Immunodeficiency and severe susceptibility to bacterial infection associated with a loss-of-function homozygous mutation of MKL1

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Condensed Title: Human MKL1-deficiency
Key Points

- MKL1 deficiency results in actin cytoskeletal disruption in myeloid and lymphoid cell lineages.
- MKL1 deficiency impairs neutrophil migration associated with downregulation of myosin II.

Abstract

Megakaryoblastic Leukemia 1 (MKL1), also known as MAL or MRTF-A, is a co-activator of serum response factor (SRF), regulating transcription of actin and actin cytoskeleton-related genes. MKL1 is known to be important for megakaryocyte differentiation and function in mice but its role in immune cells is unexplored. Here we report a patient with a homozygous nonsense mutation in the \textit{MKL1} gene resulting in immunodeficiency characterised predominantly by susceptibility to severe bacterial infection. We show that loss of MKL1 protein expression causes a dramatic loss of F-actin content in lymphoid and myeloid lineage immune cells and widespread cytoskeletal dysfunction. MKL1-deficient neutrophils displayed reduced phagocytosis and almost complete abrogation of migration \textit{in vitro}. Similarly, primary dendritic cells were unable to spread normally or to form podosomes. Silencing of \textit{MKL1} in myeloid cell lines revealed that F-actin assembly was abrogated through reduction of G-actin levels and disturbed expression of multiple actin-regulating genes. Impaired migration of these cells was associated with failure of uropod retraction likely due to altered contractility and adhesion, evidenced by reduced expression of the MYL9 component of myosin II complex and over expression of CD11b integrin. Together, our results show that MKL1 is a non-redundant regulator of cytoskeleton-associated functions in immune cells and fibroblasts and that its depletion underlies a novel human primary immunodeficiency.
Introduction

Primary immunodeficiency (PID) resulting from disorders of neutrophil function predispose to bacterial and fungal infections. Defined mechanisms include defects of respiratory burst, integrin activation and neutrophil granules, but many conditions in this heterogeneous group of PIDs remain uncharacterised. Although actin regulation is known to be central to several neutrophil functions, such as phagocytosis, adhesion and migration, cytoskeletal defects causing neutrophil disorders in humans are rare. Mutations in the cytoskeletal regulators Rac2 and Wiskott-Aldrich syndrome protein (WASP) result in immunodeficiencies associated with reduced filamentous actin (F-actin) assembly from globular actin (G-actin) specifically in haematopoietic cells. Although both proteins are expressed in neutrophils, only Rac2 deficiency produces clinically significant neutrophil dysfunction, highlighting the importance of specific cytoskeletal regulators in this cell type.

MKL1 and MKL2 are members of the myocardin-related transcription factor (MRTF) protein family. MRTFs are held in an inactive state in the cytoplasm in a reversible complex with G-actin. Stimulation of Rho GTPases promotes incorporation of G-actin into F-actin filaments, releasing MRTFs from G-actin and allowing their import to the nucleus, where MRTFs function as coactivators of serum response factor (SRF) and stimulate SRF-mediated transcription of actin and actin cytoskeleton-related genes. In contrast with myocardin, which is restricted to cardiomyocytes and smooth muscles cells, MKL1 and MKL2 are widely expressed. While MKL2- and myocardin-knockout mice are embryonically lethal, MKL1-knockout mice have a less severe phenotype, demonstrating premature involution of mammary glands, partial embryonic lethality due to abnormal cardiogenesis associated with myocardial cell death and reduced platelet counts in peripheral blood.

Several studies indicate that MKL1 is required for maturation and migration of megakaryocytes and its absence can only partially be compensated for by the presence of MKL2. However, the role of MKL1 in myeloid or lymphoid lineage immune cells has not been reported. Here we describe for the first time human MKL1-deficiency caused by a homozygous mutation in a child with severe bacterial infections. Loss of MKL1 resulted in low levels of F-actin and impaired cytoskeletal functions of neutrophils and myeloid lineage dendritic cells, as well as lymphoid lineage cells. These findings define MKL1-deficiency as a novel PID and elucidate a non-redundant role for MKL1 in human immune cells.

Material and Methods

Human neutrophil isolation and peripheral blood dendritic cell cultures
Blood samples were obtained with informed consent from the parents of the patient in accordance with the Declaration of Helsinki and with approval from local ethics committees (04/Q0501/119 and 06/Q0508/16). Neutrophils were isolated from healthy donor or patient blood. Briefly, 2mL of a 5% dextran-saline solution was added to 10 mL of blood and gently mixed by inversion before let to sediment for 30 minutes. The plasma layer was collected and overlaid on an equal volume of Ficoll-Paque and the centrifuged at 1800 rpm for 10 minutes. The PBMC layer was taken for CD14-positive cells selection while the neutrophil pellet was resuspended in distilled water for 20 seconds before addition of 2x saline solution to restore isotonocity. The neutrophils were then centrifuged at 1200 rpm for 7 minutes and resuspended in warm complete RPMI.

CD14-positive cells were magnetically labelled and positively selected on LS columns (Miltenyi Biotec). The column flow-through, representing the unlabeled lymphocyte fraction, was collected for further experiments. CD14 positive cells were eluted from the column and seeded on day 0 at 1x10^6 cells/well in 6-well plates in complete growth media (RPMI +10% FCS + Pen/Strep) supplemented with 100ng/ml rhGMCSF (granulocyte-macrophage colony stimulating factor) and 25ng/mL rhIl-4 (recombinant human interleukin-4). Fresh cytokines were added on day 3 and cells were harvested for use on day 6 or 7.

**THP1 and HL-60 cell culture**

THP1 cells were cultured in suspension in RPMI-Glutamax supplemented with 10% FCS and Pen/Strep. Cells were maintained at 0.5-1x10^6 cells/mL. For dendritic cells differentiation, THP1 cells were cultured in the presence of 10ng/mL rhIL-4 and 10ng/mL rhGM-CSF for 6 days. Cells were split on days 2 and 5 with addition of fresh cytokines. Both adherent and suspension cells were harvested for use on day 6 or 7.

HL-60 cells were maintained in culture at a concentration between 0.1 and 1.5x10^6 cells/mL in RPMI supplemented with 10% FCS and 25mM HEPES. To differentiate the HL-60 into neutrophil-like cells (dHL-60), 1x10^6 cells were cultured for 5 days in media supplemented with 1.3% of DMSO in a T25 flask.

**Migration experiments:**

Depending on the cells used for the experiment, coverslips were coated with a solution of Fibrinogen at 25mg.mL^{-1} (primary neutrophils) or with a solution of Fibronectin at 100μg.mL^{-1} (dHL-60) for one hour at 37°C degrees. The coverslips were washed two times with PBS before being used and dHL-60 were washed three times in a warm HBSS/2% Human albumin/100mM HEPES solution. 1x10^5 neutrophils or dHL-60 were let to adhere on the coated coverslip for 20 to 30 minutes. The coverslip was then washed once with the media.
used for the migration without chemokine to remove non adherent cells. A 100nM fMLP solution was used for neutrophils/dHL-60 experiments. In order to stabilise the chemokine gradient in the Dunn chamber, 80 uL of a 1% agarose gel containing the chemokine was poured into the outer well of the chamber and let to polymerise for 2 minutes. The inner well was filled with 50 μL of the media without chemokine and then the chamber was assembled by laying down the coverslip on which the cells were adhering. The chamber was then sealed with wax. Cells were the imaged at 37°C on an Axiovert 135 microscope equipped with a motorised stage by capturing one image per minute during one hour the neutrophil/dHL-60.

Migration of EBV transformed LCLs was performed in µ-Slide Chemotaxis 3D (Ibidi) in 3D collagen matrix according to manufacturer’s instructions. Cells were stimulated with MIP-3α (100 ng.mL⁻¹) one hour prior to imaging. Images were taken over 2 hours on a Leica SP5 confocal using a 20x lens. The movie was quantified using ImageJ and Ibidi analysis software (http://ibidi.com/xtproducts/en/Software-and-Image-Analysis/Manual-Image-Analysis/Chemotaxis-and-Migration-Tool).

Fibroblast migration was performed using the Oris cell migration assay (Platypus Technologies, CMA1.101) accordingly the manufacturer instruction. Briefly, after sealing the Oris Cell Seeding Stoppers in each well, 40 000 cells were seeded in each well and let to adhere for 24 hours. The Cell seeding stoppers were then gently removed, the cell washed twice with PBS and fresh media was added. Cell migration was then monitored at 37°C for 18 hours using Axiovert 135 microscope equipped with a motorised stage by capturing one image every 15 minutes. Migration was quantified by measuring the area invaded by the cells using imageJ/Fiji.

**Supplemental methods**

Details of the reagents, shRNA, cell transduction, confocal microscopy, flow cytometry, phagocytosis assay, Western blot qRT-PCR and cell migration analysis are provided in the supplemental data available on the website of *Blood*.

**Results**

Human MKL1-deficiency is associated with severe bacterial infections, neutrophil dysfunction and actin cytoskeletal abnormalities
We report a girl born to second cousin consanguineous parents (Figure 1a) who presented in the first year of life with Pseudomonas septic shock associated with meningitis, malignant otitis externa, and more than thirty cutaneous and subcutaneous abscesses. This followed a primary chicken pox infection that was considered to have a normal course with many lesions that had crusted over by two weeks and which did not require hospitalisation. Around two weeks after the onset of chickenpox, she presented with another pustule in her ear that was initially thought to be a recurrence of VZV. However, she developed skin lesions and became acutely unwell with pseudomonas septicaemia. She was very slow to respond to appropriate antibiotic therapy, and received a month of intravenous antibiotics before being discharged home on oral ciprofloxacin. She recovered and was commenced on antibiotic prophylaxis and empiric immunoglobulin replacement therapy. Despite this, she continued to have intermittent deep skin infections, characterised by purple painful lesions, usually in the peripheries, that responded to oral antibiotics. In her past history, she had received seven days of intravenous antibiotics for possible sepsis at day 2 of age, had a history of pseudomonas ear infections and failed to thrive. BCG vaccination, given in the neonatal period, resulted in a large ulcerated abscess, requiring several months of oral antimycobacterial treatment.

Her initial investigations revealed intermittent mild thrombocytopenia (platelets typically ranging from 50-150 x10^9/L). Complement function, MBL levels, CD11a and CD18 expression, neutrophil oxidative burst activity, lymphocyte subsets, naive and memory T-cell numbers, and T-cell proliferation to PHA were normal. Her immunoglobulins levels were also unremarkable; vaccine responses were not measured (Table S1). Only her T-cell proliferation to anti-CD3 antibody was defective, suggesting a specific defect downstream of the T-cell receptor. In view of a clinical suspicion of neutrophil dysfunction, neutrophil phagocytosis was tested using pre-opsonised E.Coli and found to be abnormal on two occasions (Figure 1 b, c). As this suggested a defect of cytoskeletal function, neutrophil chemotaxis in response to fMLP was also tested using a Dunn Migration Chamber. In comparison with healthy donor neutrophils, patient neutrophils showed a severe migratory defect, with >90% of cells unable to migrate. The few cells that migrated did so with a very slow velocity (Figure 1d, e & video 1 and 2) although chemotaxis (migration along the chemokine gradient) was preserved (Figure 1f), suggesting retention of functional signal transduction in response to fMLP. In fact, patient cells demonstrated enhanced directional persistence as shown by the chemotactic index (Figure 1f, g and Supplemental movies 1 and 2), suggesting impaired regulation of F-actin remodelling, chemokine signalling or integrin function. An EBV-immortalised lymphoblastoid cell line (LCL) of the patient also
showed impaired migration towards MIP3α (Figure 1 h). In keeping with an actin cytoskeletal defect, we found reduced F-actin levels in both myeloid and lymphoid cells (Figure 2 a-c).

The clinical and cellular phenotype of the patient suggested a novel disorder. Since the patient was born to a consanguineous family, we hypothesized that her disease was caused by a recessive Mendelian mutation and used whole exome sequencing to identify it. In the exome data we found 20,590 single nucleotide variants and small insertions/deletions, including 315 very rare ones, i.e. those not seen in the 6,500 NHLBI Exomes (8), 1000 Genomes database (April 2012 data release) (9) or 2,500 exomes analyzed internally using the same bioinformatics pipeline. Of these rare variants, three were homozygous (Table S2), including one nonsense variant p.K723X in the MKL1 gene, which is predicted to truncate the 931 amino-acid MKL1 protein. Sanger sequencing confirmed the presence of the mutation (Figure 2d). Western blot of patient peripheral blood mononuclear cells (PBMC) confirmed absence of the full length MKL1 protein (Figure 2e). A smaller protein band was seen in patient cells which could represent low levels of truncated MKL1.

**MKL1 – deficiency impairs neutrophil migration**

While MKL1 is known to be important for cytoskeletal regulation, to our knowledge its role in immune cell function has never been studied. To confirm that impaired neutrophil function resulted from disruption of MKL1 in our patient, we utilised shRNA lentiviral vectors to specifically silence MKL1 in the HL-60 myeloid cell line (Figure 3a) that can be differentiated to neutrophils (dHL-60). Silencing of MKL1 in HL-60 cells (dHL-60 MKL1) did not alter differentiation potential as reflected by nuclear morphological change, upregulation of CD11b and down-regulation of CD29/CD49d surface expression (Figure S1 a and c). Surface expression of the fMLP receptor FPR1 was also not affected by MKL1 silencing (Figure S1b). In spite of this, directional migration in response to fMLP was markedly abrogated, and chemotaxis was associated with enhanced directional persistence (Figure 3b, c, Video 3 and 4) (Figure 3d, e). The migratory defect in dHL-60 MKL1 cells therefore exactly mimicked the phenotype of patient neutrophils.

**MKL1 regulates cytoskeletal functions of dendritic cells.**

To determine whether MKL1 is required for cytoskeletal regulation in other myeloid lineage immune cells, we generated dendritic cells (DCs) from patient CD14+ monocytes and analysed them for cytoskeletal morphology and adhesion. We found that patient MKL1-deficient DCs were morphologically distinct from control DCs, showing reduced spreading on fibronectin-coated coverslips (Figure 4a, b), a marked reduction in total F-actin staining and a complete absence of podosomes (Figure 4c,d). Targeted shRNA silencing of MKL1 in DC
derived from the THP1 cell line (Figure 4e) recapitulated these findings resulting in marked depletion of F-actin, poor adhesion to fibronectin, reduced spreading and defective podosome assembly (Figure 4f-i).

**MKL1 alters fibroblast migration**

Significant and abnormal scarring was a prominent feature of our patient’s response to skin infections (Figure 5a), which suggested a possible additional defect of fibroblast function. To test this (and in the absence of primary fibroblasts from the patient), we examined migration of normal primary fibroblasts following lentiviral transduction with scrambled or MKL1-targeting shRNA (transduction efficiencies of 97% and 81% respectively). In a modified wound healing assay only fibroblasts expressing MKL1 shRNA demonstrated impaired migration *in vitro* over 18 hours (Figures 5b, c). This was associated with dramatically altered cell shape and loss of cortical actin and stress fibres, when compared with cells expressing scrambled control shRNA (Figure 5d). These findings indicate an important role for MKL1-mediated cytoskeletal regulation in non-haematopoietic cells.

**MKL1 regulates neutrophil actin cytoskeleton dynamics**

As seen in patient cells, dHL-60 MKL1 displayed a reduced F-actin content (Figure 6a, b and c). Quantification of the F-actin and G-actin cellular fractions by Western blot showed that SCR and MKL1 dHL-60 had a similar G/F-actin ratio but that MKL1-deficient cells displayed a reduction in both F-actin and G-actin content (Figure 6d). To test whether loss of G-actin is the cause of cytoskeletal dysfunction in MKL-1 deficiency, we reconstituted G-actin expression by transducing dHL-60 MKL1 cells with a β-actin-mCherry lentivirus vector. While G-actin reconstitution partially restored F-actin content, a significant reduction in F-actin levels persisted (Figure 6e, f), suggesting additional defects of actin regulation in cells lacking MKL1. Using cytoskeletal specific PCR arrays we observed alteration in the expression levels of multiple regulators of the actin cytoskeleton in dHL-60 MKL1 cells, *CDC42BPA*, *CTTN* and *FNBP1L* were significantly downregulated while expression of a number of other actin-associated genes were upregulated in the absence of MKL1 (Table 1, Table S3). A notable feature of dHL-60 MKL1 cells was an abnormally elongated uropod, suggesting impaired retraction during migration. When uniformly stimulated with fMLP, 43% (+/- 5%) of MKL1-silenced dHL-60 cells displayed this morphology, compared with 15% (+/- 1.5%) of control cells (Figure 7a, b). As uropod retraction is regulated by the myosin II protein complex13, we reasoned that MKL1-deficiency could impair neutrophil migration through effects on myosin II. As expected, disruption of myosin II interaction with F-actin using blebbistatin produced impaired uropod retraction similar14 to that observed in dHL-60 MKL1 cells (Figure 7c). Furthermore, expression of myosin regulatory light chain...
(MYL9/MLC), which is a component of myosin II complex, was dramatically inhibited in dHL-60 MKL1 cells (Figure 7d, e and f).

Discussion

MKL1 was first identified in association with acute megakaryoblastic leukaemia, where it is a component of a fusion protein generated by chromosomal translocation. Subsequent investigation of MKL1 function in hematopoietic cells has focused on megakaryocytes and MKL1-deficiency in mice has been shown to result in cytoskeletal defects in this cell type disrupting differentiation, migration and proplatelet formation. Our study is the first to describe human MKL1-deficiency and demonstrates that although mild thrombocytopenia is associated with the condition, the major phenotype appears to relate to immunodeficiency.

Through direct interaction with the transcription factor, SRF, MKL1 regulates the translation of cytoskeletal genes in multiple cell types to control cellular processes including migration, adhesion and differentiation. In keeping with this, we demonstrate here that MKL1 plays a non-redundant role in maintaining normal levels of F-actin in both lymphoid and myeloid lineage cells. As the infection phenotype of our patient was prominent bacterial susceptibility, we initially studied the impact of MKL1-deficiency on neutrophils. In the absence of MKL1 activity, neutrophil actin cytoskeleton is severely perturbed evidenced by defects of phagocytosis and migration. Our data suggests that the mechanisms of cytoskeletal dysfunction in MKL1-deficient cells are multifactorial. A significant component is reduced expression of G-actin that we observed both at RNA and protein levels. However, forced reconstitution of G-actin using lentivectors did not fully restore intracellular F-actin levels suggesting that other MKL1-dependent cytoskeletal-regulators are critical for normal F-actin assembly. Using qPCR arrays, we found that multiple actin-associated genes were differentially expressed, supporting a complex role for MKL1 in cytoskeletal dynamics. Somewhat surprisingly, only a minority of actin-associated genes were downregulated, although this group included cortactin (CTTN), CDC42BPA and FNBP1L all of which promote F-actin assembly through the CDC42/WASp/Arp2/3 pathway in haematopoietic cells. In keeping with the role of MKL1 as an SRF activator, our findings are reminiscent of the SRF-deficient mouse model in which neutrophils were shown to demonstrate reduced levels of F-actin associated with impaired polarisation, migration and recruitment to areas of inflammation. In both MKL1 and SRF-deficiency, neutrophil numbers appear to be unaffected suggesting that, despite causing intrinsic cytoskeletal defects, these factors do not significantly impair development or release of neutrophils from the bone marrow.
Neutrophils classically migrate in an amoeboid fashion, characterised by lamellipodial extension and subsequent uropod retraction, for which the family of small GTPases, non-muscular myosin II and integrin trafficking are critical regulators. A prominent feature of MKL1-deficiency in neutrophil-like dHL-60 cells is abnormal uropod extension suggesting a defect of either myosin complex activity that supports uropod retraction or altered CD11b expression or activation at the cell surface. The uropod phenotype of MKL1-deficient neutrophils is mimicked by chemical inhibition of myosin complex function. It was previously shown, in CD34+ progenitor cells, that loss of MKL1 results in significantly reduced expression of myosin light chain 9 (MYL9), a component of the myosin II complex. Subsequent functional analysis demonstrated that MYL9 expression is a key cytoskeletal regulator both for platelet formation and megakaryocyte migration. We observed reduced expression of myosin light chain in dHL-60 MKL1 which is in keeping with uropod dysfunction and suggests that MKL1-dependent MYL9 expression is involved in the cytoskeletal regulation during neutrophil migration. As seen in the SRF-/- mouse model, MKL1-deficient neutrophils over-expressed CD11b, which may be due to impaired integrin trafficking. In SRF-/- mice, CD11b activation is impaired despite overexpression. Further investigation is required to determine the contribution of abnormal CD11b function to cytoskeletal dysfunction in MKL1-deficiency.

Given the role of MKL1 in megakaryocyte function, we were surprised that the observed thrombocytopenia in our patient was mild and did not lead to a clinically relevant bleeding disorder. This is unlikely to relate to residual expression of a small amount of truncated protein in our patient cells, as this did not appear preserve significant cytoskeletal function as evidenced by almost complete loss of neutrophil migration and DC podosome assembly. The position of the truncation would be predicted to preserve the B1 basic box and glutamine-rich domains that mediate interaction with SRF but disrupt the C-terminal transactivation domain (TAD) of MKL1 that confers transcriptional activity. This truncation has been modelled in vitro and demonstrated to retain nuclear localisation but completely abrogate MKL1 activity, which is in keeping with our findings. It is possible that the mild effects on platelet production may relate to compensation by MKL2 as mkl1/mkl2 double-knockout mice have a significantly more pronounced platelet deficiency.

In addition to severe effects on neutrophils and mild effects on platelets, we show that MKL1 is important for cytoskeletal regulation in myeloid lineage DC and in lymphoid cells. The clinical phenotype observed may reflect the hierarchical importance of neutrophils for antibacterial defences, as seen in the CD18 integrin deficiency, Leukocyte Adhesion Defect, which presents as a neutrophil disorder although broader cellular effects exist, reflecting the
importance for CD18 in many haematopoietic cell functions. We also observed that MKL1-deficiency impaired fibroblast migration in vitro. This is consistent with the abnormal wound healing and scarring following superficial skin infections observed in our patient, and with previous reports of dysregulated fibroblast migration in vitro when MKL1/ SRF function is manipulated genetically or chemically. It is however surprising that other tissues do not appear to be grossly affected, as MKL1 is widely expressed and deficient mice exhibit partial embryonic lethality with myocardial cell necrosis. Other extra-haematopoietic manifestations of human MKL1-deficiency may therefore manifest with increasing age.

Taken together, these data demonstrate a non-redundant role for MKL1 in regulating the actin cytoskeleton in human immune cells and fibroblasts. Our findings in neutrophils, DCs and B-cells suggest a wide impact for MKL1-deficiency on both myeloid and lymphoid immune cell function that would predict complex multi-lineage immunodeficiency. The clinical phenotype of our patient to date has been dominated by bacterial infections characteristic of neutrophil dysfunction. We do not have evidence of clinically manifesting T or B-cell defects, although these may be masked by treatment with regular immunoglobulin infusion and prophylactic antibiotics. The full clinical and immunological phenotype of human MKL1-deficiency will become apparent as additional cases are described. Patients suffering severe bacterial infections associated with neutrophil migration defects should be screened for possible mutations in the MKL1 gene.

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Authorship contributions

JR and DM designed and performed experiments, analysed data and wrote the manuscript. HLZ, VP, KN, FS, JC, and DM performed experiments and analysed data. GC, GB, AJT, DM
and KG designed experiments. CC and SH referred patient. SN, AJT and SOB wrote the manuscript.

**Disclosure of Conflicts of interest**

The authors declare that they have no conflict of interest.
References


Table 1: Differential expression of actin cytoskeleton genes in MKL1 deficient cells

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Figure Legends

Figure 1: Patient neutrophils display phagocytosis and migration defects
A) Family Tree. B) and C) FITC-positive E.coli uptake by neutrophils analysed by flow cytometry. Representative data from 2 independent experiments. D) Migration of control and patient neutrophils in a Dunn Chamber in response to an fMLP gradient. Tracks show migration path over time. 41 and 110 cells were respectively analysed in the control and patient sample in one experiment. Scale bar = 10 μm. E) Frequency distribution of the velocity of patient and control neutrophils. F) Chemotactic Index of the migrating neutrophils. G) Distribution of instantaneous angle between the gradient and the cell path during migration. H) Frequency distribution of the velocity of control and patient B LCL migrating in collagen gel. 20 cells were analysed in each population in one experiment. Data is representative of two independent experiments. *** P < .001.

Figure 2: Effect of MKL1 mutation on F-Actin
A) F-Actin (Alexa-647-phalloidin) content in lymphoid and myeloid cells analysed by flow cytometry. Patient cells were stained with CFSE and mixed with unstained control cells before fixation and staining. Representative data from two experiments. B) Confocal images
of F-actin (Alexa-647-phalloidin, red) in patient cells (CFSE, green) and mixed with control cells (unstained). Scale bar = 5 μm. Data were analysed from one experiment. C) Quantification of the Phalloidin mean fluorescence intensity observed in B. D) Sequence analysis of control and patient sample leading to the identification of the MKL1 mutation. E) MKL1 expression visualised by Western Blot in control and patient PBMC. *** P < .001.

**Figure 3: Knock down of MKL1 in HL-60 neutrophil like cells mimics patient phenotype.**

HL-60 cells were differentiated into neutrophil-like cells for 5 days in culture media containing 1.3% DMSO. A) Western blot of the MKL1 expression levels in undifferentiated (HL-60) or differentiated (dHL-60) cells carrying a scrambled shRNA (SCR) or the shRNA against MKL1 (MKL1). B) Migration of SCR and MKL1 neutrophils in a Dunn Chamber in response to an fMLP gradient. Tracks show migration path over time. Scale bar = 10 μm. C) Frequency distribution of the velocity of dHL-60 SCR and MKL1 cells during migration in a Dunn chamber in response to fMLP. 115 and 156 cells were analysed for SCR and MKL1 respectively conditions in 3 independent experiments. D) Chemotactic index of dHL-60 SCR and dHL-60 MKL1 during migration in the Dunn chamber. E) Instant angle distribution during the migration of SCR and MKL1 dHL-60. *** P < .001.

**Figure 4: Patient PBMC derived DCs have morphological abnormalities and severely reduced F-actin content.**

DCs were allowed to adhere to fibronectin coated glass cover slips for 4 hours then fixed in 4% paraformaldehyde. A) Phase contrast imaging shows poor spreading of patient DCs compared to controls. B) Quantification of the percent of cells that had spread. C and D) Cells were stained for DNA (Dapi, blue), F-actin (Alexafluor-488-phalloidin, green) and Vinculin (red) and imaged were acquired by confocal microscopy. Podosome structures were clearly present in controls (F-actin cores surrounded by vinculin, in 22/29 cells) but completely absent in patient cells (0/ 121 cells). Data acquired in a single experiment. E) Western blot of MKL1 in THP-1 cells expressing the shRNA scrambled (THP-1 SCR) and against MKL1 (THP-1 MKL). F) F-actin content in THP-1 SCR and THP-1 MKL DC, analysed by flow cytometry. G) Quantification of the spreading of THP-1 SCR and THP-1 MKL DC. H and I) Confocal images of the F-actin and Vinculin demonstrate the lack of podosomes in THP-1 MKL1 DC. Bar = 20 μm (panel A) and 10 μm (panel C and I). THP-1 DC MKL1 data were acquired in 3 independent experiments. *** P < .001.

**Figure 5: Knock down of MKL1 alters fibroblast morphology and migration**
A) Clinical photos showing skin scarring and hyper-pigmentation after treatment of skin abscesses using antibiotics. B) Migration of fibroblast expressing the SCR and MKL1 shRNA at 0, 9, 18 hours. C) Quantification of the area of migration observed in B. D) Confocal images of fibroblast expressing SCR and MKL1 shRNA. Cells were stained with phalloidin-alexa647. * P < .05.

**Figure 6: MKL1 deficient dHL-60 show a lower content in F-actin**
A) Analysis of the F-actin content in HL-60 MKL1 cells by flow cytometry. HL-60 WT cells and HL-60 carrying a scrambled or anti-MKL1 shRNA and co-expressing GFP were mixed before fixation and staining for F-actin with phalloidin-647. For clarity, only SCR and MKL1 population are displayed on the flow cytometry graph. B and C) Evaluation of the F-actin content in dHL-60 after stimulation with 100nM fMLP for 1 minute in SCR and MKL1 population respectively. Data from 3 independent experiments were analysed. D) G-actin and F-actin content in HL-60 SCR and MKL1 cells. E and F) Analysis of the F-actin content in dHL-60 after stimulation with 100nM fMLP for 1 minute in WT, MKL1 and MKL1 cells expressing mCherry-G-actin. * P < .05; ** P < .01.

**Figure 7: MKL1-deficient dHL-60 show impaired uropod retraction, associated with a downregulation of MYL9**
A) Knock down of MKL1 induces a uropod retraction defect. Differentiated HL-60 were let to adhere to fibronectin coated glass coverslips for 20 minutes, washed, then uniformly stimulated with fMLP for 2 minutes and finally fixed in 4% paraformaldehyde. Cells were then stained for F-actin and MLC phosphorylation (pMLC). The dHL-60 MKL1 F-actin staining intensity was increased 3 fold in the merged image. Images were taken with a 40x lens. B) Inhibition of myosin II interaction with F-actin using blebbistatin (100µM) induced a uropod retraction defect in dHL-60 wild-type cells. Images were taken with a 63x lens. Scale bars = 10 µm. C) Quantification of the frequency of uropod extension in dHL-60 MKL1 observed in A. Over 250 cells were analysed in each condition in two independent experiments. D) mRNA expression in HL-60 SCR and MKL1 cells analysed by quantitative RT-PCR. E and F) Myosin light chain expression was evaluated by western blot. *** P < .001.
Figure 2

A

Lymphoid

Myeloid

Control Patient

**MKL1**

GAPDH

***

0 20 40 60 80

Phalloidin fluorescence

0

Phalloidin fluorescence

B

CFSE (patient cells)

F-actin

Overlay

B

Myeloid

Phalloidin fluorescence

Lymphoid

Phalloidin fluorescence

C

80

70

60

50

40

30

20

10

0

MFI (phalloidin)

Control Patient

D

E

Control Patient

MKL1

GAPDH
Figure 4

A. Control and Patient spread cells (% of total).

B. Bar graph showing comparison of spread cells (% of total) between Control and Patient.

C. Images showing F-actin, Vinculin, and Overlay for Control and Patient.

D. Graph showing cells containing podosomes (% of total) for Control and Patient.

E. Western blot analysis showing MKL1 and GAPDH protein levels for SCR and MKL1 conditions.

F. Flow cytometry showing Phalloidin fluorescence for SCR and MKL1 conditions.

G. Bar graph showing spread cells (% of total) for SCR and MKL1 conditions.

H. Bar graph showing cells forming podosomes (% of total) for SCR and MKL1 conditions.

I. Images showing F-actin, Vinculin, and Overlay for SCR and MKL1 conditions.
Figure 5

A

B

C

D

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Figure 6

A. Phalloidin fluorescence

B. F-Actin (MFI)

C. F-Actin (MFI)

D. G-actin F-actin ratio

E. Phalloidin fluorescence

F. F-Actin (MFI)
Figure 7

**A** F-actin, pMLC, Overlay

**B**

<table>
<thead>
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<th>Count (%)</th>
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<td>MKL1</td>
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**C** F-actin, pMLC, Overlay

**D**

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**E**

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**F**

Relative expression

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References:
Immunodeficiency and severe susceptibility to bacterial infection associated with a loss-of-function homozygous mutation of MKL1

Julien Record, Dessislava Malinova, Helen L. Zenner, Vincent Plagnol, Karolin Nowak, Farhatullah Syed, Gerben Bouma, James Curtis, Kimberly Gilmour, Catherine Cale, Scott Hackett, Guillaume Charras, Dale Moulding, Sergey Nejentsev, Adrian J. Thrasher and Siobhan O. Burns