Dysferlin regulates cell adhesion in human monocytes

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Running title: dysferlin function in monocytes

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Keywords: Dysferlin, monocytes, LGMD2B, focal adhesion

Background: dysferlin mutations cause progressive muscular dystrophies with strong inflammation, yet its function in immune cells is unclear.

Results: dysferlin forms a protein complex with focal adhesion proteins and its loss in monocytes results in deregulated adhesion.

Conclusion: dysferlin is involved in regulating cellular interactions in human monocytes.

Significance: dysferlin dysfunction in monocytes may contribute to pathology in dysferlinopathy.

SUMMARY

Dysferlin is mutated in a group of muscular dystrophies commonly referred to as dysferlinopathies. It is highly expressed in skeletal muscle, where it is important for sarcolemmal maintenance. Recent studies show that dysferlin is also expressed in monocytes. Moreover, muscle of dysferlinopathy patients is characterized by massive immune cell infiltrates, and dysferlin negative monocytes were shown to be more aggressive and phagocytose more particles. This suggests that dysferlin deregulation in monocytes might contribute to disease progression, but the molecular mechanism is unclear. Here we show that dysferlin expression is increased with differentiation in human monocytes and the THP1 monocyte cell model. Freshly isolated monocytes of dysferlinopathy patients show deregulated expression of fibronectin and fibronectin binding integrins, which is recapitulated by transient knockdown of dysferlin in THP1 cells. Dysferlin forms a protein complex with these integrins at the cell membrane, and its depletion impairs cell adhesion. Moreover, patient macrophages show altered adhesion and motility. These findings suggest that dysferlin is involved in regulating cellular interactions and provide new insight into dysferlin function in inflammatory cells.

Mutations in the dysferlin gene (DYSF, MIM*603009) cause the progressive late-onset muscular dystrophies Limb Girdle Muscular Dystrophy (LGMD) 2B (MIM#253601) (1), Miyoshi Myopathy (MIM#254130) (2) and Distal Anterior...
Compartment Myopathy (MIM#606768) (3), collectively referred to as dysferlinopathies. All of them are characterized by an adult onset of muscle weakness, followed by progressive muscle wasting. The muscle tissue presents with strong inflammatory infiltrates (4), and for that reason the disease is often misdiagnosed for the immune disorder polymyositis. Dysferlin is a C2 domain containing transmembrane protein that is highly expressed in skeletal muscle (5) and activated satellite cells (6), and increases with differentiation. In addition it is expressed in monocytes (7,8).

Recent studies suggest that the immune response in dysferlin deficient tissue is compromised (9,10). In muscle, dysferlin deficiency causes reduced cytokine (9,10) and chemokine (11) secretion and a subsequent local misbalance in recruitment and persistence of immune cells (9). In dysferlin mouse models, neutrophils and macrophages appear later at the site of damage compared to controls in degeneration/regeneration experiments (9). Yet after their late appearance these cells reside longer in the muscle tissue, suggesting a prolonged inflammatory response of these cells (9). Roche et al (12) showed the existence of a strong immune component in dysferlin deficiency that contributes to its slower recovery from injury.

As dysferlin is strongly expressed in monocytes (7,8), it was suggested that modified monocyte behavior might contribute to the dysferlin phenotype. Indeed a recent study indicates that dysferlin deficient macrophages have a higher phagocytosis index and are thus more aggressive than wild-type cells (13). However, the mechanism behind this observation is unclear.

We previously characterized the dysferlin protein complex in muscle and observed that dysferlin is found in complex with focal adhesion components in skeletal muscle cells (14). Focal adhesions are cellular attachment sites where the internal cytoskeleton is connected to the extracellular matrix via integrins (15). Integrins are heterodimeric transmembrane receptors that consist of an α- and a β-subunit and are central to focal adhesions(15). According to the traditional view integrins link the extracellular matrix to the intracellular cytoskeleton (15,16). However, recent studies also indicated a role for integrins in cell-cell contacts (16). Because of dysferlin’s uncharacterized role near focal adhesions and the deregulated monocyte response in dysferlinopathy patients we investigated a potential function for dysferlin in monocyte cell adhesion. We observed a striking increase in macrophage motility in dysferlinopathy patients, in line with an altered adhesion propensity.

**EXPERIMENTAL PROCEDURES**

*Cell isolation and culture* - THP1 cells were grown at 37°C and 5% CO2 in RPMI medium supplemented with 1% L-glutamine and 10% FCS (all Gibco). PBMCs were isolated by a Ficoll gradient. CD3-positive T-cells, CD14-positive monocytes and CDx-positive B-cells were subsequently isolated with antibody-coated magnetic beads (Invitrogen) according to manufacturer’s protocol. Cells were counted and resuspended in RPMI medium for further experiments. Monocytes were *in vitro* differentiated according to protocol (17), using established cytokine cocktails. Differentiation was monitored by FACS analysis of reported surface markers (CD11b, or ITGAM).

*Antibodies and reagents* - THP1 cells were differentiated by the addition of PMA (sigma) at 20nM to the culture medium. Cells were differentiated for 3-5 days for further experiments. The following matrixes were used in this study: Fibronectin, rat collagen, poly L-lysine. Matrix compounds were dissolved 1:30 in PBS, incubated on plastic 6-well plates at 37°C for 2h, washed with PBS. Adhesion was monitored by light microscopy (bright field). The following antibodies were used in this study: MaDYSF (1:300 for western and immunofluorescence, hamlet, Novocastra), RaAHNAK (KIS, gift of dr. J. Baudier), MaITGB3 (1:10,000,
R&D), MaITGB1 (1;10,000, gift of WJ Pannekoek), GaPARVB (1;1,000, Santa Cruz), MaVINC (1;5,000 Sigma), MaACTN3 (1;5,000 Sigma).

Western blot detection - Cells were counted, dissolved in sample buffer and boiled for 10 min. Resulting protein homogenates were separated on SDS-page gels and transferred onto nitrocellulose (for AHNAK) or PVDF membranes. Protein loading was standardized to cell count and monitored with Ponceau S staining of the blot directly after transfer. After antibody detection blots were analyzed with an Odyssey scanner (Licor). Protein bands were quantified with ImageJ, and Odyssey analysis software.

RNA isolation and cDNA synthesis RNA was extracted with a RNA extraction kit (Macherey-Nagel) according to manufacturer’s protocol. Subsequent cDNA synthesis was performed with a kit and random hexamer primers (Fermentas) according to manufacturer’s instructions. 1 µg RNA was used as input for cDNA reactions.

Quantitative RT-PCR - All primers were designed with the webtool Primer3 (URL), with mispriming against human database. Quantitative PCR reactions were performed with SYBRgreen, in 15 µl reaction volume with 3 ng cDNA input. All primers had comparable efficiencies between 95% and 105%. All measurements were performed in triplo. Statistical analysis was performed according to the method Pfaffl(18). GAPDH was used as a reference gene.

DNA plasmids and transfection – The following shRNA plasmids out of the Sigma Mission shRNA library were used in this study: non-target SHC002, and Dysferlin TRCN0000000967. The Dysferlin shRNA was validated by co-expression with Dysferlin expression vector. 10^6 THP1 cells were washed in PBS and dissolved in nucleofactor buffer V. 0.5 µg DNA plasmid was added. Transfection was achieved by electroporation with an Amaxa Nucleofactor device. Transfected cells were resuspended in culture medium.

Immunostaining - Differentiated THP1 cells were fixed in formalin solution for 10 min, following by permeabilization in 0.3% Triton (Sigma) for 5 min. Cells were washed, blocked in 1% BSA (Sigma) for 10 min, and incubated with 1st antibody 2h RT. Cells were subsequently washed 3x and incubated with 2nd antibody for 1h RT. Cells were washed in PBS and mounted onto slides with AquaPolymount supplemented with DAPI. For the visualization of the very transient focal adhesions we used CSK buffer. THP1 cells were differentiated at coverslips for >3 days using PMA. Cells were fixed with fresh CSK buffer (0.3% Triton, 10 mM Pipes pH 7.0, 50 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 300 mM sucrose, 4% paraformaldehyde) for 15 min. This treatment washed out all of the soluble cell components but fixes the cytoskeletal parts including the focal adhesions. The coverslips were subsequently washed 2x in PBS and blocked with 1% BSA in PBS for >10 min. Next the coverslips were stained with the antibodies as mentioned above. For the endocytosis assays, cells were incubated for 1 h on ice with ITGB3 antibody at 1;10,000. Non-bound antibody was washed of with PBS, and fresh medium was added. Cells were cultured at 37°C for 1 h. Endocytosis was stopped by putting the cells on ice. Surface antibody was washed of with 0.2 mM glycine (pH 2.5). Next cells were prepared as otherwise. As a control, cells were analyzed directly after antibody incubation, confirming that all surface-bound antibody could be washed of by the glycine treatment (not shown). To inhibit the Integrins differentiated THP1 cells were incubated in 1 µg/ml GRGDS (Sigma), or a control peptide (SDGRG, Sigma). For the ITGB3 stimulation cells were incubated with ITGB3 antibody at 37°C.

Immunoprecipitation - To obtain total cell lysates differentiated THP1 cells were lysed by scraping in triton buffer (50 mM TrisHCl, pH 7.5, 150 mM NaCl, 0.2% Triton X100, 1x protease inhibitor cocktail (Roche)) after a PBS wash. Cultured cells were prepared freshly by washing in PBS.
and lysed by scraping on ice in lysis buffer. All homogenates were spun down at maximum speed, 4°C, 20 min. To generate subcellular fractions, differentiated THP1 cells were trypsinized and washed with PBS. The pellet was lysed in sucrose buffer (320 mM Sucrose, 5 mM HEPES (pH 7.4), 5 mM EDTA, 2x Protease inhibitor cocktail) with a dounce homogenizer. Lysates were spun down subsequently at 1,000g (P1, containing insoluble debris), 10,000g (P2, containing mitochondria and peroxisomes) and 100,000g (P3, enriched for cell membrane, endoplasmic reticulum and vesicles (microsomes)). Pellet fractions were dissolved in triton buffer for further immunoprecipitations. Protein A sepharose CL-4B (GE Healthcare) was washed 3x in lysis buffer and used to preclear the homogenates for 1h, at 4°C tumbling. Sepharose was removed and antibody added (50 µg HCAb) for O/N incubation at 4°C, tumbling. Thereafter washed sepharose was added and incubated for 2h, 4°C, tumbling. Homogenates were spun down at 500g and supernatant stored as non-bound fraction. The sepharose was washed 5x 3x short, 2x long (>20min tumbling at 4°C). Finally, all fluid was removed and protein eluted by boiling in sample buffer.

**RESULTS**

**Dysferlin expression is enhanced in differentiating monocytes.** Previous reports show that dysferlin is expressed in monocytes and macrophages (7,8,13). To confirm this observation we purified B cells, T cells, and monocytes from freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors and investigated dysferlin protein levels on western blot. Dysferlin is expressed only in monocytes, conform prior observations, while its interaction partner AHNAK (19) is expressed in all three cell types (Figure 1A). We next differentiated freshly isolated monocytes in vitro to a pro-inflammatory (M1) or contra-inflammatory (M2) phenotype, and again tested for dysferlin and AHNAK expression on western blot (Figure 1B). Both proteins are increased with differentiation in both M1 and M2 macrophages. Interestingly, the monocyte-like leukemic THP1 cell model shows a similar increase in dysferlin and AHNAK upon differentiation (Figure 1B). Finally we measured RNA expression levels of dysferlin and AHNAK in THP1 cells (Figures 1C and 1D). Again, we found a strong upregulation of dysferlin. AHNAK
however, is upregulated to a much smaller extent suggesting that the increase in AHNAK protein levels is achieved through a different mechanism than that for dysferlin. We conclude that dysferlin and AHNAK are increased with monocyte differentiation.

**Dysferlin expression is insensitive to cell-matrix contact formation.** In skeletal muscle, dysferlin functions as a calcium-sensitive membrane repair protein (20). In a THP1 cell scratch-wounding assay dysferlin displayed a calcium dependent recruitment to the lesion site (data not shown), indicating that also in monocytes dysferlin participate in calcium-sensitive membrane repair. However, it is not likely that monocytes suffer from frequent membrane damage as do myofibers, challenging the biological relevance of the membrane repair function of dysferlin in monocytes. We therefore explored potential additional functions of dysferlin. We and others previously reported that dysferlin forms a complex with focal adhesion components, which are important in cell-cell and cell-matrix contacts (14,21). Monocytes require interactions with the extracellular matrix and surrounding cells to exert their function. THP1 cells are non-adherent prior to differentiation and become adherent upon differentiation. We therefore investigated whether dysferlin and AHNAK expression levels are sensitive to cell adherence. We first measured the mRNA expression levels of various matrix and adherence proteins in differentiating THP1 cells (Figure 2A). This showed dysferlin expression to be concomitantly upregulated with fibronectin and integrins α5, αV, β1 and β3. Interestingly, the integrins α5β1 and αVβ3 have fibronectin binding capacity. We could not detect upregulated expression of other matrix proteins such as laminin (Figure 2A). This suggests that the THP1 cells adhere to fibronectin. The same genes were upregulated in *in vitro* differentiated M1 and M2 macrophages (Figure 2B). We proceeded to test THP1 cell adhesion on protein matrixes. We coated wells with PBS (mock), poly L-lysine, collagen or fibronectin. Poly L-lysine is positively charged and attracts all cells to the surface without adherence, while fibronectin and collagen are matrix proteins. Figure 2C shows that on plastic surfaces dysferlin and AHNAK proteins are only increased upon the induction of differentiation. A similar effect is seen for poly L-lysine. Fibronectin however increases AHNAK protein levels, yet not dysferlin, even in the absence of differentiation. Indeed the cells adhere onto a fibronectin matrix without achieving the spread-out differentiated phenotype (not shown). Dysferlin again only increases upon differentiation. Finally, the collagen matrix has no strong effect on either protein unless the cells were differentiated. We conclude that dysferlin expression does not respond to cell-matrix adhesion, contrary to AHNAK, which is sensitive to fibronectin adherence.

**Differentiation induced dysferlin expression is enhanced in response to cell-cell contact formation.** We next investigated dysferlin and AHNAK expression in response to cell-cell contacts. We differentiated THP1 cells at increasing cell densities, predicting that this would result in an increased frequency of cell-cell contact formation. Surprisingly, the differentiation-induced dysferlin mRNA expression levels are further increased with increasing cell density, suggesting a positive response to cell-cell contact formation (Figure 3). The integrins ITGA5, ITGAV, ITGB1 and ITGB3 show the opposite pattern, with expression levels dropping with increasing cell density (Figure 3A). Nevertheless, apart from their function in cell-matrix contacts, integrins are also important for cell-cell contacts (16,22). The dysferlin interaction partner AHNAK shows an increase in expression similar to dysferlin but followed by a sudden drop at the highest cell density tested. It is not clear why this happens, but given its function in cell interactions(23,24) its increase may be a response to the cell’s loss in integrin expression. To verify that dysferlin protein is involved in cell-cell contacts we performed immunofluorescent staining...
experiments on differentiated THP1 cells. Dysferlin strongly accumulates at cell-cell contacts (Figure 3B), together with ITGB3. Intriguingly, the proteins AHNAK and ITGB1 are also strongly present at cell-cell contacts (not shown), although their RNA expression levels are insensitive to cell density. This indicates that dysferlin plays a role at cell-cell contacts.

Loss of dysferlin protein results in deregulation of integrin expression. To obtain more evidence that dysferlin is important for cell adhesion, we transfected THP1 cells with an shRNA plasmid to knock down dysferlin (Figure 4A+B). We confirmed a substantial reduction of dysferlin protein levels by western blotting (Figure 4B). We measured RNA (Figure 4A) and protein (Figure 4B) expression levels during differentiation and observed that ITGB1 and ITGB3 and fibronectin are much stronger upregulated in the dysferlin depleted cells, while other markers such as CD11b (ITGAM, or integrin αM) appear unaffected. This shows that integrin mRNA expression is affected by the absence of dysferlin, which is suggestive of a change in cell adhesion properties. We next measured RNA expression levels in a confirmed patient with LGMD2B and a matched control, and observed a strikingly similar deregulation of fibronectin and the fibronectin binding integrins in the patient cells (Figure 4C). Intriguingly though, in the patient we did not detect an increase in ITGB3, and a decrease in ITGAM. Based on these markers the patients cells resemble more the M2 macrophages as shown in Figure 2B. Finally, we performed cell counts at 4 days post-differentiation of mock-transfected and dysferlin depleted THP1 cells. In wild-type cells, most of the cells adhere to the surface after four days of differentiation. Transfection with a non-target shRNA plasmid results in a slight decrease in adherent cells, probably due to the transfection procedure. However, depletion of dysferlin results in a strong reduction in cell adhesion (Figure 4E). We stained shRNA transfected THP1 cells for surface ITGB3. Again, this showed a clear loss of adherent cells, but no difference in surface ITGB3 could be detected between dysferlin depleted and control cells, in the adherent cells. This suggests that the localization of integrins is unaffected by dysferlin depletion, but leaves open the possibility of altered dynamics. We conclude that dysferlin is involved in Integrin mediated THP1 monocyte cell adhesion.

Dysferlin can form a complex with focal adhesion components. In skeletal muscle cells, dysferlin forms a complex with vinculin and associated focal adhesion proteins (14). In order to test whether dysferlin is physically associated with adhesion complexes we performed immunoprecipitation experiments on protein homogenates from differentiated THP1 cells. In addition to total homogenates we also analyzed cellular subfractions enriched for organelles or microsomes (Figure 5). We used two independent dysferlin-specific heavy chain antibody fragments (VHH) and one non-specific VHH, as described previously (14,25) and analyzed co-immunoprecipitation by western blot. Dysferlin is found in all fractions. Moreover, ITGB3, vinculin, paxillin and β-parvin co-immunoprecipitate with dysferlin from the microsomal fraction, suggesting that as in muscle cells, dysferlin forms a complex with a subset of focal adhesions in THP1 cells. ITGB1 does not co-immunoprecipitate with dysferlin. It should be noted that in the total cell extract no detectable amount of ITGB3 coimmunoprecipitates with dysferlin suggesting that the interaction takes place at the focal adhesion only. Also we observe that the amount of protein that is immunoprecipitated with Dysferlin is modest, suggesting that the interactions are either transient, or concern only a subset of the proteins. To further substantiate the evidence for dysferlin near focal adhesions we performed co-immunofluorescence experiments with an antibody against the focal adhesion protein vinculin (Figure 5C). Focal adhesions have a rapid turnover.
Therefore we fixed the cells with the stringent CSK buffer. This allowed for detection of vinculin in focal adhesions. Dysferlin partially colocalizes with vinculin at these focal adhesions. This modest colocalization might suggest that the interaction is transient in nature. We conclude that dysferlin can form a protein complex with focal adhesion components in monocytes.

**Dysferlin endocytosis in response to Integrin stimulation.** A recent study by Sharma et al (26) suggested a role for Dysferlin in the trafficking of the adhesion molecule PECAM in endothelium. We therefore studied the trafficking of dysferlin and ITGB3 in THP1 cells with co-immunofluorescence staining. We observed that in resting differentiated THP1 cells dysferlin and ITGB1 or ITGB3 do not strongly colocalize (Figure 6A). This confirms the IP data on whole cell lysates and supports the hypothesis that both proteins are together only at the cell membrane or on endocytic vesicles. We therefore decided to stimulate integrin endocytosis by incubating differentiated THP1 cells with small peptide (GRGDS) containing the matrix binding site for integrin (RGD). This peptide competes for the matrix-binding site. Upon binding the peptide the integrin is endocytosed (Figure 6B) and it is replaced by exocytosis of integrin molecules from an intracellular storage. We used a low concentration that removed the focal adhesions in the cell protrusions (stained by vinculin in Figure 6B), but prevented the cells from rounding up and detaching, and we stained the cells for dysferlin and ITGB3. Both proteins show a rapid response to GRGDS, and accumulate in a perinuclear compartment (Figure 6A). This recruitment is specific for dysferlin and ITGB3, as subsequent costaining for dysferlin and AHNAK showed the latter to shift towards the cell periphery (Figure 6C).

Many integrin heterodimers have the potential to bind to an RGD peptide motif (27), and all these heterodimers might be inhibited by the GRGDS treatment. However, our data thus far suggested that mainly ITGB3 is functionally linked to dysferlin. To test this we incubated cells in the presence of a monoclonal antibody specific for the extracellular part of ITGB3. This resulted in a similar recruitment of both ITGB3 and dysferlin (Figure 6D), suggesting that the GRGDS induced trafficking of dysferlin is largely caused by ITGB3 inhibition.

To prove that the integrins undergo endocytosis upon GRGDS stimulation we incubated differentiated THP1 cells with the integrin β3 antibody on ice, prior to GRGDS stimulation. Subsequent stimulation with GRGDS at 37°C resulted in intracellular ITGB3 antibody, strongly indicating that the integrins are indeed endocytosed in response to the GRGDS treatment (Figure 6E). As there is no antibody available that binds to the extracellular domain of dysferlin it is technically not feasible to confirm this in a similar experimental setup for dysferlin. We conclude that dysferlin is co-endocytosed with ITGB3.

**Dysferlinopathy macrophages move faster.** Our data thus far suggest that dysferlin locates to monocyte cell adhesion sites, and that it is involved in cell interactions. We therefore hypothesized that cell adhesion might be disturbed in monocytes of dysferlinopathy patients. To test this we isolated and cultured human primary macrophages from five molecularly confirmed LGMD2B patients, and five healthy matched control donors. All isolated cells stained positive for the marker CD68, confirming the purity of our cultures (Figure 7A). We performed live cell microscopy to monitor the cells and record their movements. Strikingly, we observed that dysferlinopathy macrophages are more motile than their healthy counterparts (Figure 7B, C). Specifically, dysferlinopathy macrophages covered more distance and had a 60% increase in average velocity (13.85µm/h compared to 8.71µm/h for controls, with p=0.00009). These data are consistent with the reduced adhesion in...
dysferlin depleted THP1 cells, and with a role for dysferlin in regulation of cellular interactions.

**DISCUSSION**

We report that dysferlin expression is upregulated in differentiating monocytes and the THP1 monocyte cell model. Dysferlin localizes to the cell membrane of monocytes and is rapidly endocytosed with ITGB3 upon inhibition of this integrin. Dysferlin forms a complex with ITGB3 and focal adhesion components at the cell periphery. Moreover, our data indicate that in the absence of dysferlin the regulation of fibronectin binding integrins is disturbed. Dysferlin depletion further enhances the increase in ITGB3 expression. This results in attenuated differentiation and adhesion of these cells, which mimics the loss of adhesion observed in dysferlin depleted HUVEC cells(26). In dysferlinopathy macrophages this reveals itself as increased cell motility. Interestingly, monocytes expressing ITGB3 show increased motility (28) compared to cells without ITGB3.

During the recruitment of mononuclear cells to inflamed tissue, these cells first need to adhere to the endothelial cell and start the process of rolling. They subsequently transmigrate through the endothelial cell layer. And finally, they migrate through the tissue to participate in phagocytosis of apoptotic cells. The attachment of circulating monocytes to endothelial cells is in part mediated by integrins that bind to endothelial VCAM molecules and thereby induce tight interaction between the two cell types(29). Such cell-cell interactions generally increase the levels of integrin expression (15) by positive feedback, thereby gradually increasing adherence to facilitate subsequent transmigration.

Our data suggest that dysferlin-depleted THP1 cells adhere less efficiently, but nevertheless increase expression of integrins. Integrins can be activated by outside-in and by inside-out signaling. Outside-in signaling refers to the process where integrins are activated through adhesion and trigger signaling cascades in the cells. Inside-out signaling denotes relocalization of integrins during for instance motility to provide direction (15). Via outside-in signaling, integrins can affect Erk, JNK and PKB and thereby affect gene expression(15). Moreover, cells have numerous feedback mechanisms that link expression of different adhesion proteins. For example, monocyte cell adhesion results in increase levels of fibronectin (30). Moreover, such feedback loops can be negative, as integrin αVβ6 can compensate for the loss of ITGB1 (31,32). Therefore the increased integrin mRNA expression in the absence of dysferlin may be explained as a compensatory mechanism. In muscle similar negative feedback loops have been described for dysferlin. Dysferlin deficient muscle cells produce increased amounts of IL1β (10) and thrombospondin (11) suggesting that dysferlin inhibits expression of these proteins.

When comparing the mRNA expression levels in dysferlinopathy patient monocytes with dysferlin depleted THP1 cells, the deregulated expression of ITGB1 is consistent, contrary to ITGB3. However, cross-talk between integrins is common and has been reported between ITGB1 and ITGB3 in monocytes. ITGB1 (α5β1) is regulated by ITGB3 (αVβ3) in phagocytosis experiments (33). Crosstalk between αVβ3 and α4β1 regulates monocyte migration on VCAM1 (34). Such crosstalk might explain the apparent discrepancy of the mRNA expression data.

We observed that dysferlin and ITGB3 trafficking is co-regulated. This is consistent with dysferlin’s role in the transport of the adhesion protein PECAM in endothelium(26). Moreover, the fact that focal adhesion components are identified in the dysferlin protein complex in both monocytes and myoblasts suggest parallels in dysferlin function between these cells. Indeed, the process of phagocytosis has been implicated to be analogous to endocytosis, and both processes require coordinated restructuring of cell membrane and the cortical actin based cytoskeleton.
Interestingly, ITGB1 and ITGB3 are involved in phagocytosis of both apoptotic cells and fibronectin coated beads by monocytes (35). Moreover, fibronectin is used by monocytes for opsonization and enables ITGB1 dependent phagocytosis (36). This suggests that the disturbed LGMD2B monocyte phagocytosis behavior might be explained by a specific subset of fibronectin binding integrins. An intriguing model would be that dysferlin deficient monocytes show increased integrin endocytosis. Given the parallels between endocytosis and phagocytosis, this might translate into the increased phagocytotic behavior that was shown in LGMD2B monocytes (13). It is tempting to hypothesize that the integrin deregulation also results in a modified phagocytotic response. This would open the way for a potential immunomodulatory treatment of dysferlinopathy, and such immunotherapies aimed at integrins are currently being investigated for other diseases (37,38).

Though recent studies provided evidence that the cause of the pathology in dysferlin deficient mouse models is intrinsic to the skeletal muscle tissue (39,40) the role of the immune component was unclear. It was suggested that the inflammation aggravates the disease (39). A defect in monocytes due to Dysferlin deficiency could result in a misappropriate immune response, and thus aggravate the muscle disease. Chiu et al reported that in dysferlin deficient mouse muscle tissue, monocytes arrive later after notoxin induced myofiber damage (9). This was explained by a deregulated communication from the myofibers to recruit the monocytes (9). Our data however provide an additional explanation. In the absence of dysferlin the monocytes and macrophages show strongly reduced adhesion and increased motility, which might influence the infiltration.

In summary, we have identified a potential involvement of dysferlin in cellular adhesion and thereby a potential mechanism for the deregulation of dysferlin deficient immune cells.

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Acknowledgements - We thank dr. J. Baudier for the KIS AHNAK antibody and dr. W.J. Pannekoek for the ITGB1 and Paxilin antibodies and for help with the focal adhesion stainings.

**FOOTNOTES**

This study was funded by the Jain Foundation.

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**FIGURE LEGENDS**

**Figure 1:** Dysferlin is expressed in monocytes and THP1 cells, and increases with differentiation. A) Fresh PBMCs were isolated from a healthy donor. CD3-positive T-cells, CD14-positive monocytes and CD19-positive B-cells (right panel) were subsequently isolated from this cell population. Cells were analyzed freshly, or cultured for 24h in culture medium, in the presence or absence of T-cell activation aCD3 antibody, to investigate the effect on protein expression. Cells were dissolved in sample buffer and analyzed on western blot for AHNAK and dysferlin levels. Arrows denote the protein bands. For the dysferlin-negative B-cells two different donors are shown. B) Monocytes were differentiated in vitro to M1 and M2 macrophages and analyzed on western blot for AHNAK and dysferlin. Less protein was loaded compared to the previous panel. Additionally, the THP1 cell model was differentiated to a macrophage-like phenotype and similarly analyzed. Arrows denote the protein bands. C) Quantitative RT-PCR was performed on differentiating THP1 cells to confirm the increase in expression on RNA levels. Uncorrected Ct-values confirm the increased expression of dysferlin in differentiating THP1 cells. Two different primer sets were used to measure dysferlin mRNA. Both the small and the long isoform of AHNAK were assayed(41). D) Ct-values were standardized to GAPDH. Relative expression levels are shown. Error bars reflect relative standard deviation of technical variation.

**Figure 2:** Dysferlin expression is insensitive to cell adhesion. A+B) Quantitative RT-PCR shows selective increased expression of fibronectin and fibronectin-binding integrins upon differentiation of THP1 cells (A) and in vitro differentiated macrophages (B). Values were standardized to GAPDH. In B) monocyte expression levels were set to 1. C) THP1 cells were
seeded on different protein matrixes and dysferlin protein expression was quantified by western blot. Dysferlin increases only upon differentiation (induced by PMA addition). AHNAK increases in response to fibronectin adhesion. Arrows denote the AHNAK and dysferlin protein bands.

**Figure 3:** Dysferlin expression is sensitive to cell-cell contact formation. A) THP1 cells were differentiated at different cell densities, and after three days RNA expression was determined by quantitative RT-PCR. Values were standardized to GAPDH. B) Differentiated THP1 cells were stained for dysferlin (green) and ITGb3 (red). Nuclei are stained in blue (DAPI). Dysferlin and ITGB3 accumulate in between the two nuclei.

**Figure 4:** Dysferlin depletion results in deregulated Integrin expression and decreased cell adhesion. A) transient shRNA mediated knockdown of dysferlin further upregulates upon induction of differentiation the mRNA expression levels of fibronectin and fibronectin binding integrins. B) A concomitant western blot shows only a minor increase in protein level. C) Freshly isolated monocytes from an LGMD2B patient and a healthy matched control were analyzed for mRNA expression levels. D+E) THP1 cells were transfected with a dysferlin targeting shRNA plasmid, a non-target shRNA plasmid, or mock and differentiated for four days. After four days cells adherent cells were counted (D) and stained for surface ITGB3 (E). In the absence of dysferlin cell adhesion is strongly reduced. There is no difference in the level of surface ITGB3 in the adherent cells.

**Figure 5:** ITGb3 and focal adhesion components co-immunoprecipitate with Dysferlin. Differentiated THP1 cells were lysed in a sucrose buffer, fractionated and subjected to an immunoprecipitation (IP) protocol with HCAb against dysferlin (F4 and H7) or non-specific control HCAb (3A). Bound (B) and non-bound (NB) fractions were analyzed on western blot for dysferlin and Focal complex proteins. A) P1 (1,000g) contains the insoluble fraction, P2 (10,000g) is enriched for heavy organelles such as mitochondria and nuclei. P3 (100,000g) is enriched for microsomes and contains cell membrane and vesicles. B) IP fractions were analyzed for dysferlin and ITGB3 levels (upper blot), ITGB1, ACTN3, PAX, VINC and the described interaction partner PARVB (lower blot). Contrary to ITGB1, ITGB3 co-immunoprecipitates from the P3 fraction, containing microsomes (H7 Bound). This same IP sample also contains ACTN3, PAX, and PARVB, suggesting complete focal complexes co-immunoprecipitate with dysferlin. C) Co-immunostaining of the focal adhesion protein vinculin with dysferlin. Two representative cells are shown. The cell in the lower panels is completely removed by the CSK buffer and only the focal adhesions remain. The cell in the upper panels has some membraneous and cytoskeletal parts remaining. The arrows point to the enhanced part.

**Figure 6:** Dysferlin and integrin β3 (ITGB3) are endocytosed in response to the Integrin inhibiting peptide RGD. A) Differentiated THP1 cells were incubated 30 min with an integrin binding peptide (RGD). Cells were stained for dysferlin and ITGB3. Prior to RGD stimulus there is no apparent co-localization between dysferlin and ITGB3. Both proteins show a dotted intracellular localization reminiscent of endosomes. Upon stimulation both proteins accumulate in an intracellular compartment. B) A schematic model of integrin trafficking and RGD stimulus. RGD prevent the integrins from binding to the extracellular matrix and thereby stimulate endocytosis. C) As in A) but now the cells were stained for dysferlin and AHNAK. Only dysferlin is recruited intracellularly. In the control situation cells were incubated with a control peptide containing the inverse amino acid sequence (SDGRG), which does not bind integrins. D) Differentiated THP1 cells were incubated with a monoclonal MaITGB3 antibody and stained for dysferlin and ITGB3. Both proteins are recruited to a perinuclear compartment. E) Integrin β3 is endocytosed upon RGD stimulus. Differentiated THP1 cells were incubated with MaITGB3 at
4°C for 1h. Cells were washed and incubated 30 min at 37°C with RGD. Surface-bound antibody was removed with glycine and cells were stained for internalized ITGB3 and dysferlin. Both proteins accumulate at the perinuclear storage compartment, indicating that ITGB3 is endocytosed.

**Figure 7:** LGMD2B monocytes move faster than control cells.
A) Macrophage culture stained with DAPI and the macrophage marker CD68. B) Graphic representation of average velocity and standard deviation in µm/h of healthy macrophages (controls) and dysferlin-null macrophages (patients) (p=0.00009). C) Pictures of the 18h tracking drawn by control macrophages (a) and dysferlin-null macrophages (b).
Figure 1

A

|          | PBMC | T-Cells | Monocytes |
|----------|------|---------|-----------|
| 0h       |      |         |           |
| 24h      |      |         |           |
| 24h CD3  |      |         |           |
| 0h 24h lps |    |         |           |

B

|          | Monocytes | THP1 Cells |
|----------|-----------|------------|
| #1       |           |            |
| #2       |           |            |
| M1       |           |            |
| M2 day0  |           |            |
| 1        |           |            |
| 2        |           |            |

C

THP1 differentiation

D

THP1 differentiation

Relative abundance (log)

Day0

Day1

Day2

Dysferlin_1

Dysferlin_2

L-AHNAK

S-AHNAK

PBMC T-Cells Monocytes

B-Cells donor1 B-Cells donor2

24h     24h 0h 24h lps

THP1 differentiation

Dysferlin

AHNAK

B-Cells donor2

0h 0h 0h CD3

M2 day0 1 2

THP1 differentiation

Relative abundance (log)

Day0

Day1

Day2

Dysferlin_1

Dysferlin_2

L-AHNAK

S-AHNAK

Figure 1

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Figure 2

A

B

C

PBS  Fibronectin  Fibronectin  Collagen  Collagen  Lysine  Lysine

PMA  PMA  PMA

Day0  Day1  Day3  Day0  Day1  Day3  Day1  Day3  Day0  Day1  Day3  Day1  Day3

250 kDa  120 kDa  250 kDa  120 kDa

ITGAM  FN  ITGA5  ITGAV  ITGB3  FN  LAMB  ELN  DYSF  L-AHNAK  S-AHNAK  AHNAK2

Day 0  Day 3

ITGAM  FN  ITGA5  ITGAV  ITGB1  ITGB3  L-AHNAK  S-AHNAK  AHNAK2

0 1 10 100 1000

0,1

0,1

0,1 1 10 100 1000

Relative abundance (log)

Relative abundance (log)

Relative abundance (log)

Loading control

Quantification

AHNAK  Dysferlin

Relative abundance

Relative abundance

Relative abundance
Figure 3

A

B

Dysferlin ITGB3

DAPI Merge

10^4

10^5

10^6

10^7

ITGAM FN ITGA5 ITGAV ITGB1 ITGB3 Dysferlin L-AHNAK S-AHNAK AHNAK2

0

2

4

6

8

10

10

30

50

70

90

110

140

170

200

Relative expression

Dysferlin

ITGB3

DAPI

Merge
Figure 4

A

B

C

D

E

Figure 4
**Figure 5**

**A**
- **P0 Total cell lysate**
  - **S1** P1 debris
  - **S2** P2 organelles
  - **S3** P3 microsomes cytosol

**Epitopes of Dysferlin Affinity Binders**
- **F4** VHH aDYSF
- **H7** VHH aDYSF
- **3A** VHH ctrl
- Western detection

**B**

|     | P1 | P2 | P3 |
|-----|----|----|----|
|     | F4 | H7 | 3A | F4 | 3A | H7 | F4 |
| NB  | B  | NB | B  | NB | B  | NB | B  |

- **250 kDa**
- **120 kDa**
- **100 kDa**
- **75 kDa**
- **55 kDa**
- **250 kDa**
- **55 kDa**
- **34 kDa**

+ **P0H73AF4NB B NB B NB B H73AF4**

- **DYSF**
- **ITGB3**
- **ITGB1**
- **ACTN3**
- **PAX**
- **VINC**
- **PARVB**

**C**

- **vinculin**
- **dysferlin**
- **merge**

- **cell 1**
- **cell 2**
Figure 7

A

B

C

Control Patient

p=0.00009
Dysferlin regulates cell adhesion in human monocytes
Antoine de Morree, Barbara Flix, Ivana Bagaric, Jun Wang, Marlinde van den Boogaard, Laure Grand Moursel, Rune R. Frants, Isabel Illa, Eduard Gallardo, Rene Toes and Silvere M. van der Maarel

*J. Biol. Chem.* published online April 4, 2013

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