Quaking promotes monocyte differentiation into pro-atherogenic macrophages by controlling pre-mRNA splicing and gene expression

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A hallmark of inflammatory diseases is the excessive recruitment and influx of monocytes to sites of tissue damage and their ensuing differentiation into macrophages. Numerous stimuli are known to induce transcriptional changes associated with macrophage phenotype, but posttranscriptional control of human macrophage differentiation is less well understood. Here we show that expression levels of the RNA-binding protein Quaking (QKI) are low in monocytes and early human atherosclerotic lesions, but are abundant in macrophages of advanced plaques. Depletion of QKI protein impairs monocyte adhesion, migration, differentiation into macrophages and foam cell formation in vitro and in vivo. RNA-seq and microarray analysis of human monocyte and macrophage transcriptomes, including those of a unique QKI haploinsufficient patient, reveal striking changes in QKI-dependent messenger RNA levels and splicing of RNA transcripts. The biological importance of these transcripts and requirement for QKI during differentiation illustrates a central role for QKI in posttranscriptionally guiding macrophage identity and function.
Monocytes serve as danger sensors within the circulation. The activation of blood-borne monocytes by inflammatory stimuli triggers their adhesion and homing to sites of tissue injury, where they differentiate into macrophages and collectively aid in the resolution of damage.1,2 However, the chronic accumulation of macrophages at these sites of injury is a hallmark of inflammatory diseases such as rheumatoid arthritis, Crohn’s disease and atherosclerosis.3–7

Dynamic changes in gene expression are associated with monocyte to macrophage differentiation, where PU.1 (ref. 8), Signal Transducer and Activator of Transcription (STATs)9 and CCAAT/Enhancer Binding Protein (C/EBP)10 are key transcription factors that drive this alteration in cellular phenotype and function.11,12 Importantly, numerous studies have identified critical roles for both microRNAs (miRNAs) and RNA-binding proteins (RBPs) in posttranscriptionally regulating monocyte13 and macrophage14 biology. However, the posttranscriptional regulation of monocyte to macrophage differentiation has generally been limited to studies detailing miRNA-based targeting of individual transcription factors or effector molecules that either stimulate or delay this phenotypic conversion.15–17

In contrast to miRNAs, RBPs mediate both quantitative and qualitative changes to the transcriptome, interacting with pre-mRNAs to influence (alternative) splicing, transcript stability, editing, subcellular localization and translational activation or repression.18–20 This broad arsenal of RNA-based control points enables RBPs to modulate the proteome in response to immunogenic stimuli,17 shifting inflammatory cells from an immature or naive state to a mature or activated state, as has previously been established in lymphoid cells.21,22 In recent times, we discovered that expression of the RBP Quaking (QKI) is induced in human restenotic lesion-resident vascular smooth muscle cells (VSMCs), where it directly mediates a splicing event in the Myocardin pre-mRNA that governs VSMC function.23 This finding prompted us to investigate whether QKI could similarly serve as an inflammation-sensitive posttranscriptional guide during monocyte to macrophage differentiation. Alternative splicing of the QKI pre-mRNA yields mature transcripts of 5, 6 or 7 kb that encode distinct protein isoforms, namely QKI-5, -6 and -7 (refs 24,25). QKI-5 possesses a nuclear localization signal in the carboxy-terminal region and is found in the nucleus of cells. In contrast, QKI-6 and QKI-7 are found in the cytoplasm. However, QKI-6 and QKI-7 can also translocate to the nucleus.23,26 The presence of a KH-family homology domain confers QKI with the capacity to bind RNA, albeit dimerization is required to bind with high affinity to the QKI response element (QRE) sequence (NACUAAY N1-20 UAAY) on target RNAs.29–33 Importantly, aberrant QKI expression is associated with inflammatory diseases such as schizophrenia, cancer, and restenosis.25

Here we show that the RBP QKI plays a critical role in regulating the conversion of monocytes into macrophages in, for example, atherosclerotic lesions. Our studies pinpoint QKI as a dynamic regulator of pre-mRNA splicing and expression profiles that drive monocyte activation, adhesion and differentiation into macrophages, and facilitates their conversion into foam cells.

Results

Human atherosclerotic lesion macrophages express QKI. We previously observed QKI expression in VSMCs and in leukocyte foci within human coronary restenotic lesions. Based on this observation, we used laser-capture microdissection to harvest CD68+ macrophages from early and advanced atherosclerotic lesions of human carotid arteries. QKI mRNA was 4.2-fold enriched in macrophages derived from advanced as compared with early atherosclerotic lesions (Fig. 1a). Next, using immunohistochemistry, we assessed QKI protein expression in human tissue sections at various stages of atherosclerotic lesion development, namely early pathological intimal thickening (PIT), fibrous cap atheroma (FCA) and intraplaque haemorrhaging (IPH). Although QKI was detectable in CD68+ myeloid cells of PIT, it was abundantly expressed in macrophage-rich FCA and IPH lesions (Fig. 1b). Furthermore, QKI-5, -6 and -7 were detectable in the nuclear, perinuclear and cytoplasmic regions of intimal macrophages in both FCA and IPH, respectively (Fig. 1c). We conclude that the accumulation of macrophages in human atherosclerotic lesions is associated with increased mRNA and protein expression of all three QKI isoforms within the macrophage.

A reduction in QKI decreases lesional macrophage burden. To investigate whether decreased QKI expression in monocytes and macrophages could influence atherosclerotic lesion formation, we transplanted bone marrow (BM) from QKI viable (qkv) mice, which express reduced levels of QKI protein, or their wild-type (wt) littermate controls (LM) into athrogenic LDLR−/− mice. Although qkv knockout mice die as embryos, the qkv mouse harbours a spontaneous ~1 Mb deletion in the QK promoter region that leads to reduced levels of QKI mRNA and protein.37 Indeed, macrophage colony-stimulating factor (M-CSF)-mediated conversion of LM and qkv BM-derived monocytes to macrophages showed subtly reduced QKI-5 mRNA and protein levels, and almost a complete ablation of QKI-6 and -7 protein (Fig. 1d,e). Following BM transplantation, the LDLR−/−/qkv and LDLR−/−/LM mice were fed a high-fat diet for 8 weeks, to induce atherosclerotic lesion formation. Interestingly, the long-term reduction of QKI expression during haematopoietic reconstitution limited neutrophil and monocytic repopulation (Supplementary Data 1). In keeping with this finding, immunohistochemical analysis of the aortic root revealed significantly decreased monocyte/macrophage content within atherosclerotic lesions of LDLR−/−/qkv mice (Fig. 1f), a finding that immunohistochemical analysis revealed was independent of plaque size or collagen content. These findings suggested that changes in haematopoietic and monocytic QKI expression could influence the macrophage content of atherosclerotic lesions.

QKI is induced on monocyte to macrophage differentiation. Having identified high QKI expression in macrophages in atherosclerotic lesions, we first explored whether QKI mRNA expression levels differ in macrophage precursors, namely classical (CD14+/CD16−), intermediate (CD14+/CD16+) and non-classical (CD14+/CD16+) monocytes derived from human peripheral blood (PB). All three monocyte subpopulations abundantly expressed QKI-5, -6 and -7 mRNAs as compared with glyceraldehyde 3-phosphate dehydrogenase (Fig. 2a). Moreover, QKI-5, -6 and -7 mRNA levels increased as classical monocytes progressed towards intermediate or non-classical monocytes. Interestingly, QKI-5 mRNA was the most abundantly expressed transcript in all three subpopulations. (Fig. 2a).

Next, we assessed QKI mRNA and protein levels in human PB monocytes treated with granulocyte–macrophage CSF (GM-CSF) and M-CSF, to stimulate their conversion to pro-inflammatory and anti-inflammatory macrophages, respectively (Fig. 2b). We observed remarkable increases in the expression of all QKI mRNA transcripts in mature macrophages (Fig. 2c). However, despite abundant QKI mRNA, the distinct QKI isoforms
**Figure 1 | Quaking is expressed in macrophages within atherosclerotic lesions.** (a) Pan-QKI mRNA expression levels in CD68⁺ macrophages of early and advanced atherosclerotic lesions isolated by laser-capture microdissection (n = 4). Data expressed as mean ± s.e.m.; Student’s t-test, *P < 0.05. Scale bar, 50 μm. (b) Immunohistochemical analysis of co-localization of pan-QKI and CD68 expression in preliminary intimal thickening (PIT), FCA and intraplaque haemorrhage (IPH). Dashed line denotes intimal/adventitial border. Scale bar, 50 μm. (c) Immunohistochemical analysis of QKI-5, -6 and -7 expression in PIT, FCA and IPH (top), and quantification of QKI-positive cells mm² per tissue sample (n = 5). Data expressed as mean ± s.e.m.; one-way analysis of variance (ANOVA), Bonferroni’s post-hoc test; *P < 0.05, **P < 0.01. (d) Quantitative RT-PCR (qRT-PCR) analysis of QKI mRNA expression in naïve BM-derived CD115⁺ mouse monocytes and 7 days M-CSF stimulated macrophages of either wt-littermates (LM) or quaking viable (qkv) mice (n = at least 3 mice per condition). Data expressed as mean ± s.e.m.; one-way ANOVA, Bonferroni’s post-hoc test; *P < 0.05 and **P < 0.01. (e) Western blot analysis of QKI-5, -6 and -7 expression levels in 7 days M-CSF stimulated macrophages derived from BM of wt and qkv mice. Each lane represents an individual mouse lysate (biological n = 3). (f) Immunohistochemical analysis for atherosclerotic plaque-resident macrophages (% MoMa-positive area) in aortic root sections of γ-irradiated (8 Gy) LDLR⁻/⁻ mice that subsequently were transplanted with BM from either qkv mice (qkv-BM) or littermates (LM)(LM-BM) and fed a high-fat diet for 8 weeks to develop atherosclerotic lesions (n = 12 per group). Scale bar, 200 μm. Data expressed as mean ± s.e.m.; Student’s t-test, with *P < 0.05.
mRNA isoforms in naive PB monocytes isolated using CD14 non-classical (CD14$^+$) PB monocytes cultured for 7 days in the presence of either GM-CSF or M-CSF. Scale bar, 50 μm. Expression is depicted relative to copies per glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data expressed as mean ± s.e.m.; one-way analysis of variance (ANOVA), Bonferroni’s post-hoc test; *$P$ < 0.05 and **$P$ < 0.01. (b) Phase-contrast photomicrographs of human PB monocytes cultured for 7 days in the presence of either GM-CSF or M-CSF. Scale bar, 50 μm. (c) Quantitative RT–PCR (qRT–PCR) analysis for QKI mRNA isoforms in naive PB monocytes isolated using CD14$^+$ microbeads, 7 days GM-CSF and 7 days M-CSF differentiated macrophages ($n = 3$). Expression is depicted relative to copies per GAPDH. Data expressed as mean ± s.e.m.; one-way ANOVA, Bonferroni’s post-hoc test; *$P$ < 0.05 and **$P$ < 0.01. (d) Western blot analysis of QKI protein isoforms in naive monocytes, 7 days GM-CSF and 7 days M-CSF differentiated macrophages (pan-QKI and CD14; $n = 5$). Data expressed as mean ± s.e.m.; Student’s t-test, with **$P$ < 0.01. Equivalent concentrations of whole-cell lysates were loaded per lane as determined using a BCA protein assay.

Figure 2 | QKI is highly expressed in macrophages derived from PB monocytes. (a) mRNA expression levels of distinct QKI isoforms following negative selection and FACS sorting for blood-derived human monocyte subsets, namely classical (CD14$^+$, CD16$^-$), intermediate (CD14$^+$,CD16$^+$) and non-classical (CD14$^+$,CD16$^+$). Expression is depicted relative to copies per glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data expressed as mean ± s.e.m.; one-way analysis of variance (ANOVA), Bonferroni’s post-hoc test; *$P$ < 0.05 and **$P$ < 0.01. (b) Phase-contrast photomicrographs of human PB monocytes cultured for 7 days in the presence of either GM-CSF or M-CSF. Scale bar, 50 μm. (c) Quantitative RT–PCR (qRT–PCR) analysis for QKI mRNA isoforms in naive PB monocytes isolated using CD14$^+$ microbeads, 7 days GM-CSF and 7 days M-CSF differentiated macrophages ($n = 3$). Expression is depicted relative to copies per GAPDH. Data expressed as mean ± s.e.m.; one-way ANOVA, Bonferroni’s post-hoc test; *$P$ < 0.05 and **$P$ < 0.01. (d) Western blot analysis of QKI protein isoforms in naive monocytes, 7 days GM-CSF and 7 days M-CSF differentiated macrophages (pan-QKI and CD14; $n = 5$). Data expressed as mean ± s.e.m.; Student’s t-test, with **$P$ < 0.01. Equivalent concentrations of whole-cell lysates were loaded per lane as determined using a BCA protein assay.

were poorly expressed in freshly isolated PB monocytes as compared with mature macrophages (Fig. 2d). The GM-CSF or M-CSF-induced conversion of monocytes into macrophages was associated with striking increases in QKI-5, -6 and -7 protein levels, with a more pronounced increase in all three isoforms observed with M-CSF treatment (Fig. 2d).

QKI haploinsufficiency perturbs macrophage differentiation. To further assess the role of QKI in monocyte and macrophage biology, we undertook an in-depth analysis of a unique, QKI haploinsufficient individual (Pat-QKI$^{+/−}$) and her sister control (Sib-QKI$^{+/+}$)38. This patient is the only known carrier of a balanced reciprocal translocation (t(5;6)(q23.1;q26)), where a breakpoint in one of her QKI alleles specifically reduces QKI expression by 50% in both QKI mRNA38 and QKI protein levels as compared with her sibling (Sib-QKI$^{+/+}$; Fig. 3a,b). RNA sequencing (RNA-seq) analysis (see below) confirmed altered QKI expression and furthermore revealed the precise location of the translocation breakpoint in intron 4 of QKI (Fig. 3b and Supplementary Fig. 1a).

We next compared the circulating monocytes of these two individuals for the expression of well-established monocyte cell surface markers such as CD14, CD16, CX3CR1, CCR2, SELPLG and CSF1R by fluorescence-activated cell sorting (FACS) analysis. Although monocyte subset ratios were not different (Supplementary Fig. 2a), the expression of CSF1R, the receptor that drives macrophage commitment, was elevated in Pat-QKI$^{+/−}$ non-classical monocytes as compared with Sib-QKI$^{+/+}$ (Supplementary Fig. 2b). As CSF1R is normally reduced when monocytes differentiate into macrophages, this observation points towards a potential defect in monocyte maturation in the patient.

Next, we investigated the consequences of decreased QKI expression on monocyte to macrophage differentiation. For this, we obtained freshly isolated Pat-QKI$^{+/−}$ and Sib-QKI$^{+/+}$ monocytes from venous blood and treated the cells for 7 days in the presence of either GM-CSF or M-CSF. Similar to the results in Fig. 2b, Sib-QKI$^{+/+}$ monocytes possessed the capacity to adopt the characteristic pro-inflammatory macrophage morphology, whereas monocytes from Pat-QKI$^{+/−}$ generally retained a monocytic morphology (Fig. 3c top panels). We
harvested RNA and protein from these macrophages and found that both QKI mRNA and protein levels were decreased (Fig. 3d,e). Surprisingly, this reduction in QKI did not appear to have an impact on the conversion of monocytes into anti-inflammatory macrophages (Fig. 3c bottom panels), a finding that prompted us to focus on the role of QKI in monocyte to macrophage differentiation in a pro-inflammatory setting.

**Figure 3: QKI impact on monocyte to macrophage differentiation.**

**a)** Normal genotype (Sibling: QKI+/+) vs QKI haploinsufficiency (Patient: QKI+/–). Reciprocal balanced translocation: Two intact QKI alleles joined by one QKI allele severed.

**b)** Gene expression levels of QKI-5, QKI-6, and QKI-7 in primary macrophages (4d GM-CSF) for Sib-QKI+/+ vs Pat-QKI+/–. QKI-5, QKI-6, and QKI-7 were decreased in Pat-QKI+/–.

**c)** Immunofluorescence images of Sib-QKI+/+ and Pat-QKI+/– primary macrophages (7d GM-CSF) and macrophages (7d M-CSF).

**d)** Regulated genes (4d GM-CSF) for Sib-QKI+/+ vs Pat-QKI+/–. Log2FC expression changes and p-values indicated.

**e)** Gene expression levels of pan-QKI, QKI-5, QKI-6, QKI-7, and H3-histone in primary macrophages (4d GM-CSF) for Sib-QKI+/+ vs Pat-QKI+/–.

**f)** RNA-seq derived differential gene expression (monocyte differentiation genes) 7d GM-CSF vs 7d M-CSF for Monocytes and Macrophages.

**g)** Top regulated QRE-containing genes in PB monocyte (1,583) and PB macrophage (1,486) for Sib vs Pat. PRUNE2, PHD1, and MTUS1 were significantly downregulated.

**h)** Log2FC and Gene expression changes for Monocytes and Macrophages.

**i)** Regulated QKI targets vs Other genes in Monocyte and Macrophage.

**j)** Cumulative fraction of genes with log2FC values for No QRE vs QRE in Monocyte and Macrophage.
QKI impacts transcript abundance in monocytes and macrophages. The observed increase in QKI expression during macrophage differentiation and well-established function as a splicing and translational regulator suggested that QKI is necessary for posttranscriptional control of events that lead to macrophage identity. To identify potential regulatory targets of QKI at a genome-wide level, we characterized the transcriptomes of Sib-QKI+/− and Pat-QKI+/− monocytes and GM-CSF-stimulated macrophages by RNA-seq (Supplementary Data 2). First, we assessed the expression levels of established immune-regulated genes. As shown in Fig. 3f, the mRNA levels of many monocyte to macrophage differentiation markers and genes were similarly regulated in Sib-QKI+/− and Pat-QKI+/− (CD68, APOE, ITGAM, CD14, CX3CR1, and CD163). In contrast, the expression levels of several key pro- and anti-inflammatory markers indicated an antiatherogenic shift in Pat-QKI+/− macrophages (Fig. 3f right; IL6, IL23a, CD161, ApoE, and IL10). At the genomewide level, QKI haploinsufficiency altered the abundance of 2,433 and 1,306 mRNA species in monocytes and macrophages (Fig. 3g, Supplementary Data 2 and Supplementary Fig. 3 top), respectively. Subsequently, we computationally determined the subset of mature mRNA transcripts in the genome that contain a QKI-binding sequence motif (termed QRE) (Supplementary Data 2). Our data suggested that QKI directly modulates the expression of 215 (128 up and 87 down) and 154 (100 up and 54 down) mRNAs in PB monocytes and macrophages, respectively (Fig. 3g, Venn sum of intersect). The five most differentially expressed genes in the patient relative to the sibling that harbour a QRE are shown in Fig. 3h. By selecting genes containing QREs, we identified a substantial number of putative QKI-mediated changes in transcript abundance (Fig. 3i).

Previous genome-wide studies have reported contrasting roles for QKI as both a repressor and stabilizer of target mRNAs. Intrigued by this ambiguity, we determined the consequences of QKI haploinsufficiency on mRNA transcript abundance in monocytes and macrophages. For this, we tested whether the presence of a QRE within a target mRNA was associated with increased or decreased mRNA abundance in the patient relative to her sibling (Supplementary Fig. 3 top). For this, we plotted the cumulative distribution function (CDF: y axis, as a fraction of total genes) against the transcript Log2FC (x axis: Pat-QKI+/−/Sib-QKI+/−) and stratified for either putative QKI targets (with QRE) or non-targets (no QRE). In PB monocytes, a reduction in QKI was associated with significantly lowered target mRNA expression (Fig. 3j left panel, left shift of cyan line). In contrast, in PB macrophages the expression levels of mature mRNAs containing QREs was strikingly increased in the patient relative to her sibling, as compared with those without QREs (Fig. 3j right panel, right shift of cyan line). Collectively, these studies suggested that QKI potently regulates gene expression during monocyte-to-macrophage differentiation.

QKI controls splicing in monocytes and macrophages. Given previous reports that QKI is involved in splicing of pre-mRNAs, we tested whether QKI acts similarly in monocytes and macrophages. First, we evaluated our RNA-seq analysis of Sib-QKI+/− and Pat-QKI+/− PB monocytes and macrophages for splicing changes (Supplementary Data 3). This analysis uncovered 1,513 alternative splicing events between Pat-QKI+/− and Sib-QKI+/− monocytes and macrophages, revealing events that were unique to either monocytes or macrophages, as well as common events (Supplementary Data 3). Previous observations for QKI and other RBPs suggested that when a splicing factor binds the intron downstream of an alternative exon, it promotes exon inclusion; however, when binding the intron upstream of the alternative exon, the RBP promotes exon skipping. We analysed the RNA-seq data for such a trend using the set of splicing events that change between the Pat-QKI+/− and Sib-QKI+/−, to determine the frequency of the QKI-binding motif, ACUAA, around these regulated exons, relative to a background set of exons that is expressed, but inclusion is unchanged between the two data sets. The results of these analyses are shown in Fig. 4a and Supplementary Data 4, and demonstrate ACUAA motif enrichment upstream of exons with increasing inclusion in Pat-QKI+/− (QKI repressed exons) relative to background exons, as well as an increase in ACUAA motif frequency downstream of exons with increased skipping (QKI activated) relative to background. This suggested that similar to C2C12 myoblasts, QKI promotes exon skipping by binding the upstream intron, while promoting inclusion of alternative exons by binding to the downstream intron, in monocytes and in macrophages. These data strongly support a direct, position-dependent role for QKI in regulating alternative splicing, while also providing additional protein diversity during monocyte-to-macrophage differentiation. As shown in Fig. 4b, QKI haploinsufficiency triggered alternative splicing events in PB monocytes (orange tracks) and macrophages (blue tracks). Interestingly, the presence of QKI-binding sites, as defined by either experimentally determined QKI PAR-CLIP sites and/or ACUAA motifs clearly...

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**Figure 3** | Characterization of monocyte and macrophage biology in a unique QKI haploinsufficient patient. (a) Schematic of chromosomal translocation event in the qki haploinsufficient patient (Pat-QKI+/−), reducing QKI expression to ~50% that of her sister control (Sib-QKI+/−). (b) Top: UCSC Genome Browser display of reference genome QKI locus with standard and chimeric reads for the patient and sibling. The reduced expression levels and altered 3′-untranslated region (UTR) composition in the patient RNA as compared with a sibling control is noteworthy. Patient shows increased intronic RNA extending to the point where chimeric reads map at the breakpoint to chr5. Middle: chromosome diagrams showing normal chromosomes 5 and 6 with the red line, indicating the location of the breakage fusion point. Bottom: sequence across the fusion point. The chromosomal origin of the AG dinucleotide is ambiguous. (c) Photomicrographs of sibling and patient macrophages, cultured in GM-CSF or M-CSF for 7 days, respectively. (d,e) Assessment of QKI isoform mRNA and protein expression in 4-day GM-CSF-stimulated macrophages in the sibling and patient. (f) Hierarchical clustering (Euclidean algorithm) of key monocyte differentiation genes changes in RNA-seq-derived mRNA abundance where dark blue = low expression, whereas light blue = high expression (‘+’ and ‘−’ indicates ≥1.5-fold expression change in monocytes or macrophages, respectively). (g) Venn diagrams with numbers of differentially expressed genes (minimally ±1.5-fold; patient/sibling expression) for unstimulated (top) and GM-CSF-stimulated macrophages (bottom). An expression cutoff (Pat + Sib expression ≥1CPM) was applied. (h) The most differentially expressed genes, harbouring a QRE are depicted. (i) Genome-wide scatterplot of mRNA abundance (y axis: Log10 CPM) versus the log2FC (x axis: Patient/Sibling CPM) after an expression cutoff (Pat + Sib expression ≥1 CPM) in monocytes (left) and GM-CSF-stimulated macrophages (right). Blue dots indicate QRE-containing transcripts minimally ±1.5-fold differentially expressed. Grey dots do not fulfill these criteria. (j) CDF (y axis) for QKI target (QRE containing: blue line) and non-target (non-QRE containing: cyan line) mRNAs (x axis: log2FC) in monocytes (left) and macrophages (right). Left shift indicates lower expression of QKI target genes, whereas a right shift indicates higher expression of QKI targets in the patient samples. Distributions were compared using a Wilcoxon rank-sum test.
**Figure 4 | QKI influences pre-mRNA splicing in naive PB monocytes and macrophages.** (a) SpliceTrap assessment of the proximal ACUAA RNA motif enrichment in 50-bp windows upstream and downstream of alternatively spliced cassette exons (as compared with a background set of exons; grey circles). The relationship between the frequency of exon exclusion (blue triangles) or exon inclusion (red squares) and ACUAA RNA motif enrichment over the genomic locus are depicted. (b) Sashimi plots illustrate RNA-seq read coverage for selected alternative splicing events in Pat- and Sib-PB monocytes and macrophages (blue). Splicing events (se) are highlighted by inverted brackets. The location of ACUAA motifs and QKI PAR-CLIPs are provided below. Splicing events were defined based on the genomic organization of RefSeq transcripts (bottom tracks). Full event details are provided in Supplementary Data 3. (c) PCR validation of alternatively spliced cassette exons in Sib- and Pat-QKI+/– PB monocytes and macrophages. Primers were designed to target constitutive flanking exons. PCR product size for exon inclusion (top) and exclusion (bottom) variants are provided (left). (d) Phase-contrast and fluorescence-microscopy photographs (scale bar, 50 μm) of primary human, PB macrophages of healthy controls that have been treated with FAM-labelled GapmeRs, to reduce QKI expression. (e) Quantitative RT-PCR (qRT-PCR) of QKI mRNA isoform expression in GapmeR-treated macrophages (n = 3). Data expressed as mean ± s.e.m.; Student’s t-test, with **P < 0.01. (f) PCR validation of alternatively spliced cassette exons in GapmeR-treated PB-derived macrophages. Primers were designed to target constitutive flanking exons. PCR product size for exon inclusion (top) and exclusion (bottom) variants are provided (left). A representative illustration is shown of an n = 3 donors. Data expressed as mean ± s.e.m.; Student’s t-test, with **P < 0.01 and #P = 0.08.
associated with changes in exon inclusion (for example, ADD3), alternative 5′-splice sites (PARP2), alternative 3′-splice sites (M6PR) and intron retention (for example, BICD2), thereby expanding the detection of veritable QKI-regulated events beyond cassette exons (splice event 'se' location defined by brackets). Importantly, strong correlations were observed between QKI expression levels and the magnitude of the splicing event, be it between the patient and sibling control, or between monocytes and macrophages (Fig. 4b).

Finally, we validated several alternatively spliced cassette exons, including events in ADD3, LAIR1 and UTRN by reverse transcriptase–PCR (RT–PCR), using primers in flanking exons (Fig. 4c). Collectively, our RNA-seq data analysis of this unique QKI haploinsufficient individual strongly suggested a direct role for QKI in regulating alternative splicing events that could influence monocyte to macrophage differentiation.

To extend results obtained with the QKI haploinsufficient patient, we abrogated QKI expression in naive primary human monocytes harvested from freshly drawn venous blood of healthy controls. We designed GapmeR antisense oligonucleotides that either targeted QKI for degradation (QKI-Gap), or were scrambled as a control (Scr-Gap), coupled with a 5′-FAM label to track their cellular uptake. The QKI-Gap and Scr-Gap compounds were administered to the freshly isolated monocytes, concomitant with GM-CSF for 96 h, to drive the differentiation to pro-inflammatory macrophages. In contrast to our attempts to reduce QKI mRNA levels using other well-established approaches, we observed virtually no signs of cytotoxicity or apoptosis following GapmeR treatment. Furthermore, the treatment did not hamper the capacity of monocytes to differentiate into macrophages (Fig. 4d top), while uptake efficiency approached 100% (based on FAM+ cells; Fig. 4d bottom). After 96 h, we harvested RNA from the QKI-Gap- and Scr-Gap-treated macrophages, which yielded a minimal reduction in QKI-5 mRNA levels but remarkable reductions in QKI-6 and QKI-7 mRNAs (Fig. 4e). Albeit that the GapmeR-mediated reduction in QKI expression in primary human macrophages was not as striking as that observed in the QKI haploinsufficient patient, it nonetheless enabled us to visualize and validate significant changes in several of the aforementioned QKI-mediated alternative splicing events, such as ADD3 and FcγR-IIb (FGCR2B) (Fig. 4f; n = 3 donors). It should be noted that the inability to remarkably reduce the expression of the nuclear QKI isoform, namely QKI-5, could be responsible for the discrepancy between the striking shift in splicing observed in the QKI haploinsufficient patient as compared with the GapmeR-mediated abrogation of QKI expression. Taken together, these studies clearly pinpoint QKI as a regulator of pre-mRNA splicing during monocyte-to-macrophage differentiation and implicate QKI gene dosage as a determinant of splicing event magnitude.

**QKI regulates transcript abundance in THP-1 cells.** To provide further support for a regulatory role for QKI during monocyte-to-macrophage differentiation, we tested whether QKI could similarly modulate transcript abundance and pre-mRNA splicing in a well-established monocyte cell line, namely THP-1 cells. Similar to GM-CSF-induced differentiation of PB monocytes into macrophages, the 12,13-phorbol myristyl acetate (PMA)-induced transition of THP-1 'monocytes' to 'macrophages' was associated with the following: (1) significantly increased expression of all QKI mRNA transcripts (Fig. 5a); (2) barely detectable levels of QKI protein in THP-1 'monocytes' (Fig. 5b and Supplementary Fig. 4a); and (3) significantly increased expression of QKI protein during THP-1 'monocyte' to 'macrophage' differentiation (Fig. 5b,c). Next, we stably transduced THP-1 'monocytes' with either short-hairpin RNA (shRNA) targeting QKI (sh-QKI) to specifically deplete QKI or with a non-targeting shRNA control (sh-Cont) (Supplementary Fig. 4b). Similar to GM-CSF-stimulated Pat-QKI+/− versus Sh-QKI+/− monocytes, sh-QKI THP-1 'monocytes' displayed an inability to adopt the 'macrophage' morphology following stimulation with PMA as compared with sh-Cont THP-1 'monocytes' (Supplementary Fig. 4c arrows). We subsequently determined mRNA levels using an exon junction microarray44 analysing RNA isolated from unstimulated and 3 days PMA-stimulated THP-1 sh-Cont and sh-QKI 'monocytes' and 'macrophages' (Supplementary Data 5). Next, as shown in Fig. 5d, we assessed the expression profile of established monocyte differentiation genes (for example, CD14↑, CXCL8↑, CSF1R↑, ApoE↑, CX3CR1↓, CCR2↓ and CCL2↓). Similar to QKI haploinsufficient macrophages, several markers in sh-QKI THP-1 'macrophages' displayed an anti-atherogenic phenotypic shift (IL6↓, IL23a↓, CD16A↓, CD16R↓, ApoE↓ and IL10↑) as compared with sh-Cont THP-1 'macrophages' (Fig. 5d).

At the genome-wide level, the reduction of QKI significantly altered the abundance of 359 and 573 mRNAs in THP-1 'monocytes' and 'macrophages', respectively (Fig. 5e, Supplementary Data 5 and Supplementary Fig. 3 bottom). Of these differentially expressed mRNAs, 56 and 128 were computationally predicted QKI targets based on the presence of a QRE in the mature mRNA (Fig. 5e intersect). The most differentially expressed transcripts harbouring a QRE are denoted in Fig. 5f. The expression levels of mRNAs targeted by QKI in THP-1 'monocytes' and 'macrophages' are depicted in Fig. 5g (blue dots) and Fig. 5h (blue lines), relative to those not directly affected by

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**Figure 5 | QKI influences mRNA transcript abundance during differentiation of THP-1 monocyte-like cells to THP-1 macrophage-like cells.** (a) mRNA expression of the QKI isoforms as compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in THP-1 'monocytes' and 8 days differentiated THP-1 'macrophages' (biological n = 3). Data expressed as mean ± s.e.m.; Student’s t-test; *P < 0.05 and **P < 0.01. (b) Western blot analysis of whole-cell lysates of THP-1 'monocytes' and THP-1 'macrophages'. (c) Western blot quantification of QKI protein isoforms, normalized to β-actin in THP-1 'monocytes' and THP-1 'macrophages' (n = 3). Data expressed as mean ± s.e.m.; Student’s t-test; *P < 0.01. (d) Hierarchical clustering (Euclidean algorithm) of key monocyte differentiation genes depicting changes in microarray-derived mRNA abundance THP-1 'monocytes' (left two lanes) and THP-1 'macrophages' (right two lanes), where dark blue = low expression, whereas light blue = high expression (* and/or # beside gene name is indicative of a significant ≥1.5-fold change in expression in monocytes or macrophages, respectively). (e) Venn diagrams depicting the number of microarray-derived differentially expressed genes (minimally ≥1.5-fold; sh-QKI/sh-Cont expression, q-value ≤0.05) for unstimulated THP-1 'monocytes' (left Venn diagram) and THP-1 'macrophages' (right Venn diagram). (f) The most significantly differentially expressed genes harbouring a QRE are shown. (g) Genome-wide scatterplot of mRNA abundance in THP-1 'monocytes' (left scatterplot) and THP-1 'macrophages' (right scatterplot); y axis: Log2 probe intensity versus the x axis: log2FC: sh-QKI average probe intensity/sh-Cont average probe intensity. Blue dots indicate QRE-containing transcripts that are minimally ≥1.5 fold differentially expressed (q ≤ 0.05). Grey dots do not fulfill these criteria. (h) CDF (y axis) for QKI target (QRE containing; blue line) and non-target (non-QRE containing; cyan line) mRNAs (x axis: log2FC in THP-1 'monocytes' (left plot) and THP-1 'macrophages' (right plot). Left shift indicates lower expression of QKI target genes in the sh-QKI samples, whereas a right shift is indicative of higher expression of QKI targets in the sh-QKI samples. Distributions were compared using a Wilcoxon rank-sum test.
changes in QKI levels (Fig. 5g grey dots and Fig. 5h cyan lines). Consistent with our analyses in PB monocytes, putative direct QKI target mRNAs were mostly reduced on a targeted QKI reduction in THP-1 'monocytes' (Fig. 5h, left plot), although a shift towards increased target mRNA abundance in THP-1 'macrophages' was not observed (Fig. 5h, right plot).

QKI modifies pre-mRNA splicing patterns in THP-1 cells. Having identified that QKI haploinsufficiency generates pre-mRNA splicing events that probably have an impact on monocyte and macrophage biology, we also analysed RNA isolated from sh-Cont and sh-QKI THP-1 'monocytes' and 'macrophages' for alternative splicing events using the exon
Detected splicing events are illustrated in Fig. 6b, where the expression of QKI facilitates monocyte adhesion and migration in inflammatory settings.

QKI targets monocyte activation and differentiation pathways. We subsequently determined how QKI-induced changes in mRNA transcript abundance could have an impact on Gene Ontology (GO) enrichment of coordinately regulated pathways during monocyte-to-macrophage differentiation. As shown in Table 1 and Supplementary Data 7, these GO analyses point towards a central regulatory role for QKI in immune responses to injury, processes that play a critical role in the onset and development of atherosclerosis and other inflammation-based diseases. In both monocytes and macrophages, changes in QKI expression clearly had an impact on Liver X Receptor (LXR)/Retinoid X Receptor (RXR) activation and Peroxisome Proliferator-Activated Receptor (PPAR) activation and signalling, implicating a key role for QKI in regulating cholesterol biosynthesis and metabolism. Furthermore, a reduction in QKI expression also appeared to influence T-cell and Toll-like receptor signalling, biological processes that play prominent roles in the rapid resolution of infection, while in chronic settings exacerbate inflammatory conditions. Finally, alternative cassette exons in THP-1 'monocytes' and 'macrophages' were PCR validated (Fig. 6d). Importantly, we also identified several top splicing events from THP-1 'monocytes' and 'macrophages' (Supplementary Data 6), and validated these in mRNA harvested from wt and qkv mice, including REPS1, PTPRO and FGFR1OP2 (Fig. 6e).

QKI drives foam cell formation. As QKI expression remarkably increased during monocyte-macrophage differentiation (Fig. 2c–f) and our aforementioned GO analysis revealed a strong association for changes in QKI expression and lipid metabolism (Fig. 7a), we tested whether a reduction in QKI expression influences the handling of lipids. For this, we first assessed the mRNA expression levels of a subset of established lipid-related genes in monocytes and macrophages derived from WT and qkv mice. As shown in Fig. 8a, monocytes from qkv mice are characterized by significant reductions in NR1H3 (known as LXRz) and PPARγ (PPARγ2) expression, as well as cholesterol uptake (CD36 and LDLR) and efflux (ABCG1) receptors, as compared with WT monocytes. These effects were diminished on conversion to macrophages (Fig. 8a). We subsequently assessed the expression levels of these lipid metabolism/homeostasis genes in human PB-derived monocytes and macrophages (Fig. 8b and Supplementary Fig. 5). Similar to qkv monocytes, Pat-QKI+/– monocytes were characterized by decreased NR1H3 and PPARγ expression, as well as LDLR and SCARB1 (Fig. 8b). In contrast to qkv monocytes, ABCG1 expression was potently increased. Similar to qkv macrophages, this differential gene expression profile appeared to normalize in Pat-QKI+/– macrophages as compared with Sib-QKI+/+ macrophages (Fig. 8b). Moreover, in primary human macrophages where GapmeR-mediated knockdown of QKI was realized, we observed significant increases in MYLIP/IDOL and ABCG1 expression, whereas CD36 displayed a trend towards decreased expression (Supplementary Fig. 5).

Having identified that changes in QKI expression levels had an impact on lipid-associated gene expression, we
Figure 6 | QKI expression levels influence pre-mRNA splicing during THP-1-based monocyte-like to macrophage-like cell differentiation.

(a) Schematic depicting detectable alternative splicing events with the splicing-sensitive microarray platform and number of inclusion (incl.; top lines) or exclusion (excl.; bottom lines) events observed in unstimulated THP-1 'monocytes' (left) and 3-day PMA-stimulated THP-1 'macrophages' (right) with, for example, an ss of $-1.72$, indicating a 3.3-fold more inclusion of ADD3 exon 13 in sh-QKI versus sh-Cont THP-1 'monocytes'. Full event details are provided in Supplementary Data 6. CE, cassette exon; Alt 5': MAPK7 Alt 5': FRMD1, Alternative 5'start and Alternative 5'start; RI, retained intron. (b) Scatterplots of skip (y axis) and include (x axis) probe set intensity for selected alternative splicing events in sh-Cont (blue boxes) versus sh-QKI (orange circles) in unstimulated and 3 days PMA-stimulated THP-1 'monocytes' and 'macrophages', respectively. Regression coefficients (constrained to pass the origin) are depicted as solid lines. The log2 difference in the slopes (termed separation score; $ss$) are provided to the right of the plots for each event, with for example, an $ss$ of $-1.72$, indicating a 3.3-fold more inclusion of ADD3 exon 13 in sh-QKI versus sh-Cont THP-1 'monocytes'. Full event details are provided in Supplementary Data 6. CE, cassette exon; Alt 5': MAPK7 Alt 5': FRMD1, Alternative 5'start and Alternative 5'start; RI, retained intron. (c) SpliceTrap assessment of average proximal ACUAA RNA motif enrichment in 50 bp windows upstream and downstream of alternatively spliced cassette exons as compared with a background set of exons (grey circles). The relationship between the frequency of exon exclusion (blue triangles) or exon inclusion (red squares) and ACUAA RNA motif enrichment are depicted. (d) PCR validation of alternatively spliced cassette exons in sh-Cont and sh-QKI THP-1 'monocytes' and 'macrophages'. Primers were designed to target constitutive flanking exons. PCR product size for exon inclusion (top) and exclusion (bottom) variants are provided (left). All experiments depict biological $n = 3$. PCR validation of three splicing events in wt and qk'' mouse-derived primary monocytes and 7 days M-CSF-stimulated macrophages. PCR product size for exon inclusion (top) and exclusion (bottom) variants are provided (left). Depicted is a representative PCR for at least a biological $n \geq 3$. 

Intron ACUAA Exon ACUAA Intron 

Unstimulated THP-1 'monocyte' 

3d PMA THP-1 'macrophage' 

Incl. Incl. Excl. Excl. 

220 185 95 145 210 

Bases from the 3'ss 

Bases from the 5'ss 

388 bp 

292 bp 

218 bp 

134 bp 

191 bp 

164 bp 

488 bp 

138 bp 

Add3 

PTPRO 

KIF13A 

ERBB2IP 

350 bp 

388 bp 

Add3 

PTPRO 

KIF13A 

ERBB2IP 

120 bp 

164 bp 

240 bp 

300 bp 

388 bp 

134 bp 

191 bp 

164 bp 

488 bp 

138 bp 

650 bp 

550 bp 

450 bp 

350 bp 

251 bp 

170 bp 

192 bp 

108 bp 

200 bp 

86 bp 

200 bp 

108 bp 

200 bp 

86 bp 

350 bp 

350 bp 

251 bp 

Add3 

PTPRO 

KIF13A 

ERBB2IP 

350 bp 

388 bp 

Add3 

PTPRO 

KIF13A 

ERBB2IP 

120 bp 

164 bp 

240 bp 

300 bp 

388 bp 

134 bp 

191 bp 

164 bp 

488 bp 

138 bp 

650 bp 

550 bp 

450 bp 

350 bp 

251 bp 

170 bp 

192 bp 

108 bp 

200 bp 

86 bp 

200 bp 

108 bp 

200 bp 

86 bp 

350 bp 

350 bp 

251 bp 

Add3 

PTPRO 

KIF13A 

ERBB2IP 

120 bp 

164 bp 

240 bp 

300 bp 

388 bp 

134 bp 

191 bp 

164 bp 

488 bp 

138 bp 

650 bp 

550 bp 

450 bp 

350 bp 

251 bp 

170 bp 

192 bp 

108 bp 

200 bp 

86 bp 

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KIF13A 

ERBB2IP
investigated whether lipid loading affected QKI expression levels. Indeed, treatment with either acetylated low-density lipoprotein (acLDL) or β-very low-density lipoprotein (β-VLDL) led to significant increases in QKI-5 mRNA levels, while QKI-6 and QKI-7 levels also increased, albeit not significantly (Fig. 8c). In contrast to primary monocytes and macrophages, THP-1 ‘monocytes’ did not display significant changes in lipid metabolism gene expression. However, as shown in Fig. 8d, treatment with modified LDL increased expression of cholesterol uptake genes (CD36 and VLDLR), along with significant increases in cholesterol efflux genes (ABCA1 and ABCG1). Taken together, these studies suggested that changes in QKI expression could have an impact on the net balance of genes that control lipid metabolism and hostostasis.

Finally, we tested whether these QKI-mediated changes in lipid-associated gene expression could translate into consequences for lipid uptake and foam cell formation, a phenomenon tightly associated with pro-inflammatory macrophage function7. As shown in Fig. 8e, the impact of decreased QKI expression on foam cell formation on loading with β-VLDL was clear, as sh-QKI THP-1 ‘macrophages’ displayed less extensive lipid staining as compared with sh-Cont THP-1 ‘macrophages’ (Fig. 8e). Similarly, in Pat-QKI+/– macrophages we observed significantly less lipid staining after β-VLDL treatment (Fig. 8f). Even more striking was the potent decrease in oxidized LDL staining as compared with sh-Cont THP-1 ‘macrophages’ (Fig. 8f).

**Discussion**

Genes involved in regulating the transition of monocytes into pro-inflammatory macrophages serve as excellent therapeutic targets for limiting the progression of inflammation-driven diseases such as rheumatoid arthritis and atherosclerosis3,6. Our data indicate that alongside wide-ranging changes in gene expression, the differentiation of monocytes to macrophages requires extensive alternative splicing of pre-mRNA species and...
pinpoint QKI as a novel posttranscriptional regulator of both of these processes (Fig. 9).

Expression of the transcription factor PU.1 is associated with the activation of gene expression profiles that drive the differentiation of CD34+ haematopoietic progenitor cells towards a myeloid fate, including monocytes and macrophages\textsuperscript{46,47}. Recent work by Pham \textit{et al.}\textsuperscript{48} identified that the binding of PU.1 appears to be enhanced by cooperativity with neighbouring transcription factor binding sites, such as KLF4. Importantly, PU.1 induces the expression of critical monocyte

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**Figure 7** | QKI expression levels have an impact on monocyte adhesion as well as migration and differentiation. (a) Cumulative population doublings (y axis: CPDs) were counted to assess the effect of QKI reduction on cellular proliferation over time (x axis: days). Population growth curves were compared using linear regression analysis. (b) Quantification of cellular apoptosis, where annexin V+ and propidium iodide+ cells were categorized as apoptotic, as determined by FACS analysis. (c) Quantification of sh-Cont and sh-QKI THP-1 ‘monocyte’ adhesion to collagen matrix pretreated with platelet-rich plasma under flow, mimicking in-vivo endothelial denudation. Direction of flow is indicated below the photomicrographs (n = 3). Data expressed as mean ± s.e.m.; Student’s t-test; *P < 0.05. Scale bar, 100 μm. (Also see Supplementary Movies 1 and 2). (d) Assessment of integrin-mediated adhesion. Quantification of adhesion to collagen for untreated, PMA- or TS2/16-treated sh-Cont and sh-QKI THP-1 ‘monocytes’ are plotted. TS2/16 is an antibody that turns all β1-integrins in the high-affinity conformation, inducing cellular adhesion. (e) Quantification of cellular transwell migration towards either fMLP (for THP-1 ‘monocytes’) or macrophage chemoattractant protein 1 (MCP-1; for PB monocytes from either sibling or patient (n = 4 technical replicates). Data expressed as mean ± s.e.m.; Student’s t-test; *P < 0.05 and **P < 0.01.
transcription factors, including KLF4 (ref. 49). Of note, KLF4 has been demonstrated to bind to the QKI promoter region of VSMCs, which is GC rich50. Furthermore, chromatin immunoprecipitation sequencing data derived from HL-60 cells embedded in the UCSC Encode browser revealed two experimentally determined PU.1-binding sites in the QKI promoter51 (Supplementary Fig. 6). Collectively, these findings suggest that PU.1, potentially in concert with KLF4, could be responsible for driving QKI expression during monocyte-to-macrophage differentiation.

Our findings also suggest that the abundant expression of QKI mRNAs in naïve monocytes could serve to prime these cells with the capacity to rapidly respond to pro-inflammatory triggers at sites of injury. Although we did not assess the translational kinetics of QKI mRNA into protein, our investigation of monocyte activation, adhesion and differentiation strongly suggests that the determination of pre-mRNA fate by QKI critically has an impact on the capacity of the monocyte to aid in response to vascular injury. To date, the genome-wide (alternative) splicing patterns during monocyte-to-macrophage differentiation had not been described. However, the consequences of many splicing events herein described, such as γ-adducin (ADD3), FCGR2B and VLDLR are unknown. However, given that phosphorylation of the γ-adducin C terminus triggers dissociation from spectrin and cortical actin loss52, it is plausible that the QKI-mediated exclusion of a 13 amino acid coding exon proximal to this region could have an impact on cytoskeletal dynamics in monocytes and macrophages. Furthermore, alternative splicing of exon 6 in FCGR2B could potentially have an impact on the inhibitory role of this protein in monocyte- and macrophage-mediated phagocytosis at sites of vascular injury53. Therapeutic strategies tailored to target such splicing events in monocytes could potentially deter their conversion to disease-accelerating macrophages.

The expanding repertoire of RNA species have led to the emergence of RNA-based therapeutics as a novel means of treating both rare and common diseases, such as muscular dystrophy and cancer, respectively54,55. This is based on extensive efforts geared towards identifying how changes in coding (alternative splicing) and non-coding RNAs (miRNA and long non-coding RNA) have an impact on cellular pathophysiology56. Importantly, the fate of these coding and non-coding RNAs at the cellular level are determined primarily by the more than 500 RBPs that regulate eukaryotic cell biology52. Recently, the RNA motifs to which a significant portion of these RBPs bind has been characterized52, enabling the systematic identification of RNA targets for a given RBP within cells, including QKT29–32. Our genome-wide evaluation of posttranscriptional events mediated by QKI implicate both direct and indirect posttranscriptional roles for this protein, where the absence of ACUAA motifs or QREs could nonetheless involve QKI, potentially by tethering to other RBPs, or through QKI-mediated changes in the expression of other RBPs57. Moreover, in spite of the presence of QREs within target mRNAs, monocytes and macrophages probably express a large variety of RBPs that compete with QKI for access to either identical or similar motifs with varying affinities within a short stretch of RNA nucleotides19, which could preclude the observation of a posttranscriptional event.

In conclusion, we have identified QKI as a critical posttranscriptional regulator of pre-mRNA splicing and transcript abundance in monocytes and macrophages. We propose that the RBP-induced reprogramming of the posttranscriptional landscape could generate novel targets for the effective attenuation of inflammatory diseases.

Note added in proof: After the acceptance of our paper, we were informed by Dehghan et al.58 of the CHARGE Consortium’s identification that single nucleotide polymorphisms proximal to QKI significantly associated with myocardial infarction and coronary heart disease risk.

Methods

Human immunohistochemistry studies. Early lesions are defined as fatty streaks or PTI, whereas advanced plaques consist of both FCA and IFH (fibroatheroma with early-stage or late-stage necrotic core). Scoring of plaque stage, based on characteristic thin cap fibroatheroma (vulnerable or ruptured plaques), vascularization, IFH and/or thrombi/fibrin deposits, were scored by a trained pathologist. Paraffin tissue sections from human carotid arteries were deparaffinized and rehydrated. Prior to treatment with TE buffer (pH 9 for QKI-5 and QKI-7) or citrate buffer (pH 6 for QKI 6) for antigen retrieval, sections were incubated overnight at 4 °C with primary mouse-anti-human pan-QKI (10 μg ml−1, clone N147/6; UC Davis/NIH NeuroMab Facility, Davis, CA, USA), mouse anti-human QKI-7 (10 μg ml−1, clone N195A/16; NeuroMab/Antibodies, Inc.); mouse anti-human QKI-6 (10 μg ml−1, clone N183/15; NeuroMab/Antibodies Inc.) or mouse anti-human QKI-5 (10 μg ml−1, clone N195A/16; NeuroMab/Antibodies, Inc.); mouse anti-human IFH (10 μg ml−1, clone N182/17; NeuroMab/Antibodies Inc.) or mouse anti-human KLF4 (10 μg ml−1, clone N183/15; NeuroMab/Antibodies Inc.) diluted in Tris-buffered saline containing 1% BSA and 0.1% Tween 20. Subsequently, sections were washed in Tris-buffered saline and incubated with a secondary biotinylated sheep-anti-mouse antibody (GE Healthcare, Eindhoven, The Netherlands). Next, the sections were incubated with streptavidin ABC-alkaline phosphatase (Vector Laboratories, Peterborough, UK) and colour development using the Vector red substrate kit (Vector Laboratories, Burlingame, CA, USA) followed by haematoxylin counterstaining. No primary antibody was used for the negative control. QKI/CD68 co-localization immunostaining was achieved using CD68 (Dako-KP1, DakoCytomation) and pan-QKI antibodies, with CD68 and QKI being counterstained with Vector Blue and Vector Red, respectively. For quantification, slides were analysed in a blinded manner using a Leica DM5000 light microscope (Leica Microsystems) coupled to computerized morphometry (Leica Qwin 3.5.1).

BM transplantation. Male LDLR−/− mice were housed in sterile filter-top cages and fed a chow diet (Special Diet Services, Wixom, Essex, UK). Drinking water was infused with antibiotics (83 mg l−1 ciprofloxacin and 67 mg l−1 polymyxin B sulfate) and 6.5 g l−1 sugar and was provided ad libitum. BM transplantation studies were performed as previously described with minor modifications59. Briefly, to induce BM aplasia, 10- to 12-week-old recipient mice were exposed to a single dose of 9 Gy (0.19 Gy min−1) or 200 kV (4 mA) total body irradiation, followed by haematoxylin counterstaining. No primary antibody was used for the negative control. QKI/CD68 co-localization immunostaining was achieved using CD68 (Dako-KP1, DakoCytomation) and pan-QKI antibodies, with CD68 and QKI being counterstained with Vector Blue and Vector Red, respectively. For quantification, slides were analysed in a blinded manner using a Leica DM5000 light microscope (Leica Microsystems) coupled to computerized morphometry (Leica Qwin 3.5.1).

Figure 8 | QKI regulates the expression of atherosclerosis-related mRNAs and impairs foam cell formation. (a) Quantitative RT-PCR (qRT-PCR) analysis of established atherosclerosis-related genes in wt of qk−/− derived monocytes and macrophages. Gene expression in qk−/− samples are relative to either WT monocytes or WT macrophages (n≥3). Data expressed as mean ± s.e.m.; Student’s t-test; *P<0.05. **P<0.01. (b) mRNA-seq-derived expression values to illustrate the expression of established atherosclerosis-related genes in sibling or patient monocytes and macrophages. (c) qRT-PCR analysis of QKI isoform mRNA expression in unstimulated THP-1 ‘macrophages’, or treated with β-VLDL or acLDL for 24 h. Data expressed as mean ± s.e.m.; one-way analysis of variance (ANOVA), Bonferroni’s post-hoc test; **P<0.05. (d) qRT-PCR analysis of well-known atherosclerosis-related genes in sh-cont or sh-QKI THP-1 ‘macrophages’ that were either left untreated or treated with acLDL or β-VLDL to induce foam cell formation (n=3). Data expressed as mean ± s.e.m.; Student’s t-test; *P<0.05 and **P<0.01. (e) Photomicrographs of an Oil-red-O staining to assess β-VLDL, acLDL uptake in either sh-Cont or sh-QKI THP-1 ‘macrophages’. Scale bar, 100 μm. Inset is a high-magnification image of lipid-droplet accumulation. Data expressed as mean ± s.e.m.; Student’s t-test; *P<0.05 and **P<0.01.
mice were placed on a Western-type diet, containing 0.25% cholesterol and 15% cacao butter (Special Diet Services) for 8 weeks (n = 12 per group). Immunohistochemical analysis and quantification of the aortic root using anti-Monocyte and Macrophage-2 (MOMA2) antibody (Sigma-Aldrich) for MOMA2-positive cell area (expressed as a percentage of total plaque area), VSMC content (smooth muscle α-actin-positive cells) and collagen (picrosirius red staining) was performed in a blinded manner. Haematologic chimerism of the transplanted LDLR<sup>−/−</sup> mice was validated using the following primers: qf forward primer 5'-TGTGACTTGGGACTGTCAA-3', qf reverse primer 5'-AAGGGAATTTAGCAA-3'. BM-derived WT and qk<sup>+</sup> monocytes were isolated using CD115<sup>+</sup> antibody coupled to magnetic beads (Miltenyi Biotech, Leiden, The Netherlands) and differentiated for 7 days to macrophages using mouse recombinant M-CSF (PeproTech, Hamburg, Germany) in RPMI 1640 medium (Gibco, Bleiswijk, The Netherlands).
Netherlands) containing 10% FCS (Bodinco, Alkmaar, The Netherlands) and 0.01 mm glutamine, 50 units per ml penicillin and 50 μg ml⁻¹ streptomycin.

**Lentiviral transduction of monocytes.** Human THP-1 'monocytes' (ATCC, Manassas, VA, USA) were transduced with lentiviral particles encoding either sh-QKI or sh-Cont (catalogue number: SHC202; MISSION library, Sigma-Aldrich). Stable transductants were selected using 3 μg ml⁻¹ puromycin (Sigma-Aldrich) for 72 h.

**GapmeR design.** A single-stranded RNA-DNA hybrid antisense oligonucleotide (GapmeR) was designed to target exon 2 of QKI (Eurogentec, Maastricht, The Netherlands), along with a scrambled GapmeR control. Both

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**Figure 9 | Schematic depicting how QKI posttranscriptionally regulates monocyte to macrophage differentiation and atherosclerosis development.** QKI mRNA expression increases in intermediate monocytes, reaching a peak in the non-classical monocyte (middle). Monocytes adhere to the endothelium at sites of tissue injury, leading to their activation and migration into the subendothelial space. This process requires QKI, as the targeted ablation of QKI impaired monocyte adhesion and migration, and the evident transition in cellular phenotype requires extensive reprogramming of the posttranscriptional landscape. On tissue entrance, the monocyte differentiates into a macrophage, a conversion that was associated with a potent increase in QKI protein. This increase potentiates the interaction of QKI with (pre-)mRNA targets, enhancing splicing and target mRNA repression. The loss of QKI in macrophages results in an inability to adopt the macrophage phenotype and a perturbation of lipid uptake and foam cell formation.
the GapmR and scrambled control were 22 nucleotides in length and consisted of RNA (A,C,G,U) and DNA (da, dc, dd, dt) in a 10-10-6-10 manner, with a phosphorothioate backbone (*) and a 6-FAM label on the 5’ end. GapmR sequences were as follows:5’-A’C’A’U’C’U’G’U’C’C’U’C’-3’ for the QKI-targeting GapmR and 5’-U’G’C’C’C’U’C’-3’ for the scrambled control.

Monocyte population analysis. Human monocytes were derived from healthy donors by buffy coats of healthy donors with an antibody to CD14, conjugated to magnetic beads to allow for MACS sorting (Miltenyi Biotech). Cells were cultured in RPMI media supplemented with 10% FCS and 1% L-glutamine at 37°C. Differentiation of primary CD14+ monocytes to macrophages was achieved by stimulating with 5 ng/ml M-CSF (Thermo Fisher Scientific) or 5 ng/ml M-CSF (Miltenyi Biotech). Human THP-1 monocytes were cultured in RPMI media supplemented with 10% FCS and 1% L-glutamine at 37°C and 5% CO₂, with differentiation into macrophages being induced by treating with 100 nM PMA60. TS/216 for integrin activation experiments was kindly provided by Dr Arnoud Sonneberg, Netherlands Cancer Institute, Amsterdam, The Netherlands.

Monocyte and macrophage culture. Human monocytes were isolated from buffy coats of healthy donors with an antibody to CD14, conjugated to magnetic beads to allow for MACS sorting (Miltenyi Biotech). Cells were cultured in RPMI media supplemented with 1% FCS and 0.1% L-glutamine at 37°C. Sh-Cont and sh-QKI THP-1 ‘monocytes’ towards 1 nM fMLP, 10 ng/ml GM-CSF (Thermo Fisher Scientific) or 10 ng/ml M-CSF (Miltenyi Biotech). Cell migration (adherent cells) was quantified by manual counting or ImageJ. FACS analysis was conducted on a FACSCalibur (BD Biosciences, Breda, The Netherlands). RNA was isolated from the subpopulations using Trizol reagent (Thermo Fisher Scientific, Briezvik, The Netherlands).

In vitro perfusion assay. Glass coverslips were coated with type I collagen, after which the system was perfused with platelet-rich plasma for 10 min. Next, the system was flushed with flow buffer (20 mmol l⁻¹ NaCl, 6 mmol l⁻¹ KCl, 1 mmol l⁻¹ MgSO₄, 1.2 mmol l⁻¹ KH₂PO₄, 5 mmol l⁻¹ glucose, 1.0 mmol l⁻¹ CaCl₂ and 0.5% BSA) for 2 min. Sh-Cont and sh-QKI THP-1 ‘macrophages’ or Sib-QKI THP-1 ‘macrophages’ for 24 h with either 25 μg/ml 17β-estradiol, 50 μg/ml acLDL, or 10 μg/ml oxLDL, after which the cells were fixed with 10% Formalin, and Oil-Red-O stained and haematoxylin counterstained. Oil-Red-O area per field of view was divided by the number of cells using ImageJ software.

Cellular migration assays. Transwell cellular migration studies of sh-Cont and sh-Qki THP-1 ‘monocytes’ towards 1 nM N-formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma-Aldrich) or 10 ng ml⁻¹ macrophage chemotactant protein 1 (R&D Systems, Abingdon, UK) for human primary monocytes were performed. Five-well Transwells containing two or more alternative splice junctions was detected in the isomorph-specific probes. In situations where the probe sets for two or more alternative isoforms were ‘present’ in any sample of the data, an alternative splicing event was scored. For these events, the junction probe sets that hybridized to individual isoforms were identified and the probes they contained were used for the Kruskal–Wallis test. Next, we normalized individual probe intensities and grouped them by splicing profiles. Subsequently, the data was used to test the hypothesis that the probe intensities come from independent populations. If the resulting P-value was small enough, the null hypothesis was rejected and the alternative hypothesis that the probes were differentially expressed was accepted. To determine an appropriate value for the 0.01 significance level, 12,740 Kruskal–Wallis tests on randomly selected probe sets were performed, yielding an α-value that associated with the 1% quantile of randomly selected probes (1.975486 × 10⁻³). To account for multiple testing, a Bonferroni-corrected α-value of 1.975486 × 10⁻³/12,740 × 10⁻³ = 1.530617 × 10⁻⁷ was used as a P-value cutoff for significance. The Sepscore is log2 (Include/Skip ratio) of the experimental sample over the reference sample. Exon inclusion generates a positive Sepscore, whereas exon exclusion generates a negative Sepscore.

Word (5-mer) enrichment and positional mapping. Counts of all 5-mers (ACUA) in the selected region of an exon set are compared with their counts from a background set of sequences using Fisher’s exact test, with multiple testing correction. For motif mapping, we plotted the frequency of specific motifs in 50-nucleotide windows slid along the intron sequences upstream and downstream of each selected exon set with 5-nucleotide sampling intervals. At each interval, we computed the average number of motifs in exons activated or repressed in shRNA-treated cells, and background exons in the same experiment with no splicing change. For the background set, we empirically estimated the 95% confidence interval of motif frequency (error bars). For peaks of consecutive points outside the 95% interval, we applied the Mann–Whitney–Wilcoxon test to estimate a P-value that the motif frequency at each point within the peak is greater than background. As the points were not independent, we estimated a q-value for each peak by finding the most significant P-value for any point in the peak and applying Bonferroni correction for the number of points within the peak. A second Bonferroni correction controlled for the number of possible positions at which a peak might occur, yielding the final q-values. To explain further; the effect of QKI depletion on every assessed exon was calculated from the RNA-seq or the splicing-sensitive microarray: that is, whether a reduction in QKI causes inclusion or exclusion from the final mature mRNA transcript.

The intronic regions around differentially spliced exons were analysed for the presence of ACUA motifs (5-mers). By doing this for every alternative spliced exon, we could detect an enrichment of ACUA motifs in the upstream introns of the exons that were included in a ‘QKI-deficient’ cell; QKI is not binding upstream; thus, the exon is included, as measured by RNA-seq or splicing-sensitive microarray.

RNA-seq library preparation. RNA-seq data are deposited in GEO under the accession number GSE47979. For each sample, the non-ribosomal fraction of a total RNA was isolated using the Trizol Kit (Life Technologies, Madison, Wisconsin, USA). RNA-seq library preparations were performed on a Bioanalyzer 2100 bioanalyzer (Agilent Technologies, Santa Clara, USA). Samples meeting a RNA integrity number criteria of > 8 were used for further analysis.

Mapping and analysis of RNA-seq data. All samples were mapped using TopHat2 (refs 63,64) with Bowtie2 (ref. 64) as the underlying alignment tool. The input Illumina fastq files consisted of paired-end reads with each end containing 100 bp (except for 2 samples with 50 bp of paired-end reads). For equivalency,
100-bp reads were trimmed to 50 bp before mapping. The target genome assembly for the human samples was GRCh37/UCSC-hg19 and Tophat was additionally used with its "GTF" parameter with data from the hg19 UCSC KnownGenes track. For multiple-mapped fragments, only the highest scoring mapping determined by Bowtie2 was kept. Only mappings with both read ends aligned were kept. Potential PCR duplicates (mappings of more than one fragment with identical positions for both read ends) were removed with the samtools "rmdup" function, keeping only one of any potential duplicates. The final set of mapped paired-end reads for a sample were converted to position-by-position coverage of the relevant genome assembly using the bedtools 'genomeCoverageBed' function. To determine the count of fragments mapping to a gene, the position-by-position coverage was summed over the exonic positions of the gene. This gene total coverage was divided by a factor of 100, to account for the gene. The final set of mapped paired-end reads for a sample were converted to position-by-position coverage of the relevant genome assembly using the bedtools 'genomeCoverageBed' function. To determine the count of fragments mapping to a gene, the position-by-position coverage was summed over the exonic positions of the gene. This gene total coverage was divided by a factor of 100, to account for the gene.

For mapping of reads that are chimeric to the reference genome, we identified the translocation breakpoint on chr6 we used STAR-Fusion (https://github.com/STAR-Fusion). Non-splice junction reads from the macrophage samples that mapped from the QKI gene on chr6 to any location on chr5 were inspected and several were found, which mapped from QKI intron 4 to a site that is strongly transcribed from chr5 in the patient but not at all in her sibling.

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Acknowledgements

For the studies described in this manuscript, we greatly appreciate the participation and cooperation of the QKI haploinsufficient patient and her family members. R.G.d.B. and E.p.d.v.d. were supported by Netherlands Institute for Regenerative Medicine research grants (Grant No. FES09908). This research was also made possible by a private research endowment to E.p.d.v.d. L.S., S.K., J.P.D. and M.A.I. were supported by US National Institutes of Health grant GM040478 and W.S.F. was supported by training grants CIRM TG2-01157 and NIH T32 GM068466. H.K. was supported by European Union Marie Curie CIG grant (PTRCODE) and TUBITAK grant (113E159). We also gratefully acknowledge Dr Christopher Burge and his laboratory at the Massachusetts Institute of Technology, for assistance with the generation of sashimi plots from our RNA-seq data. In addition, we acknowledge Dr Arno/old Sonneberg at the Netherlands Cancer Institute for the in-depth discussions and integrain antibodies.

Author contributions

R.G.d.B., M.A.I., A.J.v.Z. and E.P.d.v.d. conceived the ideas and experiments within the study. R.G.d.B. and E.p.d.v.d. performed the majority of the experiments detailed in the study. L.S., S.K. and J.P.D. performed the RNA-seq and splicing sensitive microarray studies and analysed the data. J.P., H.G.d.B., A.S., J.M.o.G., J.M.G.d.P., A.o.K., P.H.j.v.d.Z., R.s., C.M.A.v.A. and L.B. assisted with experiments and analyses. R.G.d.B., L.S., S.B., W.Y.L., S.M.K., J.P.D. and H.K. provided bioinformatics and statistical assistance. H.E.V. provided access to the QKI haploinsufficient patient and sibling. R.G.d.B., A.J.v.Z. and E.P.d.v.d. wrote and edited the manuscript, and prepared the figures. E.A.L.B., F.A.L.B., M.A.I. and A.J.v.Z. provided critical experimental commentary, discussion and assistance with manuscript preparation and editing. T.J.R., E.A.L.B., M.A.I., A.J.v.Z. and E.P.d.v.d. provided funding for the studies.
Additional information

Accession code: RNA sequencing and microarray data were deposited in NCBI's Gene Expression Omnibus (GEO) under accession codes GSE74979 (for RNA-seq data) and GSE74887 (for the splicing-sensitive microarray data).

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: de Bruin, R. G. et al. Quaking promotes monocyte differentiation into pro-atherogenic macrophages by controlling pre-mRNA splicing and gene expression. Nat. Commun. 7:10846 doi: 10.1038/ncomms10846 (2016).

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