained with a PU.1 antibody or an isotype control as described in the main methods. 4′,6-Diamidino-2-Phenylindole Dilactate (DAPI; Thermofisher) was added 20 minutes before samples were run on the imaging cytometer at a concentration of 0.5 ng/mL. Unbound DAPI was removed by washing via centrifugation at 350 x g for 5 minutes in 100 μl wash solution (0.5 % (w/v) BSA, 5 mM EDTA and 2 mM NaNO₃ in DPBS). The supernatant was discarded, and cells were re-suspended in 30 μl of FACs wash in 1.5 mL microcentrifuge tubes. These samples were then assessed using the Amnis Imagestream® X Mark II (Merck Millipore) at 40X magnification using the highest bin mode to improve resolution. PU.1 antibody staining was assessed using the ‘Nuclear localisation’ analysis pipeline in IDEAS® software (version 6.2; Merck Millipore) per manufacturers specification.

**PU.1 manipulation in cell lines**

RAW 264.7 cells were maintained in DMEM media supplemented with 10 % (v/v) FBS and 100 units/mL (v/v) Penicillin and 100 μg/mL (v/v) Streptomycin (all Gibco) and passed every 2–3 days by gently scraping the cell monolayer. RAW264.7 cells were harvested using Accumax™ per manufacturer’s directions, and PU.1 was assessed via imaging cytometry. Jurkat cells were maintained in RPMI media supplemented with 10 % FBS (v/v), 100 units/mL (v/v) Penicillin and 100 μg/mL (v/v) Streptomycin (all Gibco) and passed every 3 days as per experimental requirements. Jurkat cells were infected with a lentivirus overexpressing the Spi1 coding sequence (Ensemble 16425.1 preceded by a CCACC Kozak sequence) and upstream of an internal ribosomal entry site (IRES) and a GFP reporter in a bicistronic transcript driven by an Sfi1 promoter, or a control vector that was identical, but lacked the Spi1 coding sequence. PU.1 expression was assessed by flow cytometric analyses and normalised PU.1 expression was calculated by removing the isotype background signal and normalising it to the average median fluorescent intensity (MFI) of pSIEW samples across all experiments (n=4).

**Figures and statistical analyses**

All statistical analyses were performed using PRISM® 6 (version 3.07 GraphPad Software Inc, RRID:SCR_002798). The tests performed are indicated at the appropriate points of the text. R (RRID:SCR_001905) is an excellent open-access alternative software for these analyses.

**Results**

**Analysis of the ~14 Kb Spi1 URE in chromatin datasets**

The likelihood of the -14 Kb URE functioning as an enhancer in microglia and pMØ was first investigated using publicly available datasets. Figure 1 shows the ATAC-Seq and H3K27ac ChiP-Seq profiles for microglia and pMØ in the Spi1 region (see methods), where the -14 Kb URE is indicated by the black bar and the Spi1 promoter by an arrow. The ATAC-Seq data peaks indicated that in both, microglia and pMØ the chromatin was open within the -14 Kb URE region and therefore accessible to being bound by transcription factors. Monomethylation of H3K4 (H3K4me1) and acetylation of H3K27 (H3K27ac) are indicative of active enhancers⁴⁶. Therefore, the H3K4me1/H3K27ac presented in Figure 1 indicates that the -14 Kb URE likely acts as an enhancer in these cell types.

**Lentiviral manipulation of PU.1 in cell lines**

Anti-PU.1 staining was confirmed to be nuclear in location (Figure 2) by imaging flow-cytometry. Figure 2A–B shows that PU.1 antibody staining (red) overlapped with DAPI nuclear staining (blue) and did not overlap with CD11b membrane staining (green). The specificity of this antibody was further confirmed in Jurkat cells which were infected with a lentivirus designed to over-express the murine Spi1 coding sequence. As detailed in Figure 2C–D the antibody detected PU.1 in Jurkats infected with the Spi1 overexpressing lentivirus, denoted by a GFP reporter, but not in cells infected with a control vector virus. Previously this antibody has been used to detect PU.1 in cells where PU.1 levels had been reduced by Spi1 shRNA targeting⁴⁷. Taken together these results indicated that the PU.1 antibody was specific and sensitive enough to detect any potential loss of PU.1 expression following removal of the -14 Kb Spi1 URE.

**PCR confirmation of URE loss**

The loxP recombination deleting the -14 Kb URE was confirmed by genomic PCR (Figure 3). As described in the methods the smaller PCR band (~450 bp) indicates an intact URE whereas the larger 650 bp band was indicative of URE loss. Total peritoneal lavage cells from untreated Spi1-ΔUREfl/fl mice had an intact URE and the Spi1-ΔUREfl/fl lavage cells lacked the -14 Kb URE consistent with the presence of an active Cre recombinase (Figure 3). Overall, these results indicated that the models were functioning as predicted.

**In vitro URE loss did not impact macrophage PU.1**

Preliminary analysis of -14 Kb Spi1 URE removal in bone-marrow derived MØ and M-CSF differentiated MØ cells supported the data from primary MØ populations (Figure 5). BMDM from Spi1-UREfl/fl or C57BL/6J mice were infected with a Cre-encoding lentivirus for 7 days before PU.1 expression was assessed. Spi1-UREfl/fl BMDM expressing Cre recombinase showed no change in PU.1 protein expression compared to BMDM infected with a control vector virus, or C57BL/6J.
Figure 1. ATAC-Seq and H3K27ac profiles of pMØ and microglia accessed through the Cistrome Database. The y-axis scale is indicated to the left of the peaks were the values selected to improve visualisation. ATAC-Seq peaks indicate that the chromatin is accessible, and the H3K27ac/H3K4me1 ChiP-Seq peaks suggest the presence of an enhancer or promoter. Together this data suggests the -14 Kb URE likely enhances Spi1 expression in pMØ and microglia.

controls (Figure 4, n=1). Similar results were obtained with MØ differentiated from Hoxb8 conditionally-immortalised bone marrow MØ precursors, generated from the Spi1-URE<sup>fl/fl</sup> mice (see methods). Again, PU.1 expression was not significantly reduced in Spi1-URE<sup>fl/fl</sup> M-CSF MØP cells infected with a Cre expressing lentivirus, compared to the control lentivirus (unpaired two-tailed t-test P=0.1166; Figure 4C, n=3).

PU.1 was unchanged in primary macrophages lacking the URE – PU.1 antibody staining was assessed in pMØ and microglia of the Spi1-URE<sup>CONST</sup> and untreated Spi1-URE<sup>EXP</sup> mice to see if removal of the -14 Kb Spi1 URE impacted the level of PU.1 protein. The antibody was able to pick up reductions in PU.1 resulting from infection with a Spi1 shRNA virus<sup>7</sup> and induced exogenous expression of PU.1 from infection with a Spi1 over-expression virus (Figure 2).

As indicated in Figure 5B Spi1-URE<sup>CONST</sup> mice, lacking the -14 Kb URE, did not have a lower PU.1 expression compared to untreated Spi1-URE<sup>EXP</sup> mice in pMØ or microglia (n=22 and n=20 per group, unpaired two-tailed t-tests P=0.868 and unpaired two-tailed Welch’s t-test P=0.249, respectively). There were concerns that the PU.1 expression in the microglia could be a result of the tamoxifen-inducible Cre becoming activated in the absence of tamoxifen, as previously observed<sup>46</sup>. Therefore, further comparisons were made with Cx3cr1<sup>CONST</sup> mice as wild-type Spi1/PU.1 controls. Figure 5C confirmed that PU.1 expression did not differ between these strains in pMØ or microglia (n=6 per group, One-way ANOVA, P=0.9334 and P=0.1045, respectively). Figure 6 shows the pMØ and microglia data presented in Figure 5B grouped by age. Age did not have a significant impact on the PU.1 expression (ordinary two-way ANOVA, genotype x age P-values were 0.2986 and 0.6774, respectively).
Figure 2. Specificity and sensitivity of anti-mouse PU.1 antibody. 

A–B First the nuclear localisation of the PU.1 antibody staining was confirmed in RAW cells using the Amnis Imagestream®X Mark II imaging cytometer (Merck Millipore) at 40X magnification. Analysis in the IDEAS™ software shows that PU.1 antibody staining (B red) was similar to the DAPI nuclear staining (blue) and did not overlap with the CD11b membrane stain (green). The magenta colour indicates the areas in which PU.1 antibody and DAPI staining overlapped. This overlap was not seen in the isotype control (A). Data shown from one experiment (n=1) though other immunofluorescence experiments in RAW264.7 cells have confirmed this localisation (n=2, data not shown).

C–D High levels of PU.1 protein were only detected in Jurkats infected with a lentivirus over-expressing the Spi1 coding sequence (Spi1 pSIEW) and not cells infected with the control vector virus (pSIEW) (paired one-tailed T-test, p = 0.0334, n=4 per group). Individual replicates are denoted by symbols and the horizontal bars represent the mean values and the error bars the standard deviation. *PU.1 expression was normalised to the average pSIEW ΔMFI of all experiments.
Figure 3. Confirmation of -14Kb URE removal in transgenic cells. A While the -14 Kb URE is present primers 1 and 2 form a polymerase chain reaction (PCR) band of approximately 450 base-pairs (bp) in size. Primers 1 and 3 were not expected to form a band given the limited extension time (1 minute per cycle) which was insufficient to produce the 3.6 Kb product. The second part shows how the PCR is altered after the -14 Kb has been excised following a Cre-LoxP recombination event. As primer 1 and 3 were expected to be in closer proximity thus allowing a PCR product of ~650 bp to be produced. The sequence binding primer 2 was lost after the URE was excised and therefore the 450 bp product was no longer formed. B Genomic DNA was isolated from *Spi1*-URE floxed MØP cells (derived from 10-week female mice) or from total peritoneal lavage cells taken from 13 week male *Spi1*-ΔURE ERT or *Spi1*-ΔURE CONST mice. The *Spi1*-URE floxed MØP cells were used as a negative control for Cre recombinase. Lavages taken from untreated *Spi1*-ΔURE ERT mice were used as an intact URE control. The DNA from *Spi1*-ΔURE CONST peritoneal lavage produced the 650 bp fragment indicating the presence of an active Cre and removal of the -14 Kb URE.

Discussion
Recent reports have shown that a lower level of *Spi1* expression is associated with a reduced risk of Alzheimer’s disease and delayed onset of this neurodegenerative condition[21]. In the brain *Spi1*/PU.1 is predominantly expressed by microglia[22]. Removal of the -14 Kb *Spi1* URE is reported to result in a marked reduction in *Spi1* expression, thus the -14 Kb *Spi1* URE floxed mouse model has the potential to investigate how a low PU.1 expression alters the microglial transcriptome. For this reason, we investigated how removal of the -14 Kb *Spi1* URE in specific macrophage populations impacted PU.1 protein expression. The PU.1 antibody specificity and sensitivity were confirmed in vitro experiments using lentiviruses to manipulate *Spi1*/PU.1 in cell lines (7 and Figure 2).

The effectiveness of the Cre-LoxP transgenic model was assessed using genomic DNA taken from MØP cell line or peritoneal lavages, which contained pMØ originating from Cx3cr1⁺ precursor cells. The PCR in Figure 3 confirmed that the floxed -14 Kb *Spi1* URE was present in the *Spi1* URE floxed MØP cells and naive *Spi1*-URE ERT cells while being absent in the *Spi1*-URE CONST cells. These results indicated the Cre-LoxP model was working as predicted, by removing the -14 Kb *Spi1* URE in Cx3cr1⁺ cells expressing a constitutively active Cre recombinase (*Spi1*-URE CONST) but not in those expressing an inactive Cre (*Spi1*-URE ERT).

PU.1 protein in pMØ and microglia was assessed via flow cytometry, and it suggested that removal of the -14 Kb *Spi1* URE did not alter PU.1 protein expression. As shown in Figure 5 and Figure 6 no meaningful changes were seen to PU.1 expression in pMØ and microglia taken from *Spi1*-URE CONST mice compared to the same cell types extracted from *Spi1*-URE ERT mice. This suggests that the -14 Kb *Spi1* URE may not be needed to maintain PU.1 in all mature MØ populations under homeostatic conditions.

This was supported by preliminary in vitro experiments (Figure 4) where Cre lentivirus did not reduce PU.1 protein expression in -14 Kb *Spi1* URE floxed BMDM or M-CSF differentiated MØP cells compared to controls.

At first glance this might appear to conflict with the epigenomic data presented above (Figure 1) and 31, who showed a reduced PU.1 expression in foetal liver cells and approximately an 80 % reduction in PU.1 expression in total bone marrow in mice lacking the -14 Kb URE. However, it was not confirmed if this bone marrow reduction in PU.1 was restricted to
either the MØ or B-cell precursors. A follow-up study suggested that while removal of the -14 Kb Spi1 URE reduced PU.1 expression in purified hematopoietic stem cells and resulted in a deficiency of B2 cells (generated from the bone-marrow) there was a proliferative effect on B1 cells residing in the peritoneal and pleural cavities. Neither report demonstrated that PU.1 protein expression was reduced in pMØ or microglia populations.

This model targeted the -14 Kb URE for removal as it is the best characterised enhancer and has been shown to maintain a high PU.1 expression through regulatory feedback loops. Figure 1 and other published work show there are other enhancer sites upstream of the Spi1 promoter where transcription factors can bind to maintain PU.1 expression in MØs that would not be impacted in this model. For example, PU.1 ChIP analysis in mouse cell lines suggests PU.1 binds the -14Kb Spi1 floxed MØP cells infected with Cre or control vector (Unpaired Two-Tailed T-Test P=0.1166). The results presented here were the result of one experiment in BMDM and representative of three similar experiments performed in M-CSF differentiated MØP cells derived from the -14 Kb Spi1 URE floxed mice.

Figure 4. Preliminary in vitro work suggests Cre lentiviral mediated-14 Kb URE removal did not alter PU.1 expression. A Cre antibody staining in infected bone-marrow derived macrophages (BMDM) confirmed that Cre recombinase protein was expressed in Cre pSFEW infected cells. Expression was normalised to the NI sample of each genotype. B In the same experiment non-infected (GFP-) Spi1-URE and C57BL/6J BMDM had similar PU.1 expression in cells infected with either a Cre or a control lentivirus (GFP+). PU.1 expression was normalised to the NI sample for each genotype (n=1). C M-CSF differentiated Spi1-URE-14 MØP cells were also infected with either a control vector or Cre over-expressing lentivirus. PU.1 protein expression was measured after infection and PU.1 expression was normalised to the non-infected cells within each sample (n=3 independent experiments utilising one Spi1-URE-14 MØP cell line). PU.1 expression did not significantly differ in -14 Kb Spi1 floxed M-CSF differentiated MØP cells infected with Cre or control vector (Unpaired Two-Tailed T-Test P=0.1166). The results presented here were the result of one experiment in BMDM and representative of three similar experiments performed in M-CSF differentiated MØP cells derived from the -14 Kb Spi1 URE floxed mice.
Figure 5. PU.1 expression in pMØ and microglia in Spi1-ΔURE<sup>ERT</sup>, Spi1-ΔURE<sup>CONST</sup> and Cx3cr1<sup>Cre<sup>CONS</sup>T</sup> mice. A Example flow cytometry plots and gating strategy used to assess PU.1 expression. B The mean PU.1 expression (horizontal line) did not appear to significantly differ in peritoneal macrophages or microglia isolated from Spi1-ΔURE<sup>ERT</sup> and Spi1-ΔURE<sup>CONST</sup> mice (Unpaired Two-Tailed t-Tests P=0.868 and Unpaired Two-Tailed Welch’s t-Test P=0.249 respectively). The results presented here are from n=22 pMØ and n=20 microglia samples per genotype from 11 independent experiments, aged between 3–14 weeks, 9 males and 12 females per group. *PU.1 expression was normalised to the average Spi1-ΔURE<sup>ERT</sup> value from each experiment (n≥2 mice per group). The mean and standard deviation are denoted by the horizontal line and error bars in both graphs. C Again PU.1 expression did not obviously differ between the Cx3cr1-Cre<sup>CONS</sup>T, Spi1-ΔURE<sup>ERT</sup> or Spi1-ΔURE<sup>CONST</sup> mice in either the pMØ (One-way ANOVA, P=0.9334) or microglia (One-way ANOVA, P=0.1045). The results presented here are from n=6 per group, from 3 independent experiments, aged 7–14 weeks, 4 males and 2 females. The Spi1-ΔURE<sup>ERT</sup> or Spi1-ΔURE<sup>CONST</sup> are included in part B of this figure. *PU.1 expression was normalised to the average Cx3cr1-Cre<sup>CONS</sup>T expression from each experiment (n=2 per group for each experiment). The mean and standard deviations are indicated on the graph by the black horizontal line and error bars respectively.
unsuitable model to study the impact of a reduced PU.1 on these MØ populations. Future work could include looking carefully at the role of the other Spi1/PU.1 enhancer sites and should focus on investigating the role of these enhancers in the maintenance of PU.1 expression in developed tissue resident MØ. Similar transgenic models where PU.1 expression is reduced in MØ may require the removal of multiple Spi1/PU.1 upstream enhancer sites.

Data availability

Underlying data

Open Science Framework: Spi1 -14 Kb URE is not required for maintenance of PU.1 expression in Macrophages. https://doi.org/10.17605/OSF.IO/M74J88.

This project contains the following underlying data:
- FlowCytometricGating.pdf
- RawData Spi1-URE study.xlsx
- PCR Raw Image Figure 3
- Amnis ImageStream data and compensation files for Figure 3

Reporting guidelines

Open Science Framework: ARRIVE checklist for ‘Spi1 -14 Kb upstream regulatory element (URE) is not required for maintenance of PU.1 expression in macrophages’. https://doi.org/10.17605/OSF.IO/M74J88.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements

We would like to offer our sincere thanks to the animal facility staff for their care of the animals used in this study. We also thank Dr W. John Watkins for his guidance regarding statistical tests.

References

1. Back J, Allman D, Chan S, et al.: Visualizing PU.1 activity during hematopoiesis. Exp Hematol. 2005; 33(4): 395–402. PubMed Abstract | Publisher Full Text
2. Heinz S, Benner C, Spann N, et al.: Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. Mol Cell. 2010; 38(4): 576–89. PubMed Abstract | Publisher Full Text | Free Full Text
3. Mak KS, Funnell APW, Pearson RCM, et al.: PU.1 and haematopoietic cell fate: Dosage matters. Int J Cell Biol. 2011; 2011: 808524. PubMed Abstract | Publisher Full Text | Free Full Text
4. Kueh HY, Champhekar A, Nutt SL, et al.: Positive Feedback Between PU.1 and the Cell Cycle Controls Myeloid Differentiation. Science. 2013; 341(6146): 670-3. PubMed Abstract | Publisher Full Text | Free Full Text
5. Karath MB, Houston IB, Janovskia J, et al.: Dose-dependent repression of T-cell and natural killer cell genes by PU.1 enforces myeloid and B-cell identity. Leukemia. 2008; 22: 1214-25. PubMed Abstract | Publisher Full Text
6. Pham TH, Minderjahn J, Schmidl C, et al.: Mechanisms of in vivo binding site selection of the hematopoietic master transcription factor PU.1. Nucleic Acid Res.
24. Butovsky O, Jedrychowski MP, Moore CS, et al.: Modest changes in Spi1 dosage reveal the potential for altered macroglial function as seen in Alzheimer’s disease. Sci Rep. 2021; 11(1): 14393.

22. Heng TS, Painter MW, Elpek K, et al.: RUNX1 regulates repressor interactions of PU.1. Blood. 2011; 117(24): 6498-508.

20. Jones RE, Andrews R, Holmans P, et al.: Spi1 -14 Kb URE is not required for maintenance of PU.1 expression and chromatin accessibility data in human and mouse. Nucleic Acids Res. 2012; 40(8): 3615–23.

19. Sosna J, Philipp S, Albay R 3rd, et al.: Regulation of B lymphocyte and macrophage homeostasis by graded expression of PU.1. Science. 2000; 288(5470): 1439-41.

18. Dagher NN, Najafi AR, Kayala KM, et al.: PU.1 regulates Alzheimer’s disease-associated genes in primary human microglia. Mol Neurodegener. 2018; 13(1): 11.

17. Olmos-Alonso A, Schetters ST, Siri S, et al.: Functional pharmacological targeting of CSFIR inhibits microglial proliferation and prevents the progression of Alzheimer’s pathology. Brain. 2016; 139(9 Pt 3): 891–907.

16. Zhang DE, Hetherington CJ, Chen HM, et al.: The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. J Exp Med. 1994; 179(1): 373-81.

15. Celada A, Borràs FE, Soler C, et al.: The Transcription Factor PU.1 Is Involved in Macrophage Proliferation. J Exp Med. 1996; 184(1): 61-9.

14. Ginhoux F, Greter M, Leboeuf M, et al.: The macrophage transcription factor Spi1 regulates corepressor interactions of PU.1. Nat Immunol. 2012; 13(8): 753-60.

13. Wang Y, Szretter KJ, Vernim W, et al.: IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. Nat Immunol. 2010; 11(10): 1052–61.

12. Wegiel J, Wiśniewski HM, Dziewiatkowska J, et al.: Reduced number and altered morphology of microglial cells in colony stimulating factor-1-deficient pups. J Neuroimmunol. 2008; 196(1-2): 135-9.

11. Linvin Y, Winter D, Blecher-Gonen R, et al.: Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment. Cell. 2014; 159(6): 1327-40.

10. Back J, Dierich A, Bronn C, et al.: The macrophage transcription factor Spi1 regulates corepressor interactions of PU.1. Nat Immunol. 2004; 5(6): 645-6.

9. Jones RE, Andrews R, Holmans P, et al.: Spi1 -14 Kb URE is not required for maintenance of PU.1 expression and chromatin accessibility data in human and mouse. Nucleic Acids Res. 2012; 40(8): 3615–23.

8. DeKoter RP, Singh H, et al.: Regulation of B lymphocyte and macrophage homeostasis by graded expression of PU.1. Science. 2000; 288(5470): 1439-41.

7. Jones RE, Andrews R, Holmans P, et al.: Spi1 -14 Kb URE is not required for maintenance of PU.1 expression and chromatin accessibility data in human and mouse. Nucleic Acids Res. 2012; 40(8): 3615–23.
Open Peer Review

Current Peer Review Status:  

Version 1  

Reviewer Report 30 May 2022

https://doi.org/10.21956/wellcomeopenres.19592.r50633

© 2022 Jung S. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Steffen Jung

Department of Immunology and Regenerative Biology, Weizmann Institute of Science, Rehovot, 76100, Israel

Studies have suggested that reduced expression of the TF PU.1 can be associated with a reduced Alzheimer’s risk. Here the authors generated animals that lack a critical enhancer element predicted to control PU.1 expression in macrophages. They convincingly show that the cell type-specific ablation using Cre and CreER mice is efficient in the respective mice. However, ablation of the -14kb upstream regulatory element (URE) did not affect PU.1 expression in the peritoneal macrophages or microglia. Collectively, these data establish that the -14 kb URE element is dispensable for steady state PU.1 expression in the two cell types tested. The conclusions are supported by solid data throughout.

These results are important to be reported.

PU.1 is both a pioneering factor but also acts as a stimulation induced TF, see the work of Natoli and colleagues (Ghisletti et al. 2010\(^1\)). The question remains whether the -14 kb URE element might be required to drive activation-induced PU.1 expression. To complete their study and rule out a regulatory function of the element the authors could analyze LPS-challenged pMF or microglia following LPS challenge. Moreover, they could include the analysis of a major target gene of PU.1 following stimulation, such as MHC II.

References

1. Ghisletti S, Barozzi I, Mietton F, Polletti S, et al.: Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity*. 2010; 32 (3): 317-28 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and is the work technically sound?  

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

*Competing Interests*: No competing interests were disclosed.

*Reviewer Expertise*: macrophage biology, microglia, monocytes

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.