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Kinase-activeTYK2 maintains tonic interferon signals and regulates terminal differentiation within the splenic DC compartment

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Kinase-active TYK2 maintains tonic interferon signals and regulates terminal differentiation within the splenic DC compartment

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Abstract (243 words)

TYK2 is a member of the JAK family of non-receptor tyrosine kinases and plays a crucial, cell type-specific role in responses to various cytokines. In dendritic cells (DCs), TYK2 has been implicated in IL-12- and IL-23-mediated T-helper 1 cell differentiation and in IL-15Rα-dependent natural killer (NK) cell maturation. Kinase-independent functions of TYK2 have also been reported in certain cell types, including NK cells and brown adipocytes. Bulk RNA sequencing and single-cell RNA sequencing of DCs isolated from the spleens of naïve *Tyk2*-deficient (*Tyk2*$^{-/-}$) and kinase-inactive (*Tyk2*^{K923E}) mice generated comprehensive transcriptomic profiles. Differential gene

expression analysis and unbiased annotation clustering were performed, and transcriptomic findings were validated by flow cytometry. In splenic cDCs, catalytically active TYK2 primarily maintained the steady-state expression of interferon-stimulated genes. Single-cell transcriptomes revealed an increased proportion of splenic pre-DCs and a signature indicative for altered maturation and differentiation following loss of TYK2 function. The impaired differentiation of splenic DCs in *Tyk2*-mutant mice was confirmed by flow cytometry. Compared with cDC1, cDC2 and plasmacytoid DCs exhibited greater transcriptional dysregulation in the absence of kinase-active TYK2. The transcriptomic and flow-cytometric profiles of *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ DCs differed only minimally, providing no evidence for kinase-independent TYK2 functions in DCs. These findings broaden our understanding of TYK2-related functions within the heterogeneous DC population of the murine spleen. The identified role of TYK2 in DC development suggests that long-term pharmacological inhibition of TYK2 should be approached cautiously in the treatment of autoimmune and inflammatory diseases.

Keywords: Tyrosine kinase 2; JAK-STAT; splenic dendritic cells; single-cell RNA sequencing; transcriptomics

Abbreviations

APC, antigen-presenting cell; cDC, conventional DC; DC, dendritic cell; DEG, differentially expressed gene; GSEA, gene set enrichment analysis; IFN, interferon; IFNR, IFN receptor; IL, interleukin; IRepG, IFN-repressed gene; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; JAK, Janus kinase; MdFI, median fluorescence intensity; pDC, plasmacytoid DC; scRNA-Seq, single-cell RNA sequencing; SEM, standard error of the mean; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TF, transcription factor; TYK2, tyrosine kinase 2; UMAP, uniform manifold approximation and projection; WT, wild type.

Introduction

Tyrosine kinase 2 (TYK2) was the first identified member of the Janus kinase (JAK) family of non-receptor protein tyrosine kinases associated to cytokine class I and II receptors [1, 2]. TYK2 mediates cellular responses to a range of cytokines and growth factors, including type I interferons (IFN-I), interleukin (IL)-12, IL-22, and IL-23 and, depending on cell type and differentiation stage, IL-10 and IL-13. Depending on the physiological or pathological context, TYK2-associated receptors can activate all seven members of the signal transducer and activator of transcription (STAT) family

[3]. Inborn errors of TYK2 in humans and gene-targeting of *Tyk2* in mice have demonstrated an essential role for TYK2 in host defence against microbial infections and in tumour immune surveillance [1, 4-6]. Human TYK2-deficient cells and genetically modified mouse models have also revealed kinase-independent functions of TYK2, including anchoring cytokine receptors at the cell surface, regulating basal mitochondrial functions and cellular differentiation, and serving as a scaffold for signalling crosstalk [4, 7-10]. Mice expressing kinase-inactive TYK2 [8, 11-13] therefore provide a valuable model for assessing the potential physiological and pathological consequences of pharmacological TYK2 inhibition, which is being developed to treat autoimmune and chronic inflammatory diseases driven by excessive TYK2 signalling [14-17].

Dendritic cells (DCs) comprise a heterogenous family of cells that includes conventional DC (cDCs), which are subdivided in cDC1 and cDC2 and plasmacytoid DC (pDCs) [18]. cDCs are major antigen-presenting cells (APCs) and are pivotal in modulating the activity of immune and stromal cells [18-23]. pDCs are specialised for robust production of type I and type III interferons, as well as other mediators, in response to pathogen-derived nucleic acids and danger signals [24-28]. DC heterogeneity has been further expanded by the identification of additional subsets in mice and men, including cDC2A and B [29], DC3 [30-33], transitional DCs (tDCs) [34, 35], lymphoid cDCs [36] and monocyte-derived DCs [37, 38]. However, these populations are not yet defined by a unified nomenclature across species, tissues, or pathophysiological contexts [20]. The various DC subsets are distinguished by their surface-marker profiles, effector molecules, and, importantly, the spatial and temporal expression of fate-determining transcription factors (TFs) [21, 39, 40].

Previous studies of DCs from *Tyk2*-deficient mice established an essential role for TYK2 in the IL-12/IL-23-driven IFNγ production and Th1 immunity [41, 42]. We previously showed that TYK2 is required in cDCs for expression of IL-15Rα, which supports NK-cell maturation [43]. Building on our recent work identifying JAK-STAT functions in homeostatic macrophages, T cells and fibroblasts of the spleen [44, 45], we hypothesized that TYK2 exerts both enzymatic and/or scaffolding functions in the responsiveness and differentiation of naïve splenic DCs.

To address this hypothesis, we analysed the homeostatic transcriptomes of splenic cDCs from *Tyk2*-deficient (*Tyk2*$^{-/-}$) and *Tyk2* kinase-inactive (*Tyk2*^{K923E}) splenic cDCs using bulk RNA sequencing. We also used single-cell RNA sequencing (scRNA-Seq)

to characterise the heterogeneity of splenic DC subsets under steady-state conditions. In addition, we assessed IL-15Rα expression in naïve and IFNβ-treated cDCs and examined the differentiation capacity of splenic DCs in the absence of TYK2 protein or kinase activity.

Both transcriptomic approaches showed that TYK2 primarily regulates IFN responsiveness in a kinase-dependent manner. TYK2 activity was unexpectedly associated with the maintenance of ribosomal structural gene expression throughout the DC lineage. TYK2-dependent transcriptional profiles displayed pronounced subset-specific patterns among pDCs and cDCs. Although expression of interferon receptor-chain (*Ifnr*) genes did not differ between genotypes, pDCs and cDCs exhibited opposing expression patterns: pDCs showed high *Ifnar* transcript levels, whereas cDCs showed high *Ifngr* expression. Finally, this study provides the first evidence that kinase-dependent TYK2 signalling is required for steady-state differentiation of splenic cDC1 and cDC2 cells.

Material and methods

Table 1: General laboratory materials

Material	**Supplier**	**Catalogue Number**
MACS Pan-DC Enrichment kit	Miltenyi Biotec	130-100-875
RPM-1640 Medium	Sigma-Aldrich	R8758-500ml
Dulbecco's modified Eagle's medium	Sigma-Aldrich	D0819-500ml
Phosphate buffer saline solution (PBS)	Sigma-Aldrich	D8357-500ml
Foetal Bovine Solution (FCS/FBS)	Sigma-Aldrich	F7524-500ml
Collagenase	Sigma-Aldrich	11088866001
Triton X100	Sigma-Aldrich	X100-1l
Penicillin-Streptomycin Solution	Sigma-Aldrich	P0781
Red blood cell (RBC) lysis buffer	Promega	Z3141
24-well cell culture plate flat-bottomed	BD Falcon	353047
Chromium™ Single-Cell 3' Library and Gel Bead Kit	10X Genomics	PN-120267

Table 2: FACS Antibodies

Target	Description	Fluorophore	Supplier and Product No.
CD11c	Used as a positive marker for cDCs and to exclude non-cDC cells, combined with MHCII	APC-Cy7, PE-Cy7, Violet-780	eBioscience, Biolegend/ 47-0114-82, 25-0114-82, 117336
MHCII	Used in combination with CD11c to positively select for cDCs	eF506 (Pacific-Blue), APC-700	eBioscience, Biolegend/48-5321-82, 107622
CD8a	Used as a marker for CD8a positive cDC1	APC	eBioscience 17-0081-82
FLT3	Used as a marker for pre-DCs/cDCs in the spleen	PE	Thermofisher 12-1351-82
CD11b	Used as a cDC2 marker	Alexa F488/M170	eBioscience 53-0112-82
CD43	Leukocyte marker (except resting B cells	PE-Cy7	BioLegend 121218
CD135	FLT3	PE	eBioscience 12-1351-82
Ly6C	Used to determine cDC2 commitment in pre-DCs	Violet 610	BioLegend 128035
Siglec-H	Used to identify pDCs/pre-DC populations (dependent on prior gating strategies)	eFluor660/APC	eBioscience 50-0333-80
Sirpa (CD172a)	cDC2 marker	PC5.5A	eBioscience 46-1721-80
Fixable Viability Dye	Used to determine the live/dead status of cells detected	eFluor506	eBioscience 65-0866-18
Streptavidin	Secondary antibody, binds to the primary antibody of IL-15Rα and IgG	APC	eBioscience 17-4317-82
IgG Isotype antibody	Goat IgG isotype biotinylated control	-	Bio-Techne BAF108

IL-15Rα antibody	Mouse IL-15R biotinylated antibody for IL-15Rα surface level determination experiments	-	Bio-Techne BAF551
CD16, CD32	Purified antibodies for FCG blocking	Unconjugated	eBioscience 14-0161-85
Lin-	DC lineage negative selection: CD3e, CD8a, CD4, B220, Ter119, NK1.1	PB450	eBioscience: 48-0033-82,48-0081-82, 48-0452-82, 48-5921-82, 48-5941-82, REA: 130-118-568

Table 3: Software

Software	**Description** (minimal version)	**URL**
FlowJo	Used for the analysis of flow cytometry data (v10)	https://www.flowjo.com/
CytExpert (Beckman Coulter)	Used for the primary acquisition and downstream analysis of flow cytometry data (v2.0)	https://www.beckman.com/flow-cytometry/research-flow-cytometers/cytoflex/software
R	Programme used for statistical analysis (v3.5)	https://cran.r-project.org/
RStudio	Graphical user interface for R (v2026.10.0)	https://posit.co/download/rstudio-desktop/
Tidyverse	Collection of R packages to increase the graphing and data handling abilities of R (v2.0.0)	https://www.rdocumentation.org/packages/tidyverse/
Seurat	R package used for the handling of scRNA sequencing datasets (v3.5)	https://rdocumentation.org/packages/Seurat/
Limma	R package used for the analyses of RNA sequencing and microarray analyses (v3.0)	https://rdocumentation.org/packages/limma/
UMAP	R package used for the calculation of uniform manifold approximation and projection (UMAP) as a	https://rdocumentation.org/packages/umap/

	nonlinear dimensionality reduction technique (v2.1)	
Clustifyr	R package used for the correlation with sequencing data with published sequencing datasets (v3.11)	https://bioconductor.org/packages/clustifyr/
ggbreak	Introduces graph breaks into a ggplot, used when y-axis values have a large separation (v0.2.2)	https://cran.r-project.org/web/packages/ggbreak/vignettes/ggbreak.html
ggpubr	R package which is a wrapper around the tidyverse ggplot2 package (v1)	https://rdocumentation.org/packages/ggpubr/
rstatix	Pipe-friendly framework for statistical tests (v1.1)	https://CRAN.R-project.org/package=rstatix
BiocManager	R package which acts as a handler for biology packages maintained on Bioconductor (v1.30.27)	https://rdocumentation.org/packages/biocmanager/
MAST	R package for differential expression analysis in scRNA sequencing data (v3.15)	https://bioconductor.org/packages/MAST/
Scater	R package used for the quality control of scRNA sequencing data (v3.7)	https://bioconductor.org/packages/scater/
DoubletFinder	R package used for the quality control of scRNA sequencing data (v2)	https://rdrr.io/github/chris-mcginnis-ucsf/DoubletFinder/f/README.md
ComplexHeatmap	R package used for producing heatmaps and clustering of rows/columns. (Clustering: Complete Linkage, Distance: Euclidean) (v2.0)	https://rdocumentation.org/packages/ComplexHeatmap
FASTQC	A quality control tool for high throughput sequencing data (v0.11.9)	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

STAR	Universal RNA-seq aligner (v2.7.1)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3530905/
Trimmomatic	Flexible Trimmer and Processing tool for Illumina NGS data (v0.3.2)	https://academic.oup.com/bioinformatics/article/30/15/2114/2390096
DESeq2	R Package: Differential expression analysis tool with a range of statistical methods (v3.0)	https://genomebiology.biomedcentral.com/articles/10.1186/s13059-014-0550-8
EdgeR	R Package: Differential expression analysis tool with a range of statistical tool and normalisation methods (v3.10)	https://academic.oup.com/bioinformatics/article/26/1/139/182458
fgsea	R Package: Provides tools for manual over-representation analysis with third-party datasets. Simple overrepresentation analysis based on hypergeometric test (v.1.12.0)	Korotkevich G, Sukhov V, Sergushichev A (2019). “Fast gene set enrichment analysis.” bioRxiv. doi:10.1101/060012, http://biorxiv.org/content/early/2016/06/20/060012
HOMER	UNIX based programme for motif discovery, next generation sequencing analysis, target gene assignment and gene functional analysis (v4.11)	http://homer.ucsd.edu/homer/index.html
CellRanger	Performs sample demultiplexing, barcode processing, single-cell 3' and 5' gene counting, V(D)J transcript sequence assembly and annotation, and Feature Barcode analysis from single-cell data (v3.1.0)	https://www.10xgenomics.com/support/software/cell-ranger/latest
igraph	R Package used for clustering high-dimensional datasets. Csardi G, Nepusz T (2006). “The igraph software package for complex network research.”	https://igraph.org

	InterJournal, Complex Systems, 1695 (v1.2.4.2)	
Reactome	Pathway annotation database and associated tools [46]	https://reactome.org/gsa/home

Mice

Tyk2$^{-/-}$ (*Tyk2*^{fl}*CMV*^{Cre} (B6.129P2-Tyk2^{tm1Biat}Tg(CMV-cre)1Cgn) [47, 48] and *Tyk2*^{K923E} (*Tyk2*^{K293E} (B6.129P2-Tyk2$^{tm3.1(K923E)Biat}$) [11] mice have been described previously. C57BL6/N mice (*WT*) were purchased from Janvier labs.

For analyses of homeostatic IL-15Rα surface expression, 11–12 sex-matched mice aged 8–10 weeks were used per genotype (35 mice in total). For IFNβ-stimulation experiments, 12–15 age- and sex-matched mice were used per genotype (41 mice in total). 18 female mice (6 per genotype) aged 8-10 weeks were used for pre-DC flow-cytometry experiments. For bulk RNA sequencing, two female littermates aged 8–12 weeks were used per biological replicate; a total of 24 mice across the three genotypes contributed to the bulk RNA-Seq datasets (4 sequencing libraries per genotype) as described previously [44]. For scRNA-Seq, spleens from two mice of each sex were pooled for each genotype, resulting in a total of six mice aged 8–10 weeks.

Isolation of homeostatic splenic conventional dendritic cells (cDCs; Lin^{-}, MHCII^{+}, CD11c^{+})

Murine DCs were isolated from spleens by collagenase digestion, red-blood-cell lysis (Promega), and pan-DC enrichment (Miltenyi Biotec), according to the manufacturers' instructions. Following enrichment, samples were centrifuged and resuspended in 100 µL phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). Single-cell suspensions were stained for 30 min at 4°C in the dark with antibodies against CD11c (PE-Cy7) and MHCII (eFluor 450 or Pacific Blue), together with a fixable viability dye (APC-eFluor 780). Cells were washed by adding 2 mL cold PBS and centrifuging for 10 min at 4,000 rpm and 4°C. Pellets were resuspended in 300 µL PBS supplemented with 2% BSA. Viable cDCs were purified by flow sorting based on high CD11c and MHCII expression.

Bulk RNA sequencing

Samples for RNA sequencing were prepared as previously described (GEO:GSE204736) [44]. For bulk RNA sequencing, 200 sorted cDCs were deposited into Triton X-100 lysis buffer and stored at −80°C. Samples were transported to the Research Center for Molecular Medicine (CeMM), Vienna, Austria for processing. RNA input (500 pg) was used for reverse transcription and PCR amplification as described [49]. Libraries were prepared from 1 ng cDNA using the Nextera XT DNA Sample Preparation Kit (Illumina), followed by size selection with SPRI beads (Beckman Coulter). Sequencing was performed at the Biomedical Sequencing Facility at CeMM using an Illumina HiSeq 3000/4000 platform with a 50-bp single-end configuration.

Raw sequencing reads were assessed using FastQC [50] and processed with Trimmomatic [51] to remove adapter sequences and low-quality bases. Filtered reads were then aligned to the mouse reference genome (GENCODE Basic set mm10/GRCm38 release 93) using STAR with sensitive parameters [52]. Reads mapping to annotated exons were quantified using htseq-count [53] and differential expression analysis was conducted using the DESeq2 package with Benjamini-Hochberg adjusted p-values [54]. Known motif enrichment analysis at the promoters of differentially expressed genes (DEG) and gene ontology enrichment analyses were performed using Homer [55] and Metascape [56], respectively. Over-representation analysis against published RNA-Seq datasets was conducted using the fgsea R package [57]. Graphs were generated using R. TMM normalisation was performed using EdgeR [58]. Heat maps were generated using ComplexHeatmap, with dendrograms derived from agglomerative hierarchical clustering using complete linkage.

scRNA sequencing

DC-enriched splenocytes were prepared as described above. Cell suspensions were adjusted to a viable-cell concentration of 10^5 cells/ml in PBS containing 0.2% BSA and transported to CeMM for library preparation using the 10x Genomics Single Cell 3′ v2 platform. Libraries were sequenced on an Illumina HiSeq 4000 instrument.

Samples from *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice were aligned to the mm10 reference transcriptome and processed jointly using CellRanger aggr (10X Genomics) with default settings. A total of 25,621 single-cell transcriptomes were identified, with mean

of 1,060 genes detected per cell. The samples were combined into an aggregate expression matrix for downstream analyses.

Quality control, filtering and clustering

Further filtering and downstream analyses were conducted in R (version>3.5) using the Seurat and Scater packages [59-61]. Each genotype was filtered independently based on the number of detected genes per cell (nGene), the number of uniquely marked identifiers (nUMI), and the proportion of mitochondrial reads (pMito). As recommended [62], the samples were filtered based on the distribution of the features per cell, thresholds were set at the tails of each genotype's nUMI or nGene distributions, and cells were excluded if they contained a nGene or nUMI that was largely outside the expected data distribution (**Supplementary Figure 2**). Principal-component analysis was subsequently performed, and 53 additional outlier cells were removed. (**Supplementary Figure 3**). Doublets were assessed using DoubletFinder [63]. No clusters were excluded on the basis of doublet detection, likely because doublets had already been removed through the nGene and nUMI filtering thresholds. After exclusion of low-quality transcriptomes, all cells were clustered irrespective of genotype calculating the neighbourhood overlap (Jaccard index) and clustering by a shared nearest neighbour (SNN) modularity optimisation-based clustering algorithm, as recommended by default in Seurat workflows (resolution=0.8, dimensions=20). The combined dataset yielded 25 transcriptionally distinct clusters, designated clusters 0–24. Uniform Manifold Approximation and Projection (UMAP) was used to visualise all filtered cells.

Annotation of single-cell clusters

Clusters were annotated using canonical DC markers [29, 64, 65], markers of migratory DCs [29, 37, 66], proliferation-associated markers [67] and markers of non-DC populations [68]. Annotations were refined by correlating the average expression profile of each cluster with published bulk RNA-Seq profiles of splenic immune-cell populations (GEO:GSE109125; ImmGen) [69, 70] and a published scRNA sequencing dataset of cDCs (GEO: GSE137710) [29] using the Clustifyr package [71].

cDC1, cDC2 and pDCs were identified by the expression of DC subset-specific marker expression. cDC1 were characterised by expression of *Xcr1*, *Cd8a*, *Cadm1*, *Clec9a*, *Naaa*; cDC2 by *Sirpa*, *Itgam*, *Irf4*, Clec4a4; and pDCs by *Bst2*, *Siglech*, *Irf7*, *Irf8* DC-

subset annotations were further supported by correlation with published bulk and single-cell RNA-Seq datasets [29, 69, 70]. Non-DC clusters were identified by a low expression of MHCII genes, including *H2-Ab1* and *H2-Dma*, together with lineage-specific marker genes. For example, the T cell cluster expressed *Cd3e*$^{+}$, *Cd27*$^{+}$, *Cd28*$^{+}$, *Cd2*$^{+}$; B cell clusters expressed *Ms4a1*$^{+}$ (*Cd20*$^{+}$), *Cd2*$^{+}$, *Cd19*$^{+}$, *Cd22*$^{+}$; and the monocyte cluster expressed: *Adgre4*$^{+}$, *Csf1r*$^{+}$, *Cd14*$^{+}$. Clusters corresponding to T cells, B cells, monocytes, NK cells, adipocytes, and endothelial cells were considered outside the scope of the DC-focused analysis and were excluded from downstream analyses.

Proliferating cells were identified through the high expression of mitotic markers, including *Top2a*, *Pcna*, *Mcm6*, and *Mki67* [29, 66]. Cell-cycle scores were calculated using Seurat [59, 60] and a reference dataset of murine haematopoietic progenitors [72]. Rather than regressing cell-cycle effects, proliferating cells were excluded from downstream analyses to minimise the risk of artefactual signals [62, 73].

Differential gene expression and enrichment analyses

Differential expression analysis of scRNA-Seq data was performed in Seurat using MAST [74]. P-value adjustment was performed using Bonferroni correction based on the total number of genes in the dataset. Expression profiles of *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} cells were compared with those of *WT* cells within each cluster. When clusters were unequally represented between genotypes, an appropriate *WT* reference cluster was selected, such as the corresponding cDC2 or pDC population. Comparisons between *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} cells were also performed. Because the two *Tyk2*-mutant genotypes showed highly similar expression profiles relative to *WT*, the primary scRNA-Seq analysis focused on *Tyk2*$^{-/-}$ versus *WT* comparisons.

For integrated analysis of genotype- and DC subset-specific genes, differentially expressed genes were selected from the *Tyk2*^{K923E} versus *WT* and *Tyk2*$^{-/-}$ versus *WT* comparisons that met a low significance threshold ($|\log_2 FC|>0.5$, padj<0.05) in at least one comparison based on the fold-change of expression. DEG were transferred to a matrix of genes labelled by their cluster and genotype comparison. The matrix was embedded in two dimensions using the UMAP algorithm implemented in the umap R package with default settings [44, 75]. Gene clusters were identified using the cluster_walktrap function from the igraph package.

Functional annotation of differentially expressed gene groups was performed by gene set enrichment analysis (GSEA) using Metascape [56]. For both bulk and single-cell RNA-Seq analyses, Metascape was run with default settings. Enrichment results were imported into R, and heat maps were generated using ComplexHeatmap and the Tidyverse suite. Over-representation analyses were performed using fgsea to compare enrichments with published sequencing datasets (see Supplementary Data).

Flow cytometric analysis of IL-15Rα cell surface levels

For homeostatic IL-15Rα measurements, total splenocytes were stained in the dark for 20 min at 4°C with fluorescent antibodies, Fc-blocking reagent, and an IL-15Rα antibody or isotype control. Antibodies and Fc-blocking reagent were used at a 1:100 dilution, and the IL-15Rα antibody or isotype control was used at a 1:80 dilution. Following a PBS wash, cells were incubated with APC-conjugated streptavidin (1:10 dilution) for 20 min at 4°C in the dark. Cells were washed, resuspended in 100 µL PBS, and analysed using a Beckman Coulter CytoFLEX flow cytometer. For IFNβ-stimulation experiments, splenocytes were processed through red-blood-cell lysis and enriched using a mouse pan-DC enrichment kit (Miltenyi Biotec). Enriched cells were washed, centrifuged, and cultured in DMEM supplemented with 1% penicillin–streptomycin and GM-CSF (40 ng/µL), with or without IFNβ at a final concentration of 1,000 U/mL. Cells were incubated for 20 h at 37°C and subsequently stained for IL-15Rα as described above. Flow cytometry data were analysed using CytExpert (Beckman Coulter), FACSDiva (BD), FlowJo (TreeStar) and R. Samples were excluded if fewer than 500 events were acquired or if the median fluorescence intensity (MdFI) of the IgG isotype control exceeded that of the IL-15Rα antibody. MdFI values were plotted by genotype and DC subset. Bars represent mean values, and error bars indicate the standard error of the mean (SEM). Statistical significance was assessed using two-way analysis of variance (ANOVA) with genotypes and experimental batch as factors and p-value adjustment by Tukey's honestly significant difference post-hoc test (Tukey-HSD).

Determination of the frequency and commitment of splenic pre-DCs

To confirm the presence of pre-cDC2 populations in the spleen, murine splenocytes were analysed by flow cytometry. Lineage-negative cells were defined as Lin^-:CD3, CD8, CD4, $B220^-$, $Ter119^-$, $NK1.1^-$. Subset-primed pre-DCs were identified as Lin^-,

CD11c$^+$, MHCII$^-$, CD43$^+$, CD135$^+$, CD172a$^+$ cells. Non-subset primed pre-DCs, corresponding to late common DC progenitors (CDPs) were identified as Lin$^-$, CD11c$^+$, MHCII$^-$, CD43$^+$, CD135$^+$, CD172a$^+$, SiglecH$^+$, Ly6C$^-$ cells as described previously [76]. The frequencies of pre-DCs and their proposed lineage commitment, including pre-cDC1, pre-cDC2, and late common DC progenitors, were determined as a percentage of viable cells. Viable cells were identified by the absence of autofluorescence and staining with a fixed viability dye. Statistical comparisons were performed using one-way ANOVA followed by Tukey-HSD. Graphs were generated in R using ggplot2; axis breaks were introduced with the ggbreak package when required to optimise visualisation.

Results

Kinase-active TYK2 governs a two-tiered tonic interferon signature

We expanded on our recent systems-wide analysis of STAT proteins and other JAK-signalling components in splenic immune cells [44] by performing an in-depth characterisation of splenic DCs using bulk RNA-Seq, scRNA-Seq, and flow cytometry (**Figure 1**). FACS-purified splenic cDCs (lineage-negative, CD11c$^+$, MHCII$^+$) from *WT*, *Tyk2*^{K923E}, and *Tyk2*$^{-/-}$ mice were subjected to bulk RNA-Seq **(Figure 1, 3rd row)**. Differential expression analysis across the three pairwise genotype comparisons identified significantly deregulated genes, defined as padj<0.05 and |log$_2$FC|>1.

Compared with *WT* cDCs, *Tyk2*^{K923E} cDCs (*KEWT*) displayed 92 significantly deregulated genes (49 downregulated, 43 upregulated). In *Tyk2*$^{-/-}$ cDCs 100 differentially expressed genes (DEG) relative to *WT* (*KOWT*) cDCs were identified (56 downregulated, 44 upregulated). Comparison of kinase-inactive *Tyk2*^{K923E} with *Tyk2*$^{-/-}$ (*KOKE*) cDCs revealed 47 DEG (27 downregulated, 20 upregulated) **(Figure 2A, Supplementary Data 1)**. The *KEWT* and *KOWT* comparisons shared 49 DEG (23 downregulated, 26 upregulated), all of which changed in the same direction relative to *WT* **(Figure 2B)**. These genes therefore depend on TYK2 kinase activity. The majority of the 23 shared downregulated genes were IFN-stimulated genes (ISGs), including members of the *Oas* and *Ifi* gene families. Notably, the genes encoding STAT1 and IRF9, components of the ISGF3 transcription-factor complex, were also moderately downregulated, consistent with impairment of an IFN-dependent feed-forward transcriptional circuit [77-80]. In murine embryonic fibroblasts and a variety of other

immune cells, however, TYK2 deficiency leads to strongly diminished STAT1 protein or RNA levels [11, 78, 81].

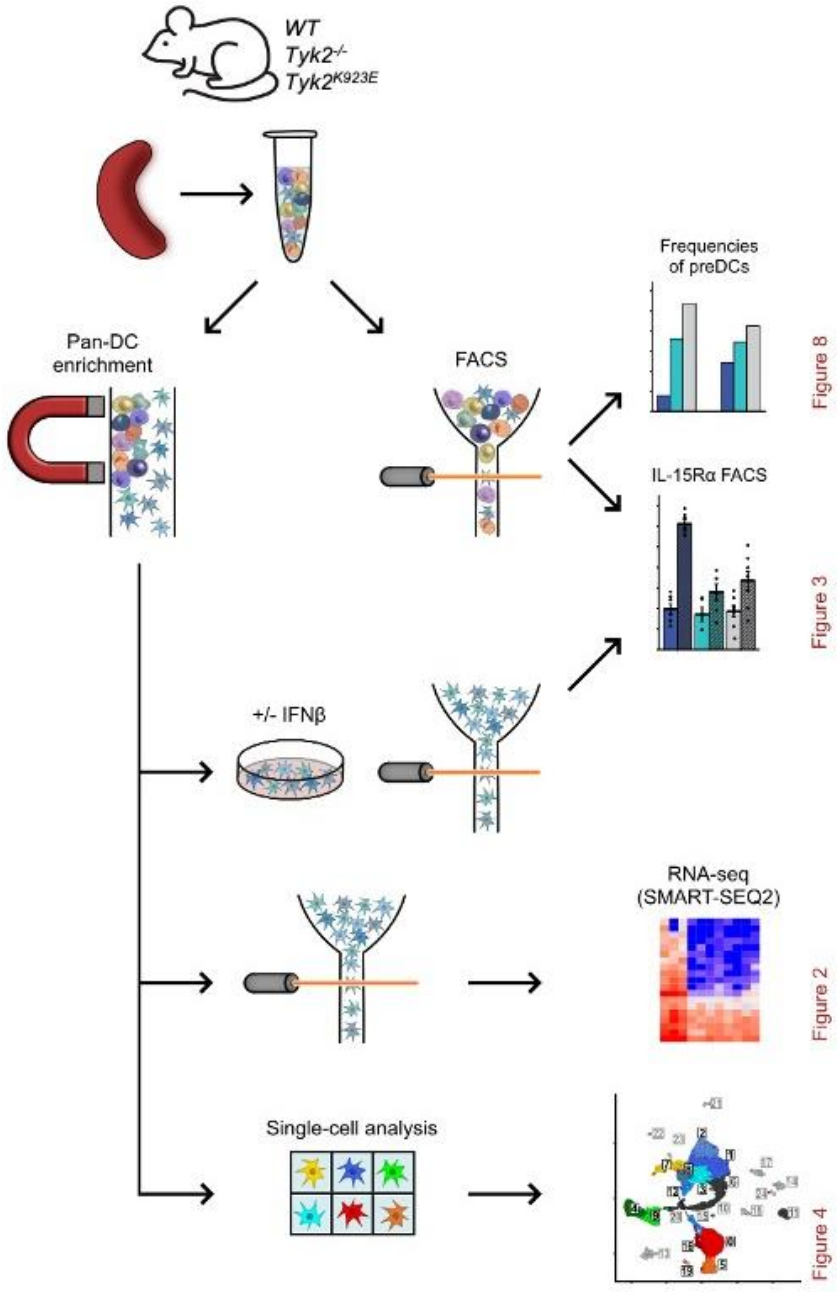


Figure 1 | Schematic diagram of the experimental workflows. Spleens were harvested from *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice aged 8-12 weeks. Splenocytes were processed into single-cell suspensions (1st row, right). Determination of homeostatic IL-15Rα surface levels (Figure 3) and frequency of undifferentiated DCs (Figure 8) was performed by FACS. (2nd row) IL-15Rα surface levels of cDCs upon IFNβ stimulus (Figure 3) were determined by FACS in pan-DC-enriched cDCs (MHCII^{hi}, CD11c^{hi}). (3rd row) For bulk RNA-Seq (Figure 2), pan-DCs enrichment was combined with FACS sorting for MHCII^{hi} and CD11c^{hi} cDCs. (4th row) Pan-DC enriched splenocytes were used for scRNA-Seq (Figure 4).

The downregulated genes exhibit two patterns of deregulation: Pattern 1 genes were highly expressed in *WT* cDCs and showed reduced, but not abolished, expression following loss of TYK2 or its kinase activity. Pattern 2 genes displayed moderate expression in *WT* cDCs but were nearly completely lost in *Tyk2*-mutant cells. This organisation resembles the previously described distinction between “robust” ISGs, which respond to low tonic IFN levels, and “tuneable” ISGs, which require stronger IFN stimulation [82, 83]. However, in our cDC dataset both patterns contained genes that previously classified as “robust” ISGs (Pattern 1: *Isg20*, *Irf9*, *H2-Q7;* Pattern 2: *Oas* family, *Ifit* family, *Ifi44*) and “tuneable” genes were not detectable. Therefore, the extent of basal ISG expression in homeostatic cDCs appears to depend not only on the previously proposed robust-versus-tuneable classification, but also on additional gene-specific regulatory mechanisms. In line with our previous findings [78], the weakened but not abrogated IFN responses in the absence of TYK2 kinase activity [11, 81, 84] also did not account for the expression pattern.

Genes repressed by TYK2 – i.e. genes upregulated in both *KEWT* and *KOWT* comparisons – showed more modest and heterogeneous deregulation **(Figure 2B)**. Several of these genes (*Gpsm2*, *Slc15a2*, *Ppm1l*, *Kpna2*) are classified as IFN-repressed genes (IRepG) [85, 86] or Type I IFN-inhibited genes (TIIG) [87]. Promoter analysis [88] revealed STAT1,3,5 binding sites in several TYK2-repressed genes (*Cyp7b1*, *Dll4*, *Hid1*, *Kpna2*), supporting a role for STAT-dependent transcriptional repression [3, 86, 89]. Bulk RNA-Seq analysis thus showed that the predominant effect of kinase-active TYK2 in homeostatic splenic cDCs is the maintenance of a tonic IFN response programme without effects of kinase-inactive TYK2 **(Figure 2D, Supplementary Data 2-4)**. This conclusion is further supported by pathway (GSEA, Metascape [56]), TF motif (HOMER [55]), and over-representation analyses, which linked the DEG to IFN-associated pathways and to published transcriptional profiles of IFNα-treated cDCs and cDCs lacking IFNAR1 [79, 90]. *Tyk2*-mutant DCs show also an enrichment for ‘interleukin 27-mediated signalling pathway’ (GO:0070106), which firstly is in line with our previous report on requirement of TYK2 for full-fledged IL-27 production [91] and secondly is based on the substantial overlap of IL-27 and IFN response pathway genes [92-94].

Due to the small number of genes and their variable annotations, no firm conclusions about biological functions of TYK2-repressed genes and TYK2 kinase-independent genes are drawn (**Figure 2C, D, Supplementary Data 2-4**).

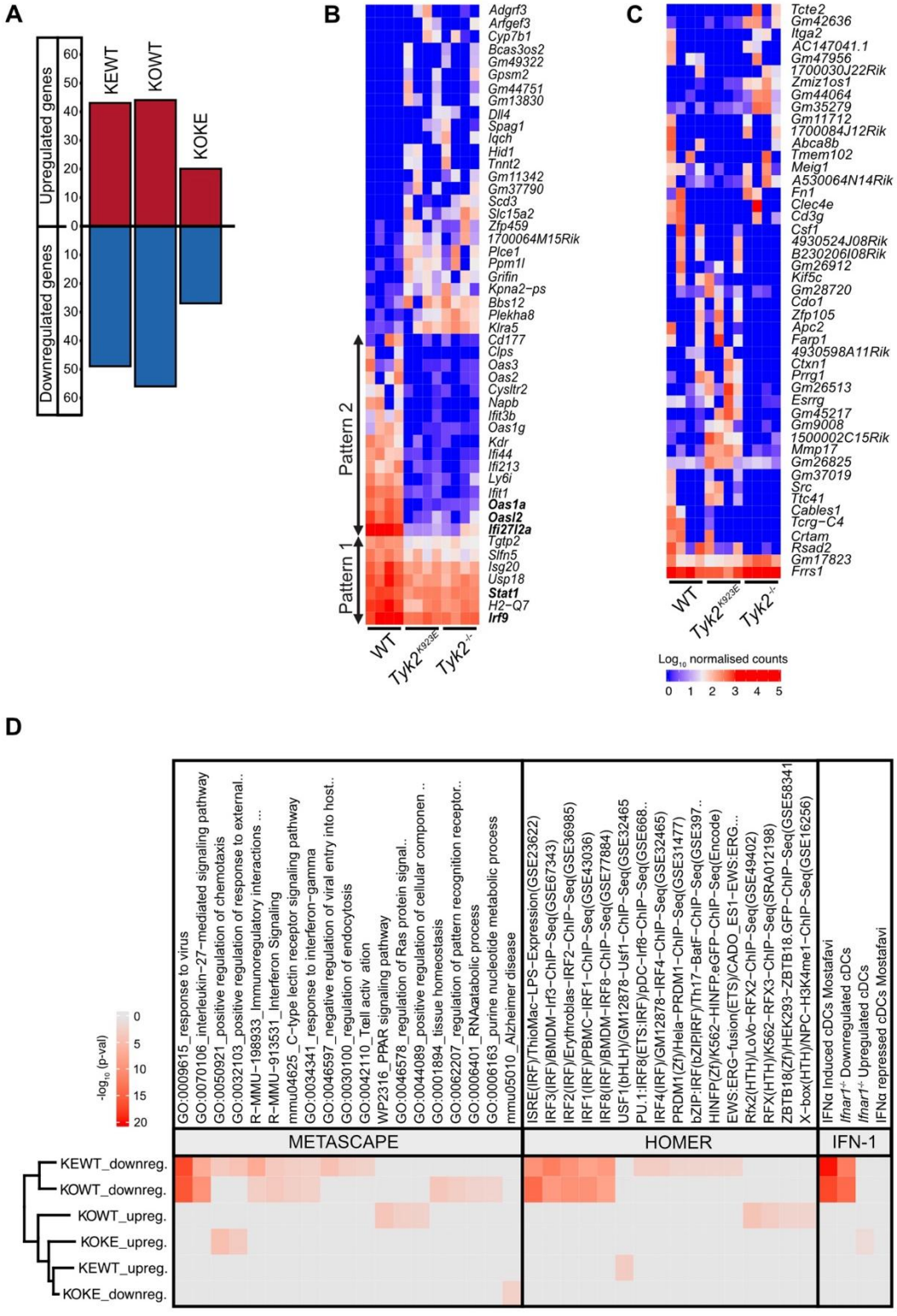


Figure 2 | Transcription profiles of splenic cDCs of WT, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice. (A) Number of significantly deregulated genes (padj<0.05 and |log$_2$FC|>1) for the comparisons *Tyk2*^{K923E} vs *WT* (*KEWT*), *Tyk2*$^{-/-}$ vs *WT* (*KOWT*) and *Tyk2*^{K923E} vs *Tyk2*$^{-/-}$ (*KEKO*). (B) Heatmap of log$_{10}$ normalised counts of genes that are significantly deregulated in *KOWT* and *KEWT*. Labelled gene patterns 1 and 2 show a two-tiered level of downregulation, genes in bold letters are published robust ISG [82]. (C) Heatmap of genes that are significantly deregulated in *KOKE* comparisons. Heatmap scales expression from low (blue) to a high (red). (D) Enrichment analysis was performed on all pairwise genotype comparisons separated by direction of deregulation. Gene set and known promoter motif enrichment analysis was performed using Metascape and HOMER, respectively. Over-representation analysis was

performed to determine the correlation with published IFNα-induced cDCs or cDCs upon loss of IFNAR1 [79, 90]. Significance for annotated pathways was pval<0.05.

In summary, the homeostatic transcriptional profiles of *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} cDCs were highly similar and showed mainly a two-tiered reduced ISG expression in comparison to *WT*. Kinase-active TYK2 exerted also transcriptional repression on selected genes.

Kinase-active TYK2 is required for basal and IFNβ-induced cell surface expression of IL-15Rα

We previously reported that NK cell maturation requires TYK2-dependent IL15/IL-15Rα trans-presentation [43] and *Tyk2*^{K923E} mice show a partial restoration of NK cell maturation [9]. We therefore tested whether TYK2 scaffolding functions are capable to alter cDC IL-15Rα surface levels. In our RNA sequencing datasets, the expression of IL-15 receptor components (*Il15*, *Il15ra*, *Il2ra*, *Il2rb*) were barely detectable and contained no obvious differences between *WT* and *Tyk2*-mutant DCs **(Supplementary Figure 1A, B)**. We next examined the effect of TYK2 cell surface protein level of IL-15Rα in splenic cDC **(Figure 1, 1st and 2nd row, Supplementary Figure 1C, D)**. Under homeostatic conditions, total splenocytes from WT, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice were analysed by flow cytometry for IL-15Rα expression in total cDCs (CD11c^{+}, MHCII^{+}), cDC1 (CD8a^{+}) and cDC2 (CD11b^{+}) under homeostatic and upon *ex-vivo* IFNβ-stimulated conditions **(Figure 1, 1st and 2nd row, Supplementary Figure 1C and D)**. Homeostatic *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} cDCs as well as their subsets show significantly lower surface IL-15Rα levels **(Figure 3A-C)**. Since *Il15rα* (*Cd215*) is an ISG [95] we *in vitro* cultured cDCs for 20 h in the presence or absence of IFNβ (1,000 U ml^{-1}) before IL-15Rα staining. All *WT* cDCs and subsets showed a strong upregulation of IL-15Rα upon IFNβ stimulation. This IFN response was less pronounced in *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} cells **(Figure 3D-F)**, demonstrating again the amplifying role of TYK2 in IFN-I signalling [81, 84].

In conclusion, TYK2 kinase activity contributes to both basal and full-fledged IFNβ-induced IL-15Rα surface expression in splenic cDC subsets.

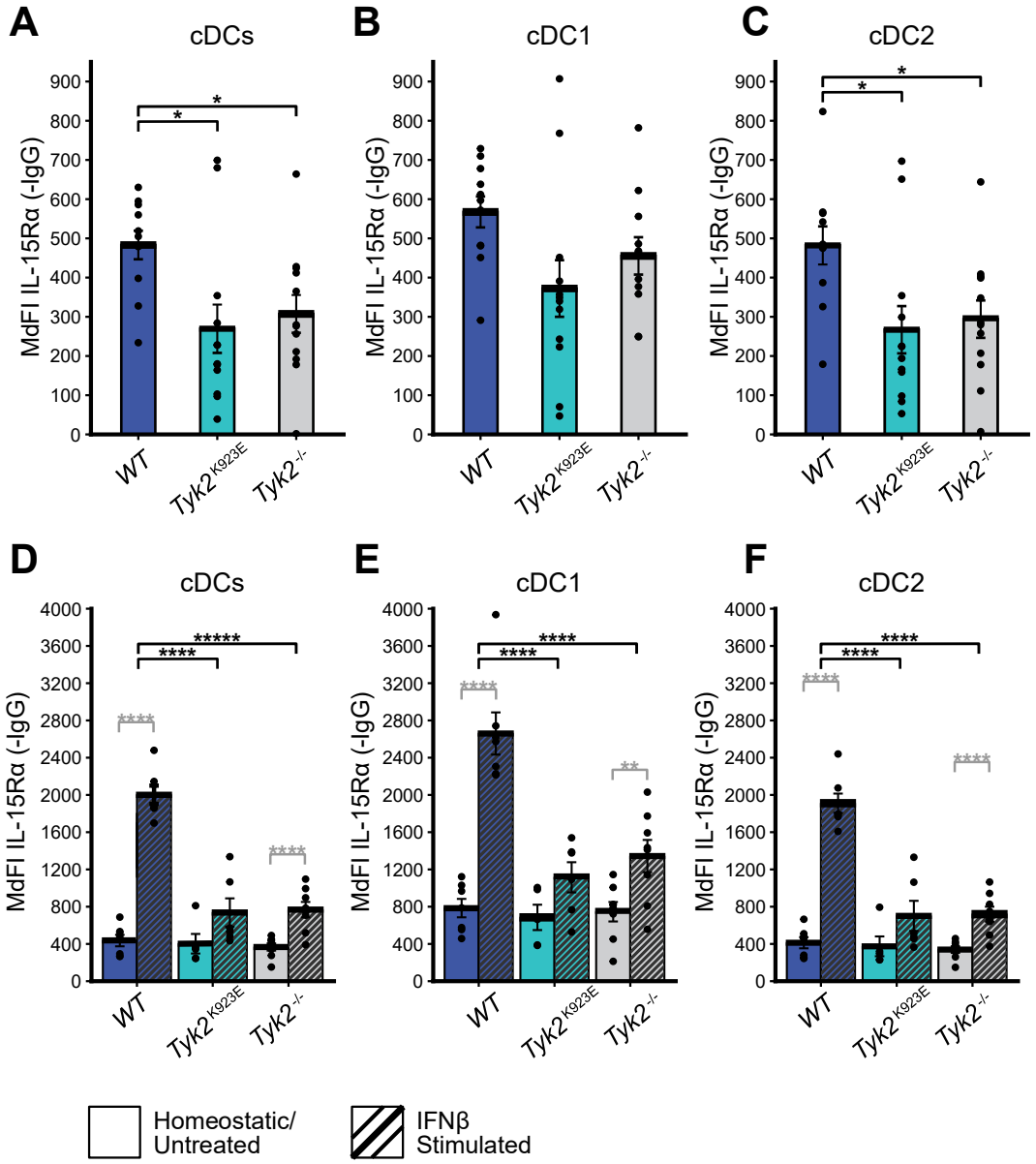


Figure 3 | Surface expression levels of IL-15Rα in total cDC and cDC1 and cDC2 subsets in homeostasis and upon IFNβ stimulation. (A-C) Splenocytes from *WT*, *Tyk2*^{K923E}, and *Tyk2*$^{-/-}$ by flow cytometry for IL-15Rα level in cDCs (CD11c^{+}, MHCII^{+}) as well as cDC1 (CD8a^{+}) and cDC2 (CD11b^{+}). (D-E) Splenocytes were pan-DC enriched and *in vitro* cultured in GMCSF medium with or without IFNβ for 20 hr. Significance is denoted by: *=padj<0.05, **=padj<0.005, ****=padj<0.00005. All statistical tests performed using ANOVA (see Methods).

Dissection of splenic pan-DCs into DC subsets by single-cell RNA-sequencing

Previous studies from our laboratory and others did not identify a substantial role for TYK2 in the differentiation or maintenance of DC subsets [41-43, 96-101]. However, bulk RNA-Seq of *Tyk2*-mutant cDCs revealed a prominent deregulated ISG signature. This has been also reported for *Ifnar1*$^{-/-}$ splenic cDC1 and cDC2 with a greater number of deregulated ISGs observed in cDC2 [90].

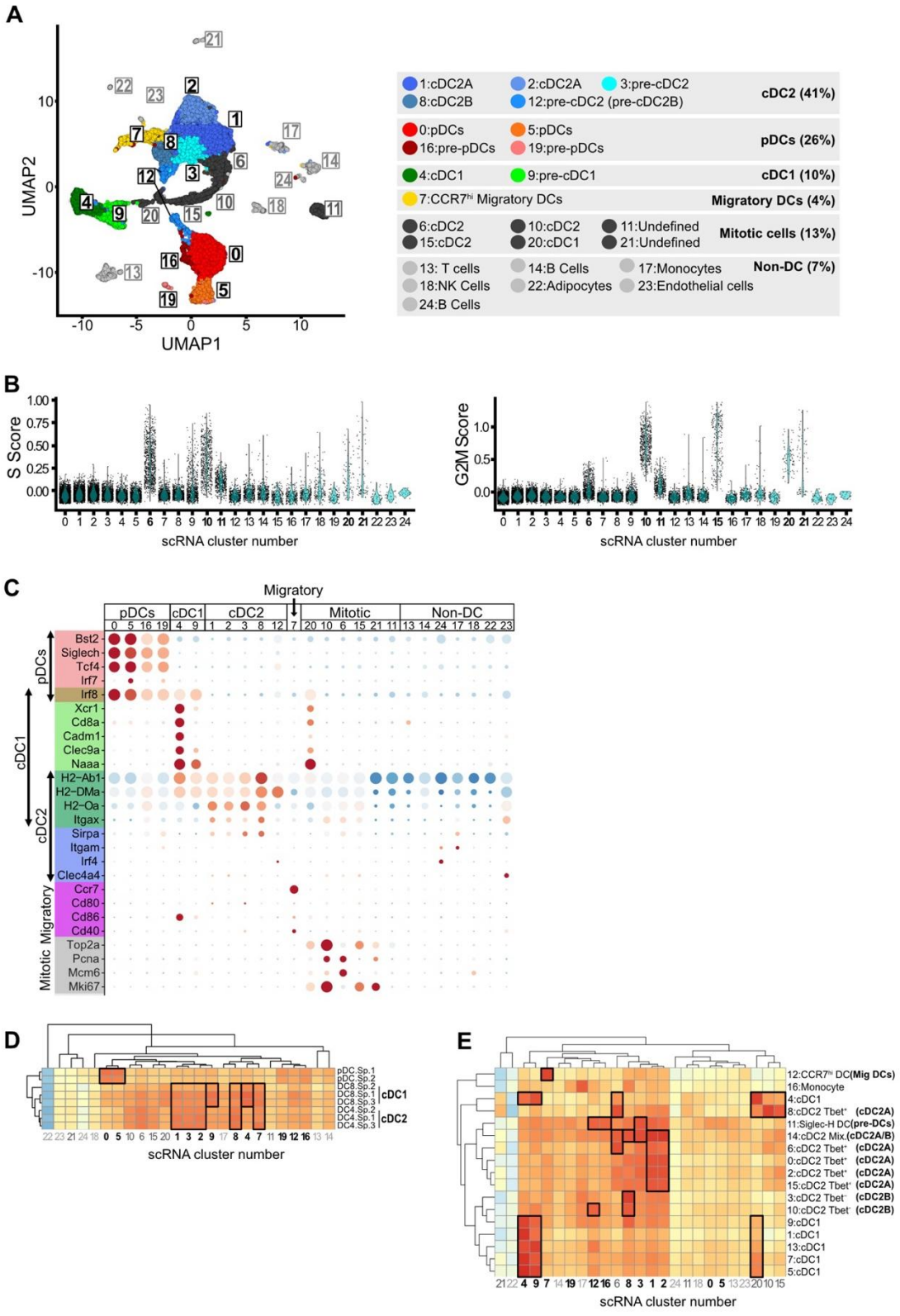


Figure 4 | Single-cell transcriptomes of murine panDC-enriched splenic cells. (A) UMAP visualization of scRNA transcriptomes and corresponding annotation with colour codes. Non-DC populations and mitotic cells were excluded from further analysis. (B) Mitotic clusters (6,10,11,15,20,21) were identified by their high expression of cell-cycle-dependent genes (S and G2/M phase). (C) Dot plot of published markers annotated to DC subset. Dot size indicates percentage of marker genes expressed in cluster (largest dot 100% and smallest dot 0%); colour scale (blue=low, red=high) indicates expression levels. (D) Cluster expression profiles correlated with the ImmGen RNA-Seq DC data set [70]. (E) Cluster expression profiles correlated with published scRNA-Seq of DCs [29]. DC cluster numbers are bold. DC annotations from published results are given their matching terminology.

We thus investigated DC heterogeneity in *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} mice by scRNA-Seq of pan-DC-enriched splenocytes **(Figure 1, 4th row)**. After processing, 25,621 single-cell transcriptomes were identified, with a mean of 1,060 genes detected per cell. Following quality control filtering and removal of outlier transcriptomes, 19,522 cells were retained for downstream analysis (**Supplementary Figure 2 and 3**): 6,064 WT cells, 6,430 *Tyk2*^{K923E} cells and 7,028 *Tyk2*$^{-/-}$ cells. Unsupervised clustering and visualisation using uniform manifold approximation and projection (UMAP) [75] identified 25 clusters **(Figure 4A)**. Cluster annotation was based on cell cycle scoring **(Figure 4B)**, expression of canonical DC markers **(Figure 4C)** and correlation with published bulk and single-cell DC transcriptomic reference datasets [29, 69, 70] **(Figure 4D, E**).

Seven clusters were classified as non-DC populations (**Figure 4A**, right panel, grey palette, clusters 13, 14, 17, 18, 22-24) because they lacked expression of DC markers [64, 102], correlated poorly with published DC transcriptomes, and showed stronger correlations with signatures of other immune cells [29, 69, 70] (**Supplementary Figure 4A, B**). Six additional clusters were identified as proliferating or mitotic DCs based on cell cycle scoring **(Figure 4A,** black palette, **4B**, clusters 6, 10, 11, 15, 20, 21**)** and expression of mitotic markers (*Top2a*, *Pcna*, *Mcm6*, *Mki67*) **(Figure 4D)**. To avoid potential artefacts associated with cell-cycle regression or batch correction, non-DC and proliferating clusters were excluded from subsequent analyses. The remaining non-proliferating DC clusters included two cDC1 clusters (green shades: 4, 9), five cDC2 clusters (blue shades: 1, 2, 3, 8, 12), four pDC clusters (red shades: 0, 5 ,16, 19), and one migratory DC cluster (yellow: 7; **Figure 4B, C)**. Correlation with published bulk RNA-Seq and scRNA-Seq reference data supported and further refined the assignment of these annotations [29, 69, 70] **(Figure 4D, E**, boxed**)**. cDC1 clusters were identified by expression of markers including *Cd8a*, *Xcr1*, *Cadm1*. Clusters 4 and 9 corresponded to cDC1 and pre-cDC1 populations, respectively. Recently, heterogeneity in the cDC1 subset was reported [103]. However, in our analysis, the cDC1 cluster 4 showed overall homogeneity (**Figure 4C**). As reported previously [29, 33, 37, 65], the cDC2 subsets represented the largest splenic DC population and showed high expression of MHCII genes (*H2-Ab1*, *H2-Dma*, *H2-Oa*), moderate expression of *Cd172a* (*Sirpa*) [29, 37] and little to no expression of cDC1 or pDC markers **(Figure 4C)**. pDC clusters expressed *Bst2*, *SiglecH*, *Tcf4*, *Irf7*, whereas high expression of *Ccr7* is indicative for migratory DCs [29, 37] (**Figure 4C**). Note that the

latter cell population is also discussed as a transient DC programme [20]. Clusters 3, 12, 16, 19 displayed mixed cDC2/pDC expression profiles and showed high correlation with Siglec-H^{+} pre-DCs, indicating subset commitment without complete differentiation [29, 37, 66, 76] (**Figure 4E**). We identified no cluster that could be clearly seen to resemble the transitional DCs (tDC, PDCA-1^{int}CD11c^{+}CXCR1^{+}) previously reported [33, 35]. However, we cannot exclude the presence of tDCs at low frequency or dispersed among related clusters.

In conclusion, the scRNA-Seq dataset of splenic DCs revealed the presence of the previously reported mature and pre-DC subsets.

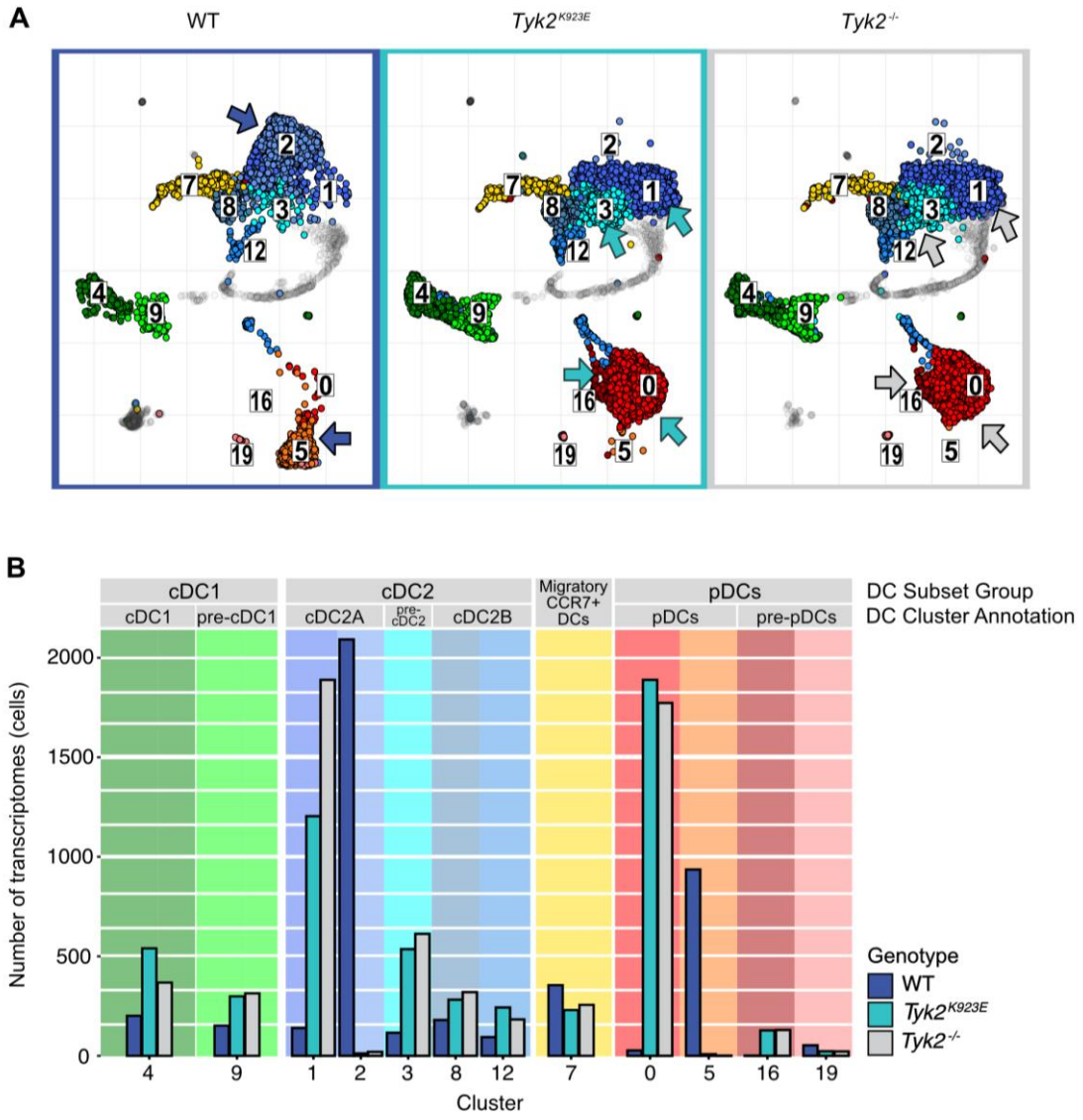


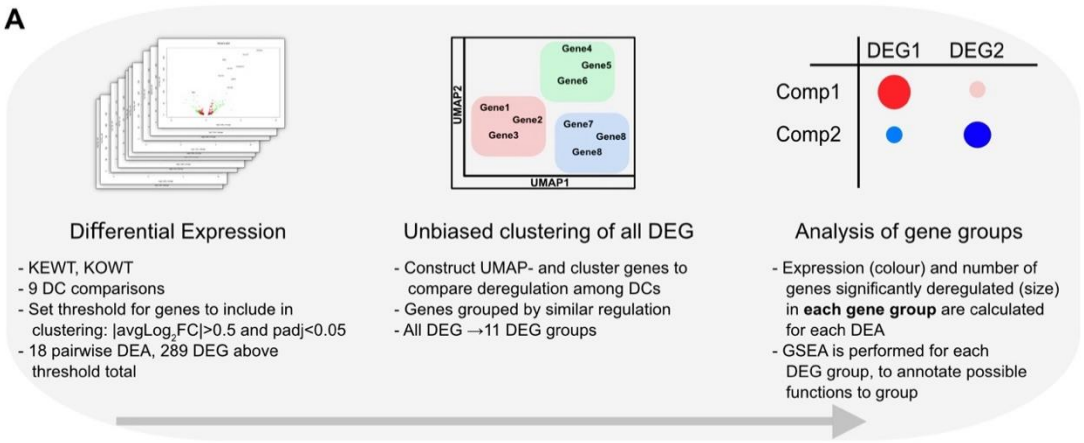
Figure 5 | scRNA-Seq DC clustering separated by genotypes. (A) Genotype-specific (*WT*, *Tyk2*^{K923E}, *Tyk2*$^{-/-}$) UMAP visualisation of DC transcriptome clusters. Areas of interest are denoted with arrows. (B) Bar charts of the transcriptome numbers of each cluster out of all non-mitotic DC transcriptomes, separated by genotype.

Loss of kinase-active TYK2 shifts pDC and cDC2 cluster representation

Genotype-specific UMAP visualisation revealed pronounced differences between *WT* and *Tyk2*-mutant DC populations, particularly among cDC2 (shades of blue) and pDC (shades of red) clusters **(Figure 5, Supplementary Data 5-7)**. *WT* cDC2 cluster 2 accounted for 48.1% of cDC2 transcriptomes but was nearly absent in both TYK2-mutant genotypes (≤0.3%). In contrast, cDC2A cluster 1 and pre-cDC2 cluster 3 were enriched in *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice, representing 22.3% and 9.9% of cells, respectively. A similar shift was evident in the pDC compartment. In WT mice, pDC and pre-pDC transcriptomes were primarily represented by clusters 5 and 19, accounting for 21.5% and 1.2% of cells, respectively. In contrast, TYK2-mutant mice showed enrichment of pDC cluster 0, which represented more than 30% of transcriptomes, and pre-pDC cluster 16, which represented more than 2% of transcriptomes. By comparison, cDC1 (shades of green) and migratory DC (yellow) clusters were represented across all genotypes and displayed substantially smaller genotype-associated differences than pDC and cDC2 populations **(Figure 5)**. These findings indicate that loss of TYK2 or its kinase activity preferentially affects the distribution of cDC2 and pDC transcriptional states. Despite these compositional shifts, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ DC transcriptomes were highly similar. Pairwise differential-expression analyses comparing the two mutant genotypes identified only limited transcriptional differences **(Supplementary Figure 5 and Supplementary Data 10**). Moreover, the two mutant genotypes did not differ markedly in the number of annotated DC clusters and their relative cluster sizes. Thus, the transcriptional consequences of kinase inactivation largely phenocopied complete loss of TYK2 in splenic DCs.

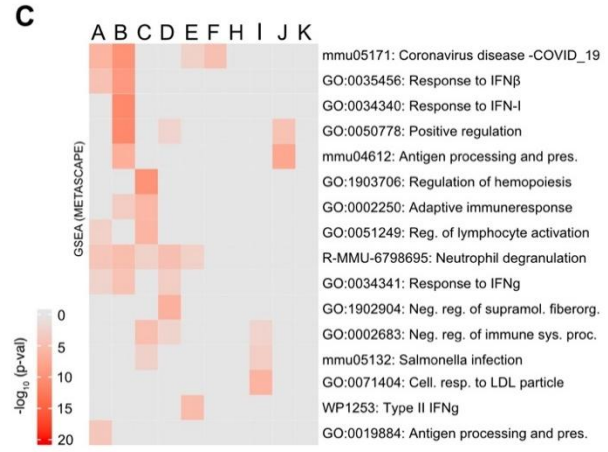
TYK2-dependent transcriptional programmes across DC subsets

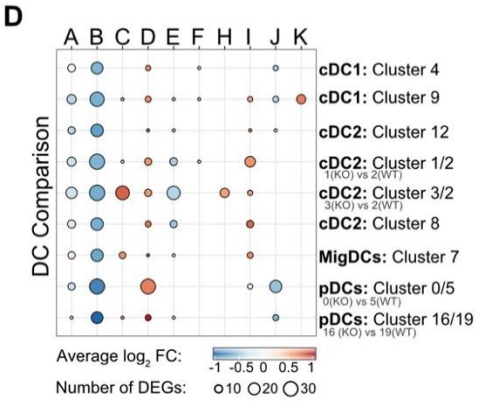
Next, we performed pairwise differential expression analysis for each of *Tyk2*-mutant vs *WT* comparisons across the five DC clusters (cDC2B clusters 8,12; (pre-)DC1 clusters 4, 9; migratory DC cluster 7) that had showed no strong genotype bias (less than 3-fold difference in cluster percentage between *WT* and *Tyk2*-mutant samples). To define shared and subset-specific transcriptional programmes regulated by TYK2, we performed pairwise differential-expression analyses between TYK2-mutant and WT DC clusters.



B

Group	Size	Top 10 genes of each DEG Group
A	67	*Dnaja1, Jun, S100a4, S100a6, Lst1, Rpl36, Bst2, Lyz2, Rpl30, Rpl37*
B	37	*Ifif27l2a, Gm42418, Hbb-bs, Ly6a, H2-Q7, Ifitm3, Ifi27, H2-K1, Hba-a1, Hspa1a*
C	28	*Cd74, Syngr2, Marcksl1, Fscn1, Sox4, Tmem176b, Tmem176a, Rogdi, Il4i1, Nfkbia*
D	39	*Pfn1, Cd209a, Gm26917, Hspa8, Xist, Gpx1, Dynll1, Gltp, Cfp, Cd7*
E	26	*Plac8, Rtp4, AW112020, Smad9l, Irf9, Rps20, Ifit3, Oasl2, Ly6e, Usp18*
F	17	*Hbb-bt, Sp140, Hmgb2, Srgn, Ccnd2, Skint3, Rplp1, Psme1, Mmp12, Rpl28*
G*	13	*Pcbp1, Aebp2, Il6st, Gpr183, B4galnt1, Glul, Slc38a2, Ppp1cc, Tmem123, Scpep1*
H	10	*Ccn2, Mthfs, Rem1, Fabp5, Ryr3, Mthfsl, Samsn1, Tecr, Rsu1, Gdi1*
I	19	*Arpc2, Ddit3, Plbd1, Klf2, Dusp1, Arpc1b, Cd9, Ywhah, Mdh2, Cd81*
J	23	*Ccl5, H2-D1, Ccl4, Gm30211, Iglc2, Pgam2, Phf11b, Trim30d, Acadl, H2-Q4*
K	10	*Ifngr1, Man2b1, Sdf2l1, Manf, Creld2, Hspa5, Pdia6, Tubb6, Arhgdia, Myl12b*





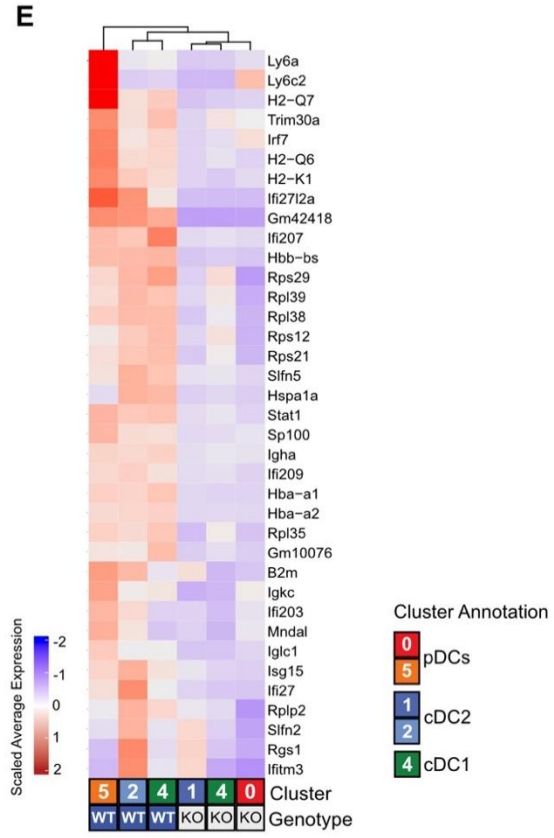


Figure 6 | Differential gene expression analysis across DC cluster comparisons. (A) Methodological scheme for UMAP clustering of DEG (*KOWT*, *KEWT*) and annotation by GSEA. (B) Lists of top10 leading genes in the 11 DEG groups (highest average absolute log_2FC); G*, group excluded from further analysis due lacking deregulation in *KOWT* comparison. (C) Heatmap of the GSEA-annotated (Metascape) DEG modules. Significance is denoted in a red-white colour scale. Pathways are listed with their identifier codes and truncated titles. Additional IFN response and non-descript pathways are not included in the heatmap (R-MMU-3371497, GO:0051235, GO:9959777, GO:0048525); see Supplementary Figure 6. (D) Dot-plot of the DEG modules group for the *KOWT* comparisons, dot size indicates percentage of genes (largest dot 100% and smallest dot 0%) and red-blue scale average log2FC expression (in each DEG. (E) Heatmap of IFN-related genes in DEG group B expressed in the indicated *WT* and *KO* (*Tyk2*$^{-/-}$) DC clusters; for pDC and cDC2 clusters that showed a large clustering bias among genotypes respective clusters were selected for comparison (e.g. cDC2: *WT* 2, *Tyk2*$^{-/-}$ 1, pDCs: *WT* 5, *Tyk2*$^{-/-}$ 0).

For clusters without pronounced genotype-dependent compositional bias (cDC2B clusters 8,12; (pre-)DC1 clusters 4, 9; migratory DC cluster 7), comparisons were made within matched clusters. For cDC2 and pDC populations with substantial genotype bias, TYK2-mutant clusters were compared with the most closely matched WT cluster of the same annotated cell type [104-106] (**Supplementary Data 8-12**).

Thus, the following pairwise differential expression analysis were performed: *Tyk2*$^{-/-}$ (pre-)cDC2 clusters 3 and 1 vs *WT* cDC2 cluster 2; *Tyk2*$^{-/-}$ pDC cluster 0 vs *WT* pDC cluster 5; *Tyk2*$^{-/-}$ pre-pDC cluster 16 vs *WT* cluster 19. Across these comparisons, gene-set enrichment analysis (GSEA) consistently identified deregulation of IFN and immune response pathways **(Supplementary Data 17-18).** To further investigate TYK2-mediated transcriptional regulation across DC subsets, we generated a transcriptional similarity map using UMAP based clustering of DEG as described previously [44]. Genes meeting the threshold of padj<0.05 and |log2FC|>0.5 in at least one of the pairwise comparisons were included **(Supplementary Data 11)**. Genes were grouped according to similarity in their differential-expression profiles across DC subsets, yielding 11 DEG modules, designated groups A–K **(Figure 6A, left and middle panel)**. Gene groups A and B contain genes that are regulated in a TYK2-dependent manner and common to all DC clusters (**Figure 6D**). These groups were significantly enriched for IFN, antiviral, and immune-response pathways (**Figure 6B and C, Supplementary Figure 6A, Supplementary Data 15**). Additionally, they contain genes encoding members of the ribosomal protein L and S family (*Rpl* and *Rps* genes), other ribosomal subunit protein genes (*Rplp2*, *Rrbp1*) and eukaryotic initiation/elongation factors (*Eif2ak2*, *Eif2s3y*, *Eef2*) (**Supplementary Data 13-14**). The magnitude of downregulation varied among subsets, with pDCs showing the most

pronounced deregulation. Notably, loss of *Rpl* and *Rps* transcripts in the absence of TYK2 is also observed in the DEG groups E and F (**Figure 6B**). DEG group E contained downregulated ISG prominent seen in cDC2s (clusters *Tyk2*$^{-/-}$ 3, *WT* 2) (**Figure 6B**, **Supplementary Figure 6A**).

In *WT* mice, expression of selected ISGs was graded across DC subsets, with the highest expression in pDCs, intermediate expression in cDC2s, and lower expression in cDC1s. In contrast, ISG expression was uniformly reduced in Tyk2-mutant mice (**Figure 6E**). pDCs (cluster 5) showed particularly high basal expression of *Ly6* and MHC class I gene family members (**Figure 6E**, upper left panel). Given that these genes are associated with activated, type I IFN-responsive pDCs, this pattern is consistent with the established high basal IFN responsiveness of this subset [107]. As described previously for other homeostatic splenic immune cell types [78], DC subsets also showed differential expression of ISGs, with highest levels of *Ifitm3* and *Ifi27* in cDC2s and *Ifi207* in cDC1s.

pDCs showed also downregulated DEG in group J genes upon loss of TYK2, which were enriched for pathways related to ‘positive regulation of immune response’ (GO:0050778) and ‘antigen processing and presentation’ (mmu04612) (**Figure 6C and D**). This finding is consistent with the established role of pDCs as APCs [108].

DEG group D comprised genes induced in the absence of TYK2 in pDCs, which were broadly associated with immune regulatory functions (**Figure 6C and D**). These genes have reported roles in DC activation (*Cd209a*, *Hspa8*, *Pfn1*, *Cfp* [109-112]); haematopoietic differentiation (*Xist*, *Cd7* [113, 114]); mitochondrial redox homeostasis (*Gpx1*, *Dynll1* [115, 116]); and vesicle trafficking or antigen presentation (*Gltp* [117, 118]).

Taken together, in addition to the known IFN and antimicrobial response genes upregulated in a TYK2-dependent manner in all DCs, we found genes involved in the assembly of ribosomes and basic translational processes [119-121]. Genes downregulated by TYK2 activity were specific to cDC2 and pDC subsets. They do not belong to the class of IRepG [77, 85-87], but annotate to differentiation/maturation processes, and are regulated in a manner requiring further elucidation. The observed graded levels of tonic ISG expression in *WT* pDC, cDC1 and cDC2 are in line with a previous report [90] and are likely caused by differential exposure to homeostatic IFN and availability of cell intrinsic IFN-I signalling components.

pDCs and cDCs differentially express IFN-I and -II receptor chains in a largely TYK2-independent manner

To determine whether the observed subset-specific differences in IFN responses were explained by changes in the expression of IFN-signalling components, we compared the expression of IFN receptor chains, JAKs, STATs, IRFs, and SOCS family members across DC subsets (**Figure 7A**). IFN-induced JAK-STAT signalling undergoes tight positive and negative autoregulation [77]. The classical ISG *Stat1*, *Ifr7* and *Irf9* show the expected reduced expression in the *Tyk2*-deficient DC subsets. In contrast, the other *Stat* and *Irf* family members did not show major differences between genotypes and DC subsets (**Supplementary Data 8, 14**).

In *WT* pDCs, *Irf7* is highly expressed, which reflects the observed high IFN response pattern in pDCs and the known constitutive basal expression of *Irf7* for mounting a high IFN production in this subset (reviewed [27, 122]). *Irf8* expression appears graded from highest in pDCs, medium in cDC1, and lowest in cDC2, which mirrors the central role of IRF8 in pDC and cDC1 differentiation, survival and function [123, 124].

The expression levels of *Jak and Socs* family members appear rather uniform among samples, except for the *Jak2* levels which are reduced in *WT* and *Tyk2*$^{-/-}$ pDCs and in *Tyk2*$^{-/-}$ cDC1. This might be owed to a reduced IFNγ response in pDCs (see below) and/or the IFN inducibility of the gene [80].

The IFN receptor chains also displayed DC subset-specific expression patterns. Type I IFN receptor chains (*Ifnar1*, *Ifnar2*) were robustly expressed in pDCs and weakly expressed in cDC subsets, whereas *Ifngr1* – and to a lesser extent *Ifngr2* – were highly expressed in cDCs but not detectably expressed in pDCs (**Supplementary Data 8**). Consistent with the described dispensable role of TYK2 in IFN type III responses [125, 126], the levels of *Ifnlr1* and *Il10rb* are unchanged between cell subsets. The comparison with published DC transcriptome datasets [69, 70, 127] confirmed the findings in our dataset (**Figure 7B** and **Supplementary Figure 6**).

In summary, TYK2 has limited effects on expression of the analysed IFN response machinery, apart from the expected feed-forward TFs STAT1, IRF7 and IRF9. Interestingly, the IFN receptor chains show an opposing expression pattern in DC subsets, with robust expression of IFN-I receptor in pDCs and of IFN-II receptor in cDCs.

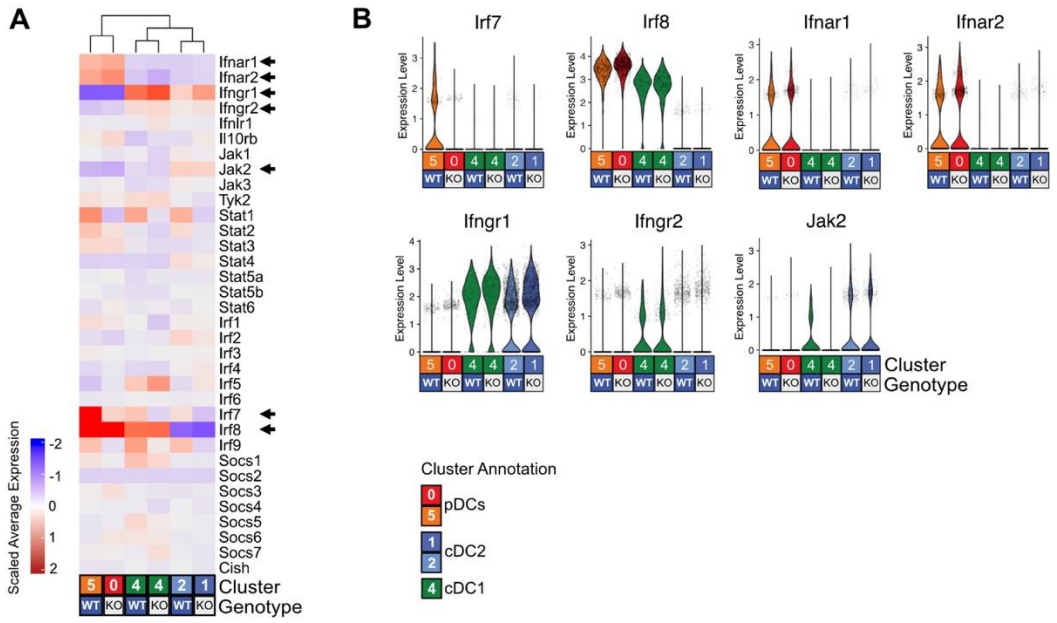


Figure 7 | Expression of IFN signalling components in *WT* and *Tyk2*$^{-/-}$ DC subsets. (A) Heatmap of the IFN response machinery with their scaled average expression among genotype separated clusters. For DC clusters with a genotype specific clustering bias (e.g. pDCs, cDC2), respective clusters were selected (e.g. cDC2: *WT* 2, *Tyk2*$^{-/-}$ 1, pDCs: *WT* 5, *Tyk2*$^{-/-}$ 0). Arrows indicate genes of interest that will be referred to in the main body of the text. Columns are separated by cluster number and genotype. Expression values are illustrated by a red-blue colour scale corresponding to a scaled average expression. (B) Violin Plots for IFN signalling components further described in the main body of the text.

Kinase-active TYK2 drives cDC2 differentiation and terminal DC maturation

DEG groups C, I, and H resulted from the comparison of *Tyk2*$^{-/-}$ pre-cDC2 (cluster 3) or cDC2A (cluster 1) and *WT* cDC2A (cluster 2) **(Figure 6B and 6C)**. They represent a pattern of genes upregulated in pre-cDC2 and upon loss of TYK2.

GSEA analysis of DEG group C revealed significant enrichment in the regulation of haematopoiesis (**Figure 6C**, GO:1903706, [128]). To further describe the genes regulated in a TYK2- and a differentiation-specific manner, we generated heatmaps of the genes in both groups. DEG group C genes appear higher expressed in pre-cDC2 (cluster 3) compared to cDC2A (clusters 1, 2), both in a TYK2-independent (*Tmem176a*, *Tmem176b*, *Mcemp*, *Icosl*, *Clptm1*, *Gpx4*, *Tubb5*) and -dependent (*H2-DMb2*, *Cd74*, *Serpina3g*, *Rogdi*, *Marcksl1*, *Nfkbia*, *Fscn1*, *M6pr*, *Tspan13*, *Flt3*, *Syngr2*, *Gsr*, *Apobec3*) manner (**Figure 8A**). The *Tyk2*-independent DEG grossly annotate to differential cDC maturation and activity genes (e.g. [29, 129-133]). The substantial proportion of upregulated in the absence of TYK2 in pre-cDC2 cluster by function as follows: most importantly, the Fms-related receptor tyrosine kinase 3 (*Flt3*, *Cd135*), the NFkB inhibitor alpha (*Nfkbia*) and the NFkB subunit *RelB* indicate a

differentiation/maturation activity [131, 134-138]; *Apobec3*, *Cd74*, *Fscn1*, *Il4i1* and *Marcksl1* facilitate cDC motility and responsiveness to stimulus [112, 139-145]; and *Cd74*, *Rodgi*, *Serpina3g*, *Syngr2* and *Tspan13* are cDC2 or other subset markers [76, 127, 146-149].

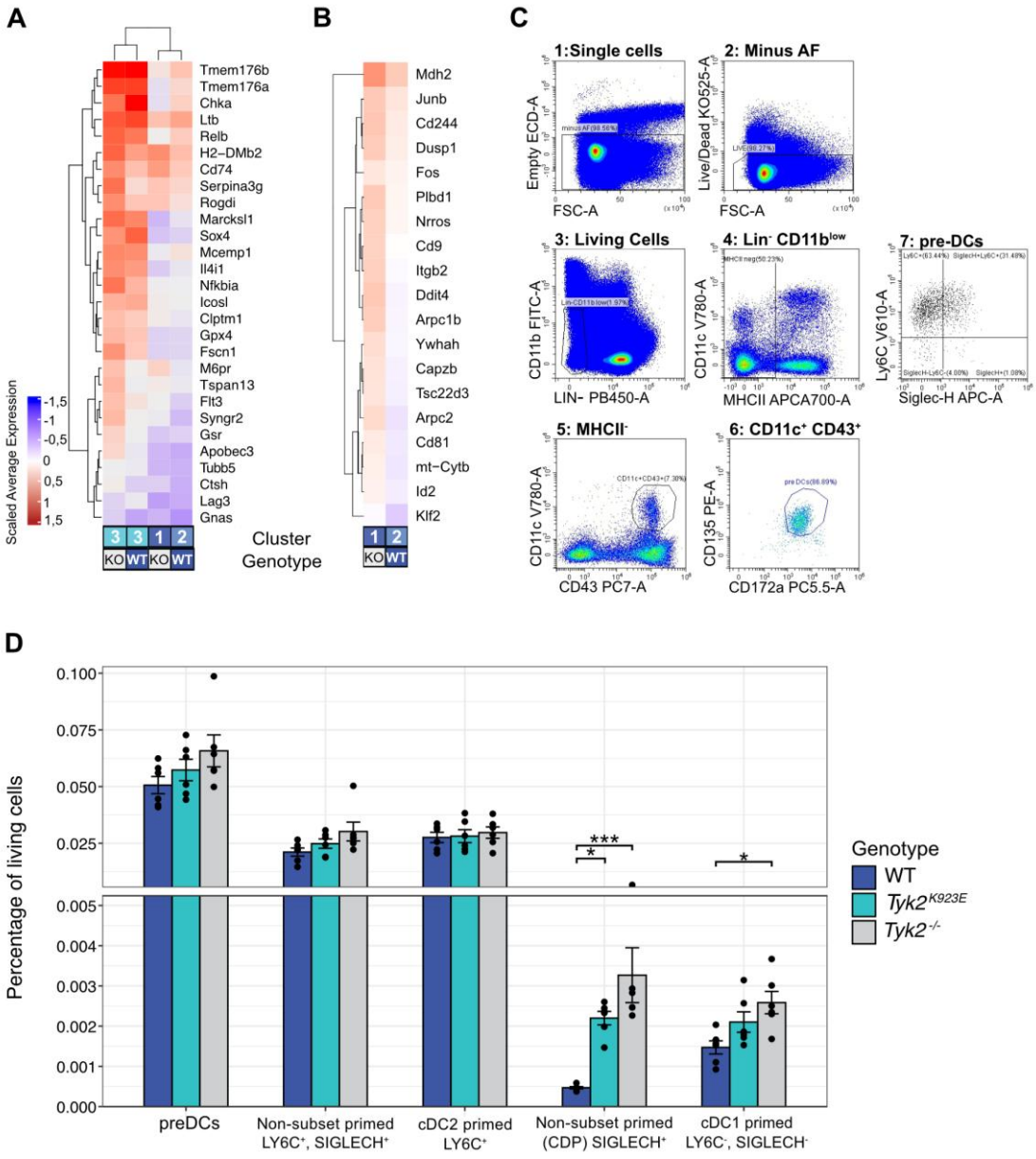


Figure 8 | Analysis of TYK2-repressed genes in DEG groups C, I and determination of the frequency of undifferentiated DCs. (A) Heatmap of pre-DC-/differentiation-related genes of DEG group C in pre-DC cluster 3 and cDC2A clusters 1,2 grouped by genotype (*WT*, *KO*: *Tyk2*$^{-/-}$). (B) Heatmap of DEG group I gene expression in *WT* (cluster 2) and *Tyk2*$^{-/-}$(1) cDC2A. Red-blue colour scale illustrates scaled average expression values. Note that no statistical tests were performed for cluster 3 genotype comparisons; they are displayed for inference only. (C) Gating scheme to identify pre-DCs from splenocytes and to determine potential subset commitment (Ly6C, SiglecH). (D) Bar plot of the percentage of each pre-DC subset out of the total number of living cells. Significance is denoted by: *=padj<0.05, ***=padj<0.005.

The DEG of group I genes upregulated in the absence of TYK2 in cDC2 extend the findings described so far (**Figure 8B**): ID2 is a TF crucial for DC differentiation [21, 150, 151] and the TF family member genes *Fos*, *Junb* and *Klf2* are central for DC diversity, identity and function [152-156].

Analogous to DEG group C the plethora of the remaining genes (*Arpc1b*, *Arpc2*, *Cd9*, *Cd81*, *Cd244*, *Ddit4*, *Dusp1* [MKP-1], *Mdh2*) are involved in DC migration [157-160] or regulation of DC functionality [161-165]. Group H genes were not assigned to an enriched pathway and reflected a non-IFN-mediated response associated with cellular metabolism and signalling/communication [166, 167], potentially accompanying the differentiation-altered cDC2 sate observed upon loss of TYK2.

The TYK2-dependent transcriptional profiles suggested differentiation and maturation activities in pre-cDC2 and other DC subsets. This was supported by the increased size of pre-DCs clusters in the absence of kinase-active TYK2 (see above, **Figure 5**).

To validate the observations up to now, we performed flow cytometry to identify pre-DCs (**Figure 1**, 1st row) as lineage negative Lin^{-}, CD11b^{low}, MHCII^{-}, CD11c^{+}, CD43^{+}, CD135^{+}, CD172a^{+} [76]. The subset commitment of the pre-DCs was determined by the expression of Ly6C and SiglecH **(Figure 8C)**. Both *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice exhibited increased numbers of splenic pre-DCs relative to *WT* mice. In particular, non-subset-primed common DC progenitors/early pre-DCs (CDP/early-stage pre-DCs), defined as lineage-negative Lin^{-}, CD11b^{low}, MHCII^{-}, CD11c^{+}, CD43^{+}, CD135^{+} CD172a^{+}, LY6C^{-}, SIGLEC-H^{+} cells, were significantly increased in both *Tyk2*-mutant genotypes. cDC1 primed pre-DCs were also significantly increased in *Tyk2*$^{-/-}$ mice **(Figure 8D).**

Collectively, these findings indicate that TYK2 promotes the transcriptional programmes associated with cDC2 differentiation and function. The increased frequency of pre-cDCs/non-subset-primed CDPs in mice lacking TYK2 or its kinase activity further supports a role for kinase-active TYK2 in terminal splenic DC differentiation.

Discussion

JAK-STAT signalling cascades and crosstalks regulate cellular homeostasis, differentiation and pathogen- or danger-induced responses [1, 44]. Here, we provide a detailed analysis of TYK2 function in murine splenic DCs using bulk RNA-Seq, scRNA-Seq, and flow cytometry. Our data identify kinase-active TYK2 as an important

regulator of tonic IFN signalling, IL-15Rα surface expression, DC-subset transcriptional programmes, and splenic DC maturation.

Bulk RNA-Seq of homeostatic splenic cDCs showed that the principal transcriptional consequence of TYK2 deficiency or kinase inactivation is the disruption of an IFN-associated gene-expression programme (**Figure 2**). This result is consistent with earlier observations in macrophages, NK cells, and T cells, in which TYK2 contributes substantially to tonic IFN responses under unperturbed conditions [78, 168] (**Figure 2B and D**). A recent meta-study of human TYK2 structure-function variants underpins the central requirement for TYK2 activity in IFN-I responses [169]. Loss of TYK2 or its kinase activity in mice and men leads to a partially reduced cytokine signalling strength at the TYK2-engaging receptors [10-12, 81, 84, 170]. The observed two-tiered ISG-expression pattern in cDCs likely reflects differences in signalling requirements among individual IFN-responsive genes. Some genes can sustain relatively high and stable expression in response to low tonic IFN concentrations, including feed-forward TFs such as STAT1 and IRF9 or antiviral effectors. Other genes may require stronger or more complex signalling inputs [78, 82]. Despite the established kinase-independent scaffolding functions of TYK2 in several cell types [6], our bulk RNA-Seq (**Figures 2A, C, D**), scRNA-Seq (**Figures 4, 5**) and flow cytometry analyses **(Figures 3, 8D)** did not reveal a clear kinase-independent role for TYK2 in splenic DCs.

At the single-cell level, *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} mice showed the greatest impact on cDC2 and pDC populations. *Tyk2*-mutant mice had marked changes in the representation of cDC2 and pDC clusters, including increased representation of transcriptionally less differentiated populations. In contrast, cDC1 and migratory DC clusters were comparatively less affected (**Figure 5**). This demonstrates that TYK2 contributes to the maintenance of normal splenic DC differentiation and homeostasis (see below). The detailed analysis of the DC subset single-cell transcriptomes revealed a broad downregulation of genes annotated to IFN and immune responses in the absence of kinase-active TYK2 (**Figure 6B-D)**. In accordance with our previous study [78], the TYK2-dependent gene expression also showed DC subset-specific patterns, most prominent in cDC2s and pDCs. This is in line with the notion that immune cell responses to IFNs and other cytokines are largely shaped by tonic signals, signalling crosstalks and feed-forward and feedback loops controlling lineage identities, cytokine response machinery levels and chromatin accessibility [171-176].

In pDCs and cDC2, we provide the first evidence that TYK2 is required to maintain the steady-state levels of ribosomal protein genes and genes involved in translation (**Figure 6B**). The regulation of ribosomal protein genes and the ribosomal synthesis is tightly regulated by multiple signalling pathways including IFN-I in response to mitogens and nutrients [177-179]. The mechanistic link between ribosome biogenesis, translational regulation and TYK2 activity is unclear.

The *Ifnar* and *Ifngr* receptor chain genes were differentially expressed in cDCs and pDCs, with high levels of *Ifnar* transcripts in pDCs and high expression of *Ifngr* in cDC1 and cDC2 (**Figure 7, Supplementary Figure 6B and C**). The homeostatic *Ifnr* expression pattern seems to be independent of TYK2, despite the respective genes harbour STAT-binding motifs in their promoters [88]. Under direct stimulus or microbial challenge, IFN-I and -II is reported to silence *Ifngr1* expression in myeloid cells [180-182]. Furthermore, the Mostafavi data set [79] classifies *Ifnar1* as an IRepG. It is tempting to speculate that this translates into wiring the DC subsets to a specific IFN type dependence/utilisation. However, this would be a simplification, since IFNR biogenesis and cell surface levels are heavily regulated by post-transcriptional processes and depend on tissue and stimulus contexts [183-186]. This is exemplified by the notion that despite strikingly different *Ifnar* transcript abundance under tonic signalling conditions both cDCs and pDCs respond to IFN-I for a full-fledged differentiation capacity and functionality [90, 187]. In line with the observed DC-specific *Ifnr* expression pattern, regulation of pDCs under challenged conditions is more dependent on IFN-I and of cDCs more on IFN-II [188, 189]. In addition, the number of IFNγ-induced genes in pDCs appears substantially lower compared to cDC1 and cDC2 [174]. In more complex settings, reverse IFN-I or -II effects have been also reported [190, 191]. Finally, a fate-switch from pDCs to cDCs based on reprogramming that is blocked by IFN-I and promoted by tumour necrosis factor has been described under pathological conditions and in healthy elderly people [192]. Spatial and combined -omics methodologies, especially Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq) and integrated scRNA-Seq with intracellular protein activity recording (INs-seq) [193, 194] are in place to further unravel the commitment of DC subsets to IFNR levels and utilisation [195, 196].

In line with the observed TYK2-dependent shifts in DC populations, we found a (pre-) cDC2-specific and TYK2-dependent transcriptional signature indicative for altered maturation and differentiation (**Figure 8A, B**). The DEG do not largely comprise of

TYK2-STAT target genes and are likely to be deregulated by signalling crosstalk, pleiotropic cytokine effects, and/or compensatory pathways [176, 197-199]. Although key regulatory genes such as *Flt3* and *Id2* were implicated, the precise intrinsic and extrinsic mechanisms responsible for pre-DC accumulation remain to be determined. no clear transcriptional signature for DC differentiation or function was detectable [39, 200, 201]. Flow cytometry confirmed an increase in pre-DCs in the absence of TYK2 (**Figure 8D**). Our findings in genetically engineered mice with loss of TYK2 or kinase-inactive TYK2 are in line with studies of a pharmacological TYK2 inhibitor in human and mouse cells, where the inhibitor led to augmented differentiation of monocytes to DCs [96].

Our findings also have potential translational implications. Pharmacological TYK2 inhibition is being developed and used for immune-mediated inflammatory diseases [202]. The present data suggest that sustained reduction of TYK2 activity could influence DC maturation and steady-state composition, in addition to attenuating pathological cytokine signalling. Our observations support careful evaluation of long-term effects of TYK2-targeted therapies on DC development and immune homeostasis.

Supplementary Data

Supplementary data tables can be found in Supplementary_Data_1_18.xlsx. The list given below refers to the sheet number presented in the files index.

Supplementary Data 1: Bulk DEA – Differential expression analysis (DESeq2) for the bulk RNA DC sequencing.

Supplementary Data 2: Bulk Metascape – Gene set enrichment analysis (GSEA) of the differential expression analysis performed prior

Supplementary Data 3: Bulk Metascape enrichment – Metascape provided enrichment summary of the pathways

Supplementary Data 4: Bulk HOMER known motif analysis – Known enrichment motifs of statistical significance

Supplementary Data 5: scRNA population – Sample of cluster sizes in scRNA analysis separated by genotype

Supplementary Data 6: Pos. Markers Cluster – Positively expressed (found in selected cluster) markers for each scRNA cluster

Supplementary Data 7: Pos. Markers Cluster and Genotype – Positively expressed (found in selected cluster) markers for each scRNA cluster split by genotype
Supplementary Data 8: *KOWT*/*KEWT* DEA – Pairwise DEA through MAST for scRNA sequencing for the *KOWT* and *KEWT* comparisons.
Supplementary Data 9: *KOWT* DEA ALT – Pairwise DEA contains alternate cluster comparisons that were not analysed due to genotype bias in clustering.
Supplementary Data 10: *KOKE* DEA - Pairwise DEA through MAST for scRNA sequencing. Contains KOKE cluster comparisons
Supplementary Data 11: Gene UMAP - UMAP1 and UMAP2 values and average log2FC values for scRNA that met the lower threshold differentially expressed gene (DEG) groups
Supplementary Data 12: Gene Metascape - Metascape gene set enrichment analysis for DEG gene groups. File provided by Metascape
Supplementary Data 13: Gene M. Enrichment - Summary Metascape enrichment file for the DEG Groups
Supplementary Data 14: DC Comp. - Pairwise DEA for DC comparisons in the *WT* and *KO* populations
Supplementary Data 15: DC Metascape - Metascape GSEA for DC comparisons
Supplementary Data 16: DC M. Enrichment - Metascape summary enrichment for DC comparisons
Supplementary Data 17: Metascape GSEA for the significant genes identified in the pairwise analysis of *KOWT* and *KEWT* comparisons. Data was not used in favour of DEG modules.
Supplementary Data 18: Metascape summary enrichment for the standard pairwise Metascape results described in Supplementary Data 17.

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and David Martin supervised and supported statistical and bioinformatical analyses and data curation. Matthias Farlik, Caroline Lassnig, Sabine Macho-Maschler, Katrin Meissl and Natalija Simonovic supervised and provided methodologies or animal experimentation. Lena Amenitsch, Therese Lederer, Hamsa Narasimhan and Stephen Shoebridge performed material preparation, data collection, data analysis and visualization. The manuscript was written by Thomas Decker, Mathias Müller, Birgit Strobl and Stephen Shoebridge.

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Data Availability. scRNA-Seq data are available from the NCBI Gene Expression Omnibus (GEO) repository accession identifier GSE286098. Bulk RNA sequencing data are assigned to GEO:GSE204736. Genome assemblies and gene annotations are available from Ensembl (https://ensembl.org). The complete list of genes analyzed, their fold change and significance and compiled lists of reference genes can be obtained in the Supplementary Data.

Declarations

Competing Interests. The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval. All mice were maintained on a C57BL/6N background under specific pathogen-free conditions in accordance with Federation of European Laboratory Animal Science Associations (FELASA) guidelines. All experiments were performed in accordance with the European Union 2010/63 EU directive and the Austrian Federal Ministry of Science and Research according to §§ 26ff animal experimentation law. All animal experiments were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna in accordance with the university's guidelines for Good Scientific Practice and authorized by the Austrian Federal Ministry of Women, Science and Research (BMWFW-68.205/0068-WF/V/3b/2015, BMBWF_GZ:2020-0.200.397, BMWFW-68.205/0093-WF/V/3b/2015,

BMBWF-68.205/0091-V/3b/2019, BMWFW-68.205/0166-WF/V/3b/2015) in accordance with current legislation.

Consent to Participate. Not applicable.

Consent to Publish. All authors reviewed their respective results contributions and contributed to the final manuscript. All authors approved this manuscript for publication.

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