**The ETS-family transcription factor PU.1 is a critical regulator of the inhibitory Fcγ Receptor IIB expression in humans**

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**Abstract**

The inhibitory Fc gamma receptor IIB (FcγRIIB) is a critical determinant of humoral immunity. By providing feedback inhibition, through inhibitory signalling or competition for antibody Fc engagement, it counterbalances and contextualises cellular responses to signals emanating from co-ligated activating receptors, such as the B-cell receptor and activating FcγR. These activities collectively suppress the emergence of B-cell-mediated autoimmune disease and immune complex-mediated pathologies. However, FcγRIIB upregulation within the tumour microenvironment limits the efficacy of monoclonal antibody (mAb)-mediated immunotherapy of cancer.

While the functional significance of FcγRIIB is well-established in mice, its physiological roles and the regulatory mechanisms governing its expression remain incompletely understood in humans. Here we characterise the molecular determinants of FcγRIIB expression in human immune models and primary cells. Our findings reveal that the ETS-family transcription factor PU.1 plays a crucial role in regulating basal and inducible FcγRIIB expression. Moreover, when co-expressed, PU.1 co-operates with the related ETS-family member SPIB to drive FcγRIIB expression. PU.1 binding to the proximal FcγRIIB promoter elicits transcription, at least in part, through recruitment of the CBP/p300 transcriptional co-activators. Interestingly, similar mechanisms are also observed at the proximal promoters of the activating FcγRI and FcγRIIA, suggesting that additional, potentially lineage specific, factors cooperate with PU.1 to drive the distinct expression patterns of these FcγR. These insights pave the way for future investigations aimed at understanding the molecular mechanisms responsible for cell lineage-specific FcγR expression and subsequently manipulating them for therapeutic purposes.

**Introduction**

Functional insights from murine models have revealed Fcγ receptor (FcγR) IIB (FcγRIIB) as a critical determinant of immune homeostasis and modulator of responses to therapeutic monoclonal antibodies (mAbs) (1-3). In the mouse, FcγRIIB is widely expressed as one of two cell surface isoforms (FcγRIIB1 or FcγRIIB2) in both haematopoietic (including monocytes, B-lymphocytes, eosinophils, basophils, and macrophages (4-6)) and select non-haematopoietic cell types (7, 8). Whilst FcγRIIB1 is principally expressed by B-cells, FcγRIIB2 represents the most dominant isoform in cells of the myeloid lineage (1, 3). In this system, FcγRIIB counterbalances and contextualises signals emanating from co-ligated activating receptors, such as the B-cell receptor (BCR) and activating FcγR (9-12). Through immunoreceptor tyrosine-based inhibitory motif (ITIM)-dependent gating of BCR signals (11), FcγRIIB increases cellular activation thresholds (9, 11) and contributes to B-cell tolerance mechanisms (10, 13, 14). FcγRIIB is particularly required for germinal centre (GC) tolerance (10, 13-16), where upregulation of its expression prevents the emergence of high affinity autoantibody responses (16, 17).

In addition to these B-cell-intrinsic effects (15, 17), FcγRIIB restrains activating FcγR-dependent immunological processes (12, 18-20). Accordingly, dysregulated FcγRIIB expression or function is widely implicated in the development of immune complex (IC)-mediated autoimmune disease (10, 13, 15-17) and in resistance to direct-targeting mAb therapies in mice (21-23). Direct-targeting mAbs (e.g. rituximab) harness activating FcγRs to delete opsonised cells via antibody-dependent cellular cytotoxicity (ADCC) and/or antibody-dependent cellular phagocytosis (ADCP) (24, 25). Of these, mononuclear phagocyte-mediated ADCP appears the most prominent *in vivo* effector mechanism in mice (19, 26, 27). FcγRIIB limits target cell depletion (20, 21) through competition with activating FcγRs for therapeutic antibody Fc engagement (28) and via ITIM-mediated inhibition of activating FcγR signalling (29). In addition to these effects, FcγRIIB is also implicated in limiting responses to immune checkpoint blocking mAbs (30-32) and, paradoxically, augmenting many immunostimulatory mAbs (e.g. anti-CD40, -OX40, 4-1BB, and DR5) (33-36). This latter function is achieved through its ability to further cross-link mAb: antigen complexes and enhance receptor clustering (33, 37-39). These effects are isotype-dependent, with those exhibiting preferential FcγRIIB-binding (e.g. mouse IgG1) the most potent agonists (33, 40).

In contrast to the mouse FcγRII, human FcγRIIB appears more selectively expressed (41). FcγRIIB is broadly detectable in B-lymphocytes, monocytes, basophils, myeloid-derived dendritic cells and hepatic Kupffer cells (4, 41-43), while other healthy tissue macrophages demonstrate a more variable pattern of expression (41, 44). FcγRIIB expression is, however, frequently elevated in primary human tumours (23, 45-47) and metastatic sites (48) including on tumour-associated monocyte and macrophage populations (23). Mirroring observations in the mouse, upregulation or dysregulation of human FcγRIIB is also associated with poor outcomes to tumour immunotherapy (45, 49) and development of IC-mediated autoimmune disease, in the form of systemic lupus erythematosus (15, 50-55). Accordingly, human FcγRIIB elicits similar restraint of BCR- and FcγR-derived signals as well as *in vitro* mAb effector mechanisms (5, 13, 29, 56, 57). Human FcγRIIB is also capable of internalising certain cell surface mAb: antigen complexes (e.g. CD20 & CD19)(58) when engaged in *cis,* leading to removal of accessible mAb and reduction of Fc available to engage effector mechanisms (19, 58-61). Consequently, FcγRIIB is also recognised as a critical negative regulator of mAb-mediated immunotherapy in humans (45, 49).

As biologically- and therapeutically-relevant FcγRIIB functions are frequently regulated through modulation of expression, a detailed understanding of the molecular regulators in multiple cell types is required. In the mouse, the polymorphic *fcgr2b* promoter (16) contains putative transcription start site (TSS) proximal glucocorticoid response, E box, and S box elements alongside AP-1 (16), AP-4 (55), and SP1 transcription factor (TF) binding sites (1, 62). However, a more robust role for the ETS-family member PU.1 (63-67) has been elucidated with additional and contrasting roles for the related factors SPIB (65, 68, 69) and SPIC (66) implied.

Despite similarities in promoter sequence between mouse and human (64), the identities of critical human TFs and promoter elements that drive FcγRIIB expression are poorly defined (1). In humans, Nishimura et. al identified a TSS proximal minimum required promoter fragment (-163: +59 bp from TSS) associated with ZNF140 and ZNF91-mediated repression under ectopic expression conditions (70). Additional studies have identified AP-1 (54) binding at position -304 and TSS proximal HIF2 binding under hypoxic conditions that co-operate to drive hypoxia-mediated FcγRIIB upregulation (23). As AP-1 binds outside the minimum required promoter fragment, the nature of critical TFs responsible for basal FcγRIIB promoter regulation remains unclear.

Given this ambiguity, we systematically dissected the human FcγRIIB promoter to identify critical regulatory elements and TF binding sites. Using human immune cells, we identify that TSS proximal PU.1 binding is essential for promoter activity in models of B-lymphocytes and monocytes. In B-cells, the related TF SPIB also exhibits redundancy with PU.1 in regulating FcγRIIB expression. As SPIB is not expressed in monocytes, these observations represent lineage-specific mechanisms of FcγRIIB promoter regulation. PU.1 is also essential for activating FcγRI and IIA expression in monocytes. However, in isolation, ectopic PU.1 expression is insufficient to drive FcγR expression. Consequently, PU.1 likely primes FcγR loci for transcription and requires co-operation with additional, potentially lineage-specific, TF to elicit FcγR expression.

**Materials & Methods**

**Human subjects & cell lines**

Anonymised leukocyte cones were obtained from informed consenting healthy adult donors attending the Southampton Blood Donor Centre (National Blood Service, Southampton, UK). The use of primary human material was reviewed and approved locally by the University of Southampton Faculty of Medicine Ethics Committee (19660.A11) and at a national level by the National Health Service/Health and Social Care Research Ethics Committee (REC) (IRAS: 186605). Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep density gradient centrifugation medium (Stem Cell Technologies) as described previously (23). Primary human monocytes were isolated by magnetic separation using negative selection Pan Monocyte Isolation kits or positive selection (Figure 5A only) CD14 microbeads (both Miltenyi Biotech). Primary human B-cells were purified by positive selection using CD19 microbeads (Miltenyi Biotech). Purified monocytes were maintained in CTL test medium (CTL) supplemented with 1 mM pyruvate, 2 mM glutamine, 100 U mL-1 penicillin, and 100 µg mL-1 streptomycin at 1x106 mL-1 in a humidified 37°C, 5% CO2 incubator. THP-1, Raji, and Ramos cell lines were obtained from the American Type Culture Collection (ATCC). BJAB and SUDHL6 were the generous gift of Prof. G. Packham. THP-1 cGAS-/- cell line was obtained from Invivogen. All cell lines were maintained at 37°C, 5% CO2 in complete RPMI (RPMI1640 (Thermo Fisher Scientific) supplemented with 1 mM pyruvate, 2 mM glutamine, 100 U mL-1 penicillin, 100 µg mL-1 streptomycin, 10% heat-inactivated foetal bovine serum (FBS)). HEK293F were obtained from Thermo Fisher Scientific and cultured in Freestyle293 medium (Thermo Fisher Scientific).

**Animals**

Human (h)FcγRIIB+/− C57BL/6 J mice, described previously (5), were maintained in local animal facilities in individually ventilated cages (IVC) under specific pathogen-free (SPF) conditions. The use of animals was approved by the University of Southampton Animal Welfare Ethical Review Board (AWERB) and was conducted under UK Home Office Project license P4D9C89EA. Experiments used both male and female mice at approximately 12 weeks of age. Groups were age and sex-matched. Mice were maintained on a 12-h light/dark cycle, with food and water available *ad libitum*. For *in vivo* administration, PLX51107 (Selleckchem) was formulated in 10% n-methyl-2-pyrrolidone, 40% polyethylene glycol-400, 5% tocopherol methoxypolyethylene glycol succinate, and 5% Poloxamer 407, as previously described,(71) and administered at 10 mg/kg *per os* by gavage once daily. Animals were monitored daily for adverse effects, with no toxicities apparent in the study. Animal tissues were extracted and disaggregated to form single cell suspensions as previously described.(23)

**Human FcγR TSS annotation and Reporter Assays**

A representative TSS position per FcγR gene was determined from Ensembl v97 (July 2019) annotation. The representative TSS chosen has the greatest number of Ensembl Havana transcripts, else the most transcripts, else is the most 5’, and was implemented using CiiiDER (72). TSS coordinates are +1-based. The human FcγRIIB TSS was defined as 161,663,160 using the chromosome 1, GRCh38.p14 Primary Assembly (NC\_000001.11), as outlined in Supplementary figure 1A. Positional information, both from this study and the wider literature, have been updated to reflect this nomenclature. A human FcγRIIB 1 Kb promoter fragment (Supplementary Table 1) was cloned from Raji genomic DNA, as described in (5), and ligated into pMCS Cypridina Luc (Thermo Fisher). Deletion constructs (Supplementary Table 1) were created by using a site-directed mutagenesis approach. PCR primers were designed to flank the proposed deletion sites. PCR of the pMCS Cypridina Luc plasmid was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The PCR product was gel purified using QIAquick Gel Extraction Kit (Qiagen) and was subsequently treated with DpnI to destroy the original plasmid (New England Biolabs), phosphorylated using T4 Polynucleotide Kinase (New England Biolabs) and self-ligated overnight using T4 DNA Ligase (New England Biolabs). The reaction mixture was used for bacterial transformation using NEB® 5-alpha Competent E. coli (New England Biolabs). Individual colonies were grown to isolate the plasmid, and deletions were confirmed by Sanger sequencing.

Raji, THP-1 cGAS-/-, Ramos, SUDHL6 and BJAB cell lines were transfected using the Neon Transfection System (Thermo Fisher Scientific). Prior to transfection, cells were grown in antibiotic-free RPMI1640 (Thermo Fisher Scientific) supplemented with 1 mM pyruvate, 2 mM glutamine, 10% FBS. Cells were seeded at 3x105 mL-1 and 48 hours later transfected using 100 µL (Raji, THP-1) or 10 µL tips (Ramos, BJAB, SUDHL6). Cells were washed with PBS and the supernatant removed following centrifugation (300 x g, 5 minutes). Cell pellets were re-suspended at 2x107 mL-1 in Buffer R alongside 5 µg of the indicated pMCS Cypridina Luc construct and 5 µg pCMV Firefly Luc (Thermo Fisher Scientific) constitutive control construct. Cells were aspirated into Neon tips and electroporated using the following parameters: 1350 V, 30 ms, 1 pulse. Following electroporation, cells were seeded at 1x106 mL-1 in antibiotic-free RPMI1640 (Thermo Fisher Scientific) supplemented with 1 mM pyruvate, 2 mM glutamine, 20% heat-inactivated FBS and grown in a humidified 37°C, 5% CO2 incubator. Luciferase activity was measured 24 hours later using Pierce Cypridina-Firefly Luciferase Dual Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s instruction and a Varioskan Flash microplate reader (Thermo Fisher Scientific). Background luminescence readings were subtracted from recordings and cypridina luciferase data normalised to the firefly luciferase internal control. Data were expressed as fold change from the full -1113: +1 bp pMCS Cypridina Luc construct. HEK293F cells were transfected with 10 µg pCMV3 or pCMV3 PU.1 (both Sino Biological) using Freestyle MAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s instruction.

**Gene Knockdown**

Raji and THP-1 cell lines were transfected as described above except 200 picomoles gene-specific siRNA (Detailed in Supplementary Table 2) or an appropriate scrambled siRNA control was utilised (Supplementary Table 2). Impacts upon target gene expression were monitored 24 and 48 hours later by qPCR and/or immunoblot. Impacts upon FcγRIIB expression were reported at timepoints exhibiting peak target gene knockdown (24 or 48 hours). Isolated monocyte gene knockdown was similarly achieved using 100 µL tips, except cells were re-suspended at 3×107 mL-1 in buffer T and electroporated at 1920 V, 25 ms, 1 pulse. Post electroporation, monocytes were cultured at 1x106 mL-1 in CTL medium supplemented with 1 mM pyruvate, 2 mM glutamine, 20% heat-inactivated FBS. Monocyte gene knockdown data are reported 48 hours post transfection.

**Flow Cytometry**

Human cell lines or purified immune cells were incubated at room temperature with complete RPMI 1640 supplemented with 10% Human AB serum (Sigma Aldrich) for 15 minutes. Cells were centrifuged, the supernatant discarded, and re-suspended in flow cytometry wash buffer (PBS, 1% BSA (Europa), 0.1% sodium azide (Sigma-Aldrich)) containing appropriate concentrations of fluorochrome-conjugated antibodies and stained for 30 minutes at 4°C. Samples were stained with anti-CD19 APC (clone: HIB19) or anti-CD14 Pacific Blue (clone: 63D3) (both Biolegend) according to the manufacturer’s recommendations in conjunction with FcγR-specific Fc-silenced mAbs. FcγR staining utilised in-house Alexa fluor 488-conjugated antibodies: anti-FcγRI (clone: 10.1, F(ab')2), anti-FcγRIIA (clone: E08 IgG1 N297Q), anti-FcγRIIB (clone: 6G11 IgG1 N297Q) in comparison to AT171-1 IgG1 N297Q or D10E6 mouse IgG1 F(ab)’2. FcγRIIA or IIB-specific mAbs were described previously (5) and provided by BioInvent International AB, FcγRI-specific mAb was generated from published sequences. Samples were then washed with flow cytometry wash buffer, centrifuged at 340 x g, supernatant discarded, and re-suspended in 100 μL wash buffer. Samples were then acquired using flow cytometry on a FACSCanto II (BD Biosciences) and analysed using FlowJo Software (BD Biosciences). Geometric mean fluorescence intensity (gMFI) was recorded and normalised to gMFI of an appropriate isotype control by subtraction (ΔgMFI).

**Chromatin Immunoprecipitation**

Chromatin was isolated from 4x106 cells per preparation using the SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signalling Technology) according to the manufacturer’s instruction, as previously described (23). Chromatin was digested using 1000 units micrococcal nuclease per preparation at 37°C for 20 minutes and samples were sonicated using an S-3000 sonicator (Misonix) for 6 cycles of 15 seconds (power setting 1.5) followed by a 30 second rest. Nuclear lysis was confirmed by trypan blue staining and appropriate DNA fragmentation assessed by agarose gel electrophoresis. 10 µg chromatin was utilised per immunoprecipitation with optimised concentrations of target-specific or isotype control antibody (Supplementary Table 3) incubated overnight at 4°C. Input control or immunoprecipitation samples were assessed for target enrichment by qPCR using site-specific primers (Supplementary Table 4). qPCR was performed as previously described(73) using either Taqman (Thermo Fisher Scientific) or Sybr-green chemistry using the 2-ΔΔcT or absolute quantification for gene expression and ChIP analysis, respectively. Assay IDs and primer sequences are detailed in Supplementary Tables 4-5. Data was acquired using a CFX connect instrument (Bio Rad) running CFX Maestro software (Bio Rad). Primer amplification efficiency calculation and data adjustment was performed utilising standard curves of ChIP input material using on-board tools of the CFX Maestro Software (Bio Rad).

**Antibodies and Reagents**

Immunoblot analysis was performed using standard techniques, as previously described (23). ChIP antibodies outlined in Supplementary Table 3 were utilised in addition to: anti-IRF3 (Cell Signalling Technology, #11904), anti-Actin (Cell Signalling Technology, #3700), and anti-FcγRIIB (Abcam, ab45143). DMOG was obtained from Sigma-Aldrich, BETi JQ-1 and PLX51107 were obtained from Selleck Chem. IL-6 and IL-10 were obtained from Peprotech.

**Results**

Given the critical roles of FcγRIIB in modulating humoral immunity (10, 17) and therapeutic mAb activity (20), we aimed to identify promoter elements that underpin human FcγRIIB expression to address the lack of current understanding. We profiled the sequence 1 kb (-1113: +1 bp) upstream of the TSS (Figure 1A-B & TSS defined in Supplementary Figure 1A) for regulatory function through a series of luciferase reporter constructs deleted for different regions of the 1kb region transiently transfected into human B-lymphocyte and monocyte model systems (Supplementary Table 1). Raji (B-lymphoma) and THP-1 (monocytic leukaemia) cell lines were selected due to previous reports describing putative differences in FcγRIIB promoter regulation (70). Promoter elements that retained the TSS and its flanking sequence but lacked the upstream regions -313: -24 or -513: -24 bp exhibited reporter activity indistinguishable from promoterless controls in both Raji and THP-1 (Figure 1B). These data identify that sequence within 313 bp of the TSS exerts critical regulatory function. To further refine these observations, we employed 5’ promoter deletion constructs to define the minimum region required for promoter activity. A 104 bp (-103: +1 bp) TSS proximal sequence demonstrated maximal reporter activity indistinguishable from, or greater than, the remaining promoter fragments (Figure 1C). When combined with 3’ deletion data (Figure 1B), these observations define the critical regulatory elements as -103: -24 bp from TSS. To further improve resolution, we subsequently modified the full length -1113: +1 bp reporter construct to incorporate ≈20 bp deletions within the -113: -24 bp TSS proximal sequence. Reporter activity was significantly reduced by disruption of any sequence located within this region; most profoundly following deletions within -83: -24 bp of TSS (Figure 1D). These collective observations were also apparent in 3 additional human B-cell lymphoma model systems (Supplementary Figure 1 B-D) confirming that modification of a 60 bp region located between -83: -24 bp from TSS is highly detrimental to promoter activity, indicative of critical regulatory function.

To identify putative TF binding sites within this TSS proximal region, we employed the predictive motif-matching FIMO algorithm (MEME suite version 5.4.1) (74), using the position frequency matrices of the human TF JASPAR 2020 CORE collection (75) of TF motifs to profile the 2 kb FcγRIIB promoter sequence (1.5 kb upstream of TSS and 0.5 kb downstream). Expression of genes encoding statistically significant motifs (p< 0.0001) was assessed in publicly available datasets and filtered for positive (≥5 TPM) expression in human B-lymphocytes, monocytes, or macrophages using publicly available datasets (Human protein atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) or Blueprint) (76, 77). We identified 12 predicted TF motifs within -83: +1 from the TSS forming two distinct clusters (Figure 2A-B). Critically, the most TSS proximal TF cluster was bisected by the Δ-43: -24 deletion responsible for the most profound suppression of reporter activity (Figure 1D & 2B). To experimentally identify TSS proximal binding of these candidate TF, we performed FcγRIIB TSS-targeted chromatin immunoprecipitation (ChIP) qPCR analysis in Raji and THP-1 cells. Notably, similar patterns of TF enrichment were observed at FcγRIIB TSS proximal locations versus control, despite differences in cell lineage, with PU.1, IRF3, SP1, and STAT1 exhibiting binding in both cell lines (Figure 2C-E). In contrast, association of SPIB with FcγRIIB TSS proximal sequence was specific to Raji (Figure 2C-E). SP4 binding could not be assessed due to a lack of available ChIP-grade antibodies at the time of study. FcγRIIB TSS-associated TF were expressed in both cell lines to variable degrees with the exception of SPIB, which was undetectable in THP-1 cells (Figure 2F). Consequently, differences in SPIB TSS binding correlated with expression level. In contrast, the remaining factors demonstrated a similar extent of TSS proximal binding between cell lines despite variable expression levels (Figure 2E-F).

Subsequently, we employed RNAi-mediated gene knockdown to determine the roles of these candidate TF in driving FcγRIIB expression. Gene knockdown was assessed in both Raji and THP-1 cells at the protein and transcript levels (Figure 3A & Supplementary Figure 2A-B). PU.1 knockdown elicited a modest reduction in baseline FcγRIIB transcript, surface protein, and total protein levels in Raji cells (Figure 3B-E). In contrast, more robust reductions in FcγRIIB expression were observed following PU.1 knockdown in THP-1 cells (Figure 3B-D). ChIP analysis determined that PU.1 appeared enriched at FcγRIIB TSS proximal locations in comparison to distal/intronic regions (Supplementary Figure 3A-B). Accordingly, gene knockdown resulted in the depletion of FcγRIIB TSS-associated PU.1 (Figure 3F). Subsequently, we investigated the impact of PU.1 knockdown upon hypoxia induced FcγRIIB upregulation in THP-1 cells, as previously described (23). PU.1 expression appeared largely unaltered by the HIF prolyl hydroxylase inhibitor, and hypoxia mimetic, DMOG and following incubation under hypoxic conditions (Figure 3G & Supplementary Figure 3C). However, PU.1 gene knockdown effectively ablated FcγRIIB upregulation at both the transcript and protein levels (Figure 3G-H & Supplementary Figure 3C-E). Thus, PU.1 appears to play a prominent role in baseline and inducible FcγRIIB expression in the monocytic THP-1 cell line, with more modest effects upon baseline expression observed in the Raji B-cell line. Similar to the limited impact of PU.1-targeting siRNA, knockdown of the related ETS-family member SPIB modestly reduced FcγRIIB levels in Raji cells (Figure 3B). Given that THP-1 cells lack SPIB expression and demonstrate an increased impact of PU.1 knockdown upon FcγRIIB expression, we reasoned that SPIB and PU.1 may exhibit functional redundancy in Raji cells and, therefore, mask effects upon FcγRIIB expression. Accordingly, co-silencing of PU.1 and SPIB expression elicited a more robust reduction in FcγRIIB at both the transcript and protein levels in Raji cells (Figure 3I-J). In contrast, silencing of other FcγRIIB TSS associated TF (IRF3, SP1, and STAT1) failed to elicit any detectable impact upon FcγRIIB expression following gene knockdown (Figure 3B) or any additional effect when combined with PU.1 gene silencing (data not shown). Consequently, the ETS-family member TF PU.1 appears essential for FcγRIIB expression in the monocytic cell line THP-1, whereas, in Raji cells, PU.1 appears to co-operate in a redundant fashion with SPIB.

Given the essential roles of PU.1 in haematopoietic lineage commitment and regulation of a multitude of immune function-associated target genes, we additionally profiled TSS proximal regions of activating FcγR loci (FcγRI, FcγRIIA, FcγRIIIA, FcγRIIIB) for TF binding motifs. Similar to FcγRIIB, FcγRI and FcγRIIA promoters exhibit putative PU.1 binding motifs located within 50 bp of TSS (Supplementary Figure 4A-D) and demonstrate TSS proximal PU.1 binding by ChIP (Figure 4A). In contrast, FcγRIIIA/B promoters lack TSS proximal PU.1 binding motifs (data not shown). Accordingly, PU.1 knockdown elicited a profound reduction in both FcγRI and FcγRIIA transcript and surface protein levels in THP-1 cells (Figure 4B-C). Similar to FcγRIIB these effects were also concordant with depletion of PU.1 from TSS proximal sequences following PU.1 knockdown (Supplementary Figure 5A). Consequently, PU.1 appears to not only contribute toward inhibitory FcγRIIB expression but also that of the activating FcγRI and FcγRIIA in THP-1 cells. As THP-1 cells lack detectable FcγRIIIA/B expression, the impact of PU.1 knockdown upon these FcγR (that also lack TSS proximal PU.1 motifs) remains to be determined.

Despite commonalities between FcγRI, FcγRIIA, and FcγRIIB regulation, additional mechanisms must also determine inhibitory/activating FcγR expression patterns, as B-lymphocytes lack activating FcγR expression. In order to determine whether PU.1 in isolation was sufficient to drive FcγR expression, we introduced exogenous PU.1 into HEK293F cells that under basal conditions express neither PU.1 nor FcγR. Ectopic expression resulted in enrichment of PU.1 at FcγRI, FcγRIIA, and FcγRIIB TSS proximal sequences and small yet significant increases in FcγR transcripts (Supplementary Figure 5B-D). However, despite supraphysiological PU.1 expression, FcγR protein could not be detected. Consequently, additional potentially cell lineage-specific factors are also required for FcγR expression.

In order to gain insight into the mechanisms of PU.1-mediated FcγR regulation, we investigated the impact of PU.1 gene knockdown upon recruitment of PU.1 interaction partners to FcγR TSS proximal locations. The prominent transcriptional co-activators and histone acetyltransferases (HAT), and known PU.1 interaction partners (78, 79), CBP/p300 were enriched at FcγRI, FcγRIIA, and FcγRIIB TSS proximal sequences and diminished following PU.1 knockdown in THP-1 cells (Figure 4D). Importantly, PU.1 knockdown did not impact total protein levels of CBP or p300 (Supplementary Figure 5E). Knockdown of either CBP or p300 similarly reduced FcγRI, FcγRIIA, and FcγRIIB transcripts but did not influence PU.1 expression (Figure 4E and Supplementary Figure 5F). Consequently, PU.1 appears to drive FcγR expression, at least in part, by recruitment of CBP/p300 to TSS proximal sequences. In addition to CBP/p300, we also assessed the contribution to FcγR expression of the potential PU.1 interaction partner (67), epigenetic reader, and bromodomain and extra-terminal (BET) family member BRD4. Similar to CBP/p300, BRD4 was enriched at FcγR TSS proximal sequences (Figure 4F) in both Raji and THP-1 cells. To explore the impact of BRD4 recruitment on FcγR expression, we employed the bromodomain-specific BET family inhibitors (BETi) JQ-1 and PLX51107 (71, 80, 81). BETi roughly halved FcγRIIB reporter activity in Raji cells (Figure 4G) and reduced surface FcγR expression (Figure 4H-I) in both Raji and THP-1 cells. Notably, the impact of BETi appeared more substantial in THP-1 cells and similarly affected activating and inhibitory FcγR. Given these observations, we examined the impact of BETi upon *in vivo* monocyte FcγRIIB expression through use of human FcγRIIB Tg mice, which express human FcγRIIB under the control of the human -1113: +1 bp TSS proximal sequence (5). BETi administration resulted in reduced hFcγRIIB levels in tissue monocytes of the liver and peritoneal cavity but not that of the spleen (Figure 4J). Collectively, these data suggest known PU.1 interaction partners CBP/p300 and BRD4 contribute toward regulation of both activating and inhibitory FcγR.

To translate our findings to primary human immune cells, we contrasted PU.1/SPIB expression patterns of purified B-cells and monocytes. Consistent with cell line data, primary human B-cells expressed PU.1 and SPIB. In contrast, monocytes demonstrated a lack of detectable SPIB and lower, yet consistently detectable, PU.1 expression than B-cell comparators (Figure 5A). PU.1 gene knockdown significantly reduced basal surface FcγRI, FcγRIIA, and FcγRIIB expression in primary human monocytes (Figure 5B-C). In contrast to THP-1 cells, approximately 5-10% of monocytes (non-classical) also express FcγRIIIA. Consistent with a lack of TSS proximal PU.1 motifs at this locus, PU.1 knockdown did not influence the frequency of FcγRIIIA+ monocytes (Supplementary Figure 6A).

Similar to our cell-line studies, human monocyte PU.1 expression levels appeared unaffected by hypoxia or the FcγR regulating cytokines IL-6 (82) or IL-10 (83, 84) (Supplementary Figure 6B-C). However, PU.1 gene knockdown effectively ablated hypoxia-induced upregulation of FcγRIIB (Figure 5D) and cytokine-mediated upregulation of FcγRI, FcγRIIA, and FcγRIIB in primary human monocytes (Figure 5E-F & Supplementary Figure 6B-C). Further correlating with our cell-line data, BETi also effectively reduced basal FcγRIIB expression levels in primary human monocytes (Figure 5G).

Collectively, our data identify PU.1, SPIB, IRF3, SP1, and STAT1 as FcγRIIB TSS proximal binding TFs in human immune cells. Of these, only PU.1 appears essential for basal FcγRIIB gene expression in both monocytes and B-cells. However, PU.1 also redundantly co-operates with the additional ETS family member SPIB in the Raji B cell-line. Moreover, we identify conserved TSS proximal PU.1 binding as a fundamental cross cell-type determinant of both activating (FcγRI and FcγRIIA) and inhibitory (FcγRIIB) FcγR expression in humans. Mechanistically, PU.1 appears to recruit additional members of the core transcriptional machinery and epigenetic modifiers/readers, namely CBP/p300 and BRD4. However, PU.1 is insufficient to drive high level FcγR expression in an inappropriate cellular context. Furthermore, PU.1 expression appears largely insensitive to tissue/microenvironment-derived FcγR regulatory stimuli. Consequently, PU.1 appears to set the potential for FcγR expression with co-operation from additional lineage-specific and/or inducible factors required to drive FcγR expression.

**Discussion**

Despite its critical roles in regulating humoral immunity (9, 10, 13), FcγRIIB poses a significant obstacle for mAb-mediated immunotherapy of cancer, due to frequent upregulation in both mouse (20-22, 30, 48) and human (45, 46, 49, 85) tumours. Accordingly, FcγRIIB blocking mAbs have been developed that potentiate responses to immunotherapy in experimental mouse models (5). Building upon these findings, the impact of clinical biosimilars upon responses to established mAb immunotherapies are currently under evaluation (NCT03571568, NCT04219254), showing promising interim findings (86-89).

As critical FcγRIIB functions often rely upon gene upregulation (13, 16), a fundamental understanding of the regulatory mechanisms that dictate its expression are paramount. In the mouse, the ETS family TFs PU.1, SPIB, and SPIC have been implicated in FcγRIIB promoter regulation (64-66). Direct binding of PU.1 to the mouse FcγRIIB promoter has been observed experimentally (64), whilst significant decreases in FcγRIIB expression were also evident in mice with reduced PU.1 expression (64, 65).

In mice, PU.1 is broadly expressed within progenitor and mature hematopoietic cells, with its expression level a key determinant of the hematopoietic lineage adopted (90-92). Germline and conditional knockout studies have identified PU.1 as a master regulator of haematopoiesis (93, 94) responsible for regulation of myriad immune-associated target genes (65, 95, 96). Indeed, PU.1 is implicated in regulation of nearly all myeloid gene promoters lacking a TATA box through recruitment of TATA binding protein (TBP), CBP/p300, BRD4 and other components of the basal transcriptional machinery (67, 78, 79). PU.1 is capable of pioneer TF activity (97, 98) but may also act alongside additional factors at low affinity sites that require a pre-prepared chromatin context for transactivation (95, 98).

In murine B-cells, SPIB appears to co-operate with PU.1 to transactivate and drive FcγRIIB expression (65). As a consequence of this functional redundancy, the latter stages of murine B-lymphopoiesis are largely unaffected by PU.1 deletion (99, 100). Instead, combined loss of PU.1 and SPIB is required to reveal functional defects in mature B-cells (65, 101). Despite this, SPIB knock-in into the PU.1 locus is unable to rescue the arrest in murine B-lymphopoiesis associated with loss of PU.1 function (102). Consequently, although SPIB and PU.1 redundancy is evident in driving mature B-cell function, they are clearly non-redundant during early lymphopoiesis (102).

Prior to the current study, the relevance of PU.1 and SPIB to human FcγRIIB promoter regulation were unclear. Nevertheless, the high similarity of PU.1 recognition motifs and FcγRIIB TSS proximal sequence between mouse and humans were highly suggestive (64). Previously, a minimal promoter fragment (-163: +59 from TSS) required for human FcγRIIB promoter activity was identified; however, no positive regulatory factors associated with this region were determined (70). Olferiev et al. identified an AP-1 binding site at position -304, as a critical positive regulator of FcγRIIB (54). However, given its location outside the minimal promoter region, AP-1 binding alone is unlikely sufficient for FcγRIIB transactivation (70). Instead, it likely requires additional TSS proximal factors to facilitate transcription.

In the present study, we refined the human minimal required promoter region to positions -103: +1 in multiple immune models and demonstrated SPIB, PU.1, IRF3, SP1, and STAT1 TF binding in this region. Nishimura et al. (70) previously discounted PU.1 binding to this region in Raji and THP-1 cells based on EMSA supershift assays. However, the conserved PU.1 recognition motif is located approximately 5 bp outside the boundaries of the regions probed in that study (64, 70). Using PU.1-targeting siRNA, we observed a significant reduction in FcγRIIB expression in human cell lines and primary monocytes. This decrease was associated with loss of PU.1 from FcγRIIB TSS proximal sequence and a concurrent reduction in the recruitment of CBP/p300 transcriptional co-activators. Importantly, siRNA-mediated knockdown of CBP/p300 also resulted in reduced FcγRIIB expression, independently of PU.1 expression. This suggests that PU.1 promotes FcγRIIB transactivation, at least in part, by CBP/p300 recruitment. Furthermore, we identified BRD4 enrichment at the FcγRIIB TSS and its inhibition via BET inhibitors JQ-1 (80) and PLX51107 (71, 81) led to a reduction in FcγRIIB promoter activity and expression both *in vitro* and *in vivo*. Although PU.1 knockdown substantially reduced FcγRIIB expression in THP-1 cells and monocytes, the effect was comparatively modest in the Raji B-cell model. This was attributable to functional redundancy with the related ETS-family member SPIB, highly expressed in human B-cells and plasmacytoid dendritic cells but not monocytes (103).

AP-1 has also been implicated in driving both basal (54) and hypoxia-inducible (23) human FcγRIIB expression through promoter binding, despite location outside the minimally required FcγRIIB promoter region (70). As PU.1-targetted siRNA ablated both basal and hypoxia-inducible FcγRIIB expression in monocytes, it is possible that AP-1 co-operates with PU.1 to aid FcγRIIB transactivation. Interestingly, a co-operative relationship, and physical interaction, has been established between PU.1 and c-JUN (an AP-1 constituent) that is required for myeloid development in mice (104). The relevance of these events to human myelopoiesis remain to be determined. In addition to FcγRIIB, PU.1 was also enriched at TSS proximal sites, and required for gene expression, of the activating FcγRI and FcγRIIA as previously described/predicted (105-107). Mechanistically, this also appeared dependent upon recruitment of CBP/p300 to TSS proximal locations.

Whilst the role of PU.1 in haematopoiesis is well defined in the mouse, evidence for an equivalent role in humans is less clear. However, a PU.1 haploinsufficiency-associated agammaglobulinemia was recently described where affected patients lack circulating B-lymphocytes and exhibit deficiencies in myelomonocytic populations (108). Consequently, PU.1 may also demonstrate analogous functions in human haematopoiesis.

Whilst these collective observations represent significant progress in our understanding of human FcγR regulation, our study is limited by a lack of assessment of the functional implications of these critical regulatory elements upon immune physiology. Furthermore, as activating FcγR are not expressed by B-cells, how cell lineage-specific activating/inhibitory FcγR expression is regulated remains unclear. Although PU.1 is critical for FcγR expression in lymphoid/myeloid models, it is insufficient in isolation to drive expression in ectopic expression models such as HEK cells. Consequently, additional, potentially lineage-specific, factors or chromatin remodelling likely contribute to human FcγR expression regulation through co-operation with PU.1. This requirement may also underpin the variability seen in human FcγRIIB expression within tissue macrophage subsets (41).

Here we identify that TSS-proximal PU.1 promoter binding is an essential determinant of basal and inducible human FcγR expression. It is anticipated that this basic understanding will form a foundation upon which future developments can build to understand the complexities of cell type-specific FcγR expression patterns and regulation. As our comprehension of the FcγR requirements that govern effective mAb therapy evolve and begin to influence their design (40), a complementary understanding of the molecular features that govern FcγR expression within tumours is paramount. This increased understanding may be leveraged to optimally deliver mAb therapeutics of the required isotype to elicit desired biological effects or to allow development of complementary drug combination strategies to potentiate mAb-mediated immunotherapy.

**Figure Legends**

**Figure 1: FcγRIIB promoter activity is dictated by a TSS proximal 57 bp sequence. A)** The structure of the low-affinity FcγR locus is depicted and its location within chromosome 1, in the region chr1:161,490,000–161,700,000, identified as a blue vertical line. **C-D)** Raji or THP-1 cGAS-/- cells were transfected with luciferase FcγRIIB promoter constructs with B) 3’ deletions, C) 5’ deletions, or D) TSS proximal deletions and a constitutive internal control. Luciferase activity was assessed after 24 hours, normalised to internal controls, and expressed as fold change from the unmodified -1113: +1 bp promoter construct. NP denotes no promoter control. Promoter diagrams depict to-scale demonstrations of deleted regions. Data denote the mean of at least 3 independent experiments each performed in triplicate. Error bars denote S.E.M. Statistical analyses were performed using one-way ANOVA and adjusted for multiple comparisons via Tukey’s test. ns = non-statistically significant, \* = p <0.05, \*\* = p <0.005, \*\*\* = p <0.0005, \*\*\*\*=p <0.00005. In all figures, bars represent mean.

**Figure 2:** **The FcγRIIB TSS proximal sequence is associated with positively enriched candidate TF. A-B)** Sequence1.5 kb upstream and 0.5 kb downstream of the FcγRIIB TSS was profiled for putative TF recognition motifs using the FIMO algorithm and position frequency matrices of the human JASPAR 2020 CORE collection. Statistically significant motifs (p< 0.0001) were filtered for positive expression in human B-cell, monocyte, or macrophage RNA seq datasets and location within the 0.1 kb TSS upstream region. Filtered TF motifs, their positional data, and p values are tabulated in **A)** and spatially represented in **B)** with deleted regions from **Figure** **1C)** highlighted. **C-D)** FcγRIIB TSS proximal sequence was profiled for candidate TF binding by ChIP using TF-specific mAbs (IgG ChIP) or an appropriate IgG control (IgG Con) in **C)** Raji and **D)** THP-1 cells. Data points represent independent experiments, each performed in triplicate. TF enrichment is expressed as % input and compared to a Histone H3 immunoprecipitation control (H3). **E)** ChIP data from **C-D)** expressed as fold enrichment over IgG control and compared across cell lines. **F)** Immunoblot analysis of whole cell protein isolates from Raji and THP-1 cell lines. Statistical analysis was performed via multiple paired **(C-D),** or unpaired **(E)** T-tests corrected for multiple comparisons using the Holm-Sidak method. ns = non-statistically significant, \* = p <0.05, \*\* = p <0.005, \*\*\*\*=p <0.00005. In all figures, bars represent mean.

**Figure 3: PU.1 and SPIB redundantly co-regulate FcγRIIB expression in a cell-specific manner.** **A)** The impact of FcγRIIB TSS-associated TF gene knockdown upon target protein expression was assessed by immunoblot in Raji cells. Blots are representative examples of at least 3 independent experiments. **B)** The impact of FcγRIIB TSS-associated TF gene knockdown upon FcγRIIB transcript levels was assessed by qPCR in Raji (blue) or THP-1 (green) cells following transfection with gene-specific siRNA in comparison to a scrambled siRNA control (denoted as siSPIB, siPU.1, siIRF3, siSP1, siSTAT1, and siScr, respectively). Data are expressed as fold change from siScr, points represent independent experiments each performed in triplicate. **C-D)** The impact of PU.1 knockdown upon FcγRIIB surface protein levels of Raji and THP-1 cells was assessed by flow cytometry, with representative flow cytometry data depicted in **C)**. Data points in **D)** represent independent experiments. **E)** The impact of PU.1 knockdown was assessed by immunoblot analysis in Raji cells. A representative immunoblot is depicted with densitometry data of 8 independent experiments adjacent. **F)** THP-1 cells transfected with siPU.1 or siScr were assessed by PU.1-directed ChIP in comparison to an isotype control (IgG) using FcγRIIB TSS-specific primers and expressed as % input. A representative example of 2 independent experiments is demonstrated. Data points represent triplicates. **G-H)** The impact of PU.1 knockdown upon inducible FcγRIIB expression was assessed in THP-1 cells by immunoblot. PU.1 targeted siRNA (siPU.1) or a scrambled siRNA control (siScr) were delivered 24 hours prior to addition of 1 µM DMOG or a DMSO vehicle control (Con) for a further 24 hours. A representative immunoblot is depicted in **G)** with densitometry data from 3 independent experiments in **H)**. **I-J)** The impact of PU.1 and SPIB gene co-silencing upon FcγRIIB expression was assessed in Raji cells following transfection with PU.1- (siPU.1) or SPIB-targeting (siSPIB) siRNA alone or in combination (Combo) compared to a scrambled siRNA control (siScr). FcγRIIB expression was assessed at the transcript level **I)** by qPCR and at the protein level **J)** by immunoblot. qPCR data represents the mean of 3 independent experiments. A representative immunoblot (left) is depicted in **J)** alongside densitometry data (right) from 3 independent experiments. Statistical analysis was performed using mixed model **(B)**, 1-way **(F, H)**, or 2-way **(D, F)** ANOVA adjusted for multiple comparisons using Dunnett’s, Sidak’s, or Tukey’s test. **E)** was assessed using Wilcoxon matched-pairs signed rank test. ns = non-statistically significant, \* = p <0.05, \*\* = p <0.005, \*\*\* = p <0.0005, \*\*\*\*=p <0.00005. In all figures, bars represent mean.

**Figure 4: PU.1 regulates FcγR expression through CBP/p300 and BRD4. A)** PU.1 association with FcγRI, FcγRIIA, or FcγRIIB TSS proximal sequence was assessed in THP-1 cells by ChIP (PU.1 ChIP) versus an isotype control (IgG ChIP). Data points represent independent experiments, each performed in triplicate, normalised for primer amplification efficiency, and expressed as % input. **B-C)** The impact of PU.1 knockdown upon FcγRI and FcγRIIA expression was observed in THP-1 cells at the **B)** transcript and **C)** surface protein levels by qPCR and flow cytometry, respectively. **C (left and centre)** depicts representative flow cytometry data; right, fold change from multiple donors. Data points represent independent experiments. **D)** CBP or p300 association with FcγRI, FcγRIIA, or FcγRIIB TSS proximal sequence was assessed by ChIP in THP-1 cells as in **A)** 48 hours post transfection with PU.1-targetted siRNA (siPU.1) or a scrambled siRNA control (siScr). Points represent triplicate values. Data is representative of 2 independent experiments. **E)** FcγR transcript levels were assessed in THP-1 cells by qPCR performed in triplicate following CBP/p300 (siCBP, sip300) gene silencing in comparison to a scrambled siRNA control (siScr); blots above represent extent of target knock-down. **F)** BRD4 association with expressed FcγR TSS proximal sequence assessed by ChIP in Raji (left) or THP-1 (right) cells in comparison to isotype (IgG ChIP) and Histone H3 (H3 ChIP) controls. Points represent independent experiments each performed in triplicate. **G)** Raji transiently transfected with -1113: +1 bp FcγRIIB promoter, or no promoter control (NP), luciferase reporter constructs subjected to 250 nM JQ-1, 625 nM PLX51107 (PLX5), or a vehicle control for 24 hours. Reporter activity was assessed and normalised as in **Figure 1A**. Points represent triplicates. **H-I)** Raji **(H)** or THP-1 **(I)** cells were treated with JQ-1 or PLX51107 (PLX5) as outlined in **(G)** for 24 hours and FcγR surface protein levels assessed by flow cytometry, presented as fold change from vehicle-treated cells. Data points represent independent experiments. **J)** hFcγRIIB Tg+/- mice were treated daily with 10 mg/kg PLX51107 (PLX5) or a vehicle control *per os* for 5 days. Mice were sacrificed 4 hours post final dose and peritoneal cavity (PC), spleen, and liver monocytes (CD11b+, Ly6C+, Ly6G-, F4/80-) were assessed for hFcγRIIB expression by flow cytometry. Geometric means (MFI) were normalised (via subtraction) to an appropriate isotype control (ΔgMFI). Data points represent individual animals. In all figures, bars represent mean. Statistical analyses were performed via T-tests (A & J) adjusted for multiple comparisons using the Holm-Sidak method or one- (E, F, H, I) or two-way (B-D, G) ANOVA adjusted for multiple comparisons using Sidak’s (B, C, E), Tukey’s (D), or Dunnett’s (F, G) tests. ns = non-statistically significant, \* = p <0.05, \*\* = p <0.005, \*\*\* = p <0.0005, \*\*\*\*=p <0.00005.

**Figure 5: PU.1 regulates FcγR expression in primary human monocytes. A)** B-cells and monocytes (Mono) purified from PBMC were profiled for PU.1 and SPIB expression by immunoblot. A representative immunoblot is demonstrated alongside densitometry data obtained from 4 donors. **B-C)** Purified monocytes transfected with PU.1 targeting siRNA (siPU.1) or a scrambled siRNA control (siScr) were assessed for expression of **B)** PU.1 by immunoblot or **C)** surface FcγR by flow cytometry in comparison to an appropriate isotype control. Data in **C)** were normalised to siScr treated cells and expressed as fold change. **D)** Purified monocytes subjected to siPU.1 or siScr for 24 hours were cultured at either 21% or 0.1% O2 for a further 24 hours. FcγRIIB levels were then assessed by flow cytometry and presented as fold change from siScr treated cells cultured at 21% O2. **D left)** depicts representative flow cytometry plots with data from individual donors summarised in **D right)**. **E-F)** PU.1 knockdown of purified monocytes was instigated, as in **D)**, and then treated with 50 ng/mL IL-6, 50 ng/mL IL-10, or a PBS vehicle control (Con) for a further 24 hours. Surface FcγR expression was assessed by flow cytometry as in **D)**. **E)** depicts representative flow cytometry plots, with data from individual donors summarised in **F)**. **G)** Monocytes were cultured in the presence of 250 nM JQ-1 for 24 hours and FcγRIIB expression level assessed by flow cytometry. **G left)** depicts representative histograms with data from individual donors summarised in **G right)**. In all experiments bars represent mean. Statistical analyses were performed via paired T-tests **(A, C, G)** adjusted for multiple comparisons using the Holm-Sidak method where appropriate or one-way ANOVA (D, F) adjusted for multiple comparisons using Sidak’s test. ns = non-statistically significant, \* = p <0.05, \*\* = p <0.005, \*\*\* = p <0.0005, \*\*\*\*=p <0.00005.

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**Data Availability:** The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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