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Research Article

Aberrant Adenosine Triphosphate Release and Impairment of P2Y2-Mediated Signaling in Sarcoglycanopathies

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ABSTRACT

Sarcoglycanopathies, limb-girdle muscular dystrophies (LGMD) caused by genetic loss-of-function of the membrane proteins sarcoglycans (SGs), are characterized by progressive degeneration of skeletal muscle. In these disorders, muscle necrosis is associated with immune–mediated damage, whose triggering and perpetuating molecular mechanisms are not fully elucidated yet. Extracellular adenosine triphosphate (eATP) seems to represent a crucial factor, with eATP activating purinergic receptors. Indeed, in vivo blockade of the eATP/P2X7 purinergic pathway ameliorated muscle disease progression. P2X7 inhibition improved the dystrophic process by restraining the activity of P2X7 receptors on immune cells. Whether P2X7 blockade can display a direct action on muscle cells is not known yet. In this study, we investigated eATP effects in primary cultures of myoblasts isolated from patients with LGMDR3 (α-sarcoglycanopathy) and in immortalized cells isolated from a patient with LGMDR5 (γ-sarcoglycanopathy). Our results demonstrated that, owing to a reduced ecto-ATPase activity and/or an enhanced release of ATP, patient cells are exposed to increased juxtamembrane concentrations of eATP and display a higher susceptivity to eATP signals. The purinoceptor P2Y2, which proved to be overexpressed in patient cells, was identified as a pivotal receptor responsible for the enhanced ATP-induced or UTP-induced Ca^{2+} increase in affected myoblasts. Moreover, P2Y2 stimulation in LDMDR3 muscle cells induced chemotaxis of immune cells and release of interleukin-8. In conclusion, a higher eATP concentration and sensitivity in primary human muscle cells carrying different α-SG or γ-SG loss-of-function mutations indicate that eATP/P2Y2 is an enhanced signaling axis in cells from patients with α-/γ-sarcoglycanopathy. Understanding the basis of the innate immune–mediated damage associated with the dystrophic process may be critical in overcoming the immunologic hurdles associated with emerging gene therapies for these disorders.

Introduction

Sarcoglycanopathies include 4 subtypes of autosomal recessive limb-girdle muscular dystrophies (LGMDR3, LGMDR4, LGMDR5, and LGMDR6) that are caused by the genetic loss-of-function of α-,

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β-, γ- or δ-sarcoglycans (SGs), respectively, and are characterized by progressive degeneration of skeletal muscle with loss of ambulation, respiratory insufficiency, and often premature death in the early 20-30s.[1,2] Currently, no treatment is available. Gene therapy/gene editing approaches have achieved relevant progress.[3-8] Inhibition of the endoplasmic reticulum quality control system or of the ubiquitin-proteasome pathway can rescue specific *SGCA* mutations.[9-11] Small molecules belonging to the class of cystic fibrosis transmembrane regulator correctors have been proposed for recovering the α-SG R98H mutant, defective in folding and trafficking.[12]

In LGMDR3-6, the mechanically weaker plasma membrane is damaged during contraction, allowing the release of intracellular antigens, infiltration of immune cells, and connective tissue replacement.[2,13] However, the molecular mechanisms that initiate and perpetuate the immune reaction in these disorders have not been thoroughly investigated yet. Priming of an inflammatory response in the absence of infectious agents is due to the so-called damage-associated molecular pattern molecules (DAMPs), including the release of intracellular ATP, primarily known for its function as an energy source.[14] When acting as DAMP, extracellular ATP (eATP) is an ideal harbinger of tissue damage. Indeed, adenosine triphosphate (ATP) is available in high concentrations within the cytoplasm of every cell and is quickly released after cell damage: eATP activates the purinergic receptors P2,[15] further divided into the subfamily of metabotropic (P2Y, evoking Ca^{2+} release from intracellular stores) and ionotropic (P2X, evoking Ca^{2+} influx from the extracellular space) receptors.[16,17] Muscle cells express both P2X and P2Y receptors and other key components of the inflammasome pathway, supporting a direct role in the development and maintenance of the inflammatory response.[18] Overexpression of *P2RX7* was immunodetected in skeletal muscle from *mdx* mice, a spontaneous model of Duchenne muscular dystrophy (DMD) and primary human myoblasts from DMD patients. Exposure to eATP in these primary cells triggers a strong and sustained increase in cytoplasmic Ca^{2+} levels.[19-21] In addition, substantially increased activity of P2Y2 was identified in primary *mdx* myoblasts stimulated with ATP or Uridine-5′-triphosphate (UTP), contributing to altered calcium signaling.[22]

In *mdx* mice, blockade of eATP/P2X purinergic signaling delayed the progression of the dystrophic phenotype, dampening the local inflammatory response.[23,24] In α-SG knockout mice (Sgca model), the use of a selective P2X7 antagonist (A438079) ameliorated clinical parameters, muscle fiber size variability, and inflammation. A438079 significantly decreased innate immune cells and upregulated the immunosuppressive regulatory T-cell subpopulation, infiltrating the dystrophic muscles.[25]

In LGMDR3, eATP effects may be further amplified: α-SG extracellular domain binds eATP and displays an ecto-ATPase activity, thus controlling eATP concentration at the cell membrane. Indeed, satellite cells isolated from skeletal muscles of Sgca mice, and differentiated to myotubes, are characterized by decreased ecto-ATPase activity when compared with wild-type cells.[26] However, eATP direct effects on muscle cells are still unknown.

We hypothesized that the reduced ecto-ATPase activation in human α-SG deficiency, and in other sarcoglycanopathies, linked to a secondary disruption of the whole SG complex,[26] might affect the magnitude and/or the duration of eATP-induced signals and modulate the activity of purinergic receptors in muscle cells. Thus, we compared ecto-ATPase activity, levels of eATP release, and response to eATP in primary cultures of myoblasts isolated from patients with LGMDR3 or from control subjects and in immortalized cells isolated from a patient with LGMDR5 (γ-sarcoglycanopathy) and a healthy control.

Materials and Methods

Cell Culture and Differentiation

Immortalized human myoblasts were isolated from the paravertebral muscle of a 16-year-old girl with γ-sarcoglycanopathy (homozygous mutation c.del525T in the γ-SG human gene [*SGCG*]) and of a 13-year-old healthy girl. Cells were immortalized by human telomerase reverse transcriptase and cyclin-dependent kinase 4 and cloned to get a pure myoblast immortalized cell line, as in a previous study.[27] After immortalization, the cells were expanded in Skeletal Muscle Cell Growth Medium Kit (PromoCell GmbH).

Primary myoblasts from 4 genetically determined patients with LGMDR3 and 4 healthy controls were isolated from the muscle biopsy conducted on the muscle vastus lateralis for diagnostic purposes (Supplementary Fig. S1). In the bioptic sample, fibrotic and fatty tissue were carefully removed, and the fragment was minced into small pieces. The explants were incubated for 24 hours in a conditioning medium at 37 °C. Then, the tissue fragments were seeded and kept at 37 °C for 15 days on 1% gelatin-coated Petri dishes in Full Aneural medium (proliferation medium; Dulbecco's Modified Eagle's Medium High Glucose containing 15% fetal bovine serum [EuroClone]; 20.2% M199 [Lonza]; 1% insulin solution of 1 mg/mL [Sigma-Aldrich]; 1% L-glutamine [EuroClone]; 10 ng/mL of recombinant human epidermal growth factor [Life Technologies]; 25 ng/mL of recombinant human fibroblast growth factor-basic [Life Technologies]; 1% penicillin-streptomycin solution [100×; EuroClone]; 1% amphotericin solution [100× EuroClone]), to allow attachment of the tissues to the plate surface and subsequent migration of cells. Next, cells were split with trypsin-EDTA (1× in phosphate-buffered saline; EuroClone) and enriched by positive selection of myoblasts, using MACS anti-CD56-conjugated magnetic microbeads and LS columns according to the manufacturer's instructions (Miltenyi Biotec).

The purity of the myoblast cell culture was checked through immunofluorescence staining with an antidesmin monoclonal antibody (1:500; Rb mAb to DesminY66, 32362 Abcam; Cambridge) and a secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H+L; 1:1000; Invitrogen). Cells were mounted with Fluoromount-G with DAPI (Southern Biotech). Ten fields of 4-well chamber slides were manually counted, quantified as mean desmin-positive cells per mean total cells, and expressed as a percentage of positive cells (Supplementary Fig. S1). Cell populations with a percentage of desmin positivity higher than 70% were used for subsequent experiments.

For experiments on differentiated myotubes, myoblasts were grown at confluence in Full Aneural medium and switched to No-Factor medium (Dulbecco's Modified Eagle's Medium high glucose containing 5% fetal bovine serum [EuroClone]; 1% insulin solution of 1 mg/mL [Sigma-Aldrich]; 1% L-glutamine [EuroClone]; 1% penicillin-streptomycin solution [100×; EuroClone]; 1% amphotericin solution [100×; EuroClone]) for 7 days. All human samples were collected after patients had signed informed consent forms in accordance with the requirements of the IRCCS G. Gaslini Institute ethics committee.

Ectocellular ATPase Activity

Myoblasts and myotubes were grown in 24-well plates, and ectocellular ATPase activity was quantified as previously described.[26] In brief, after removal of the culture medium, cells

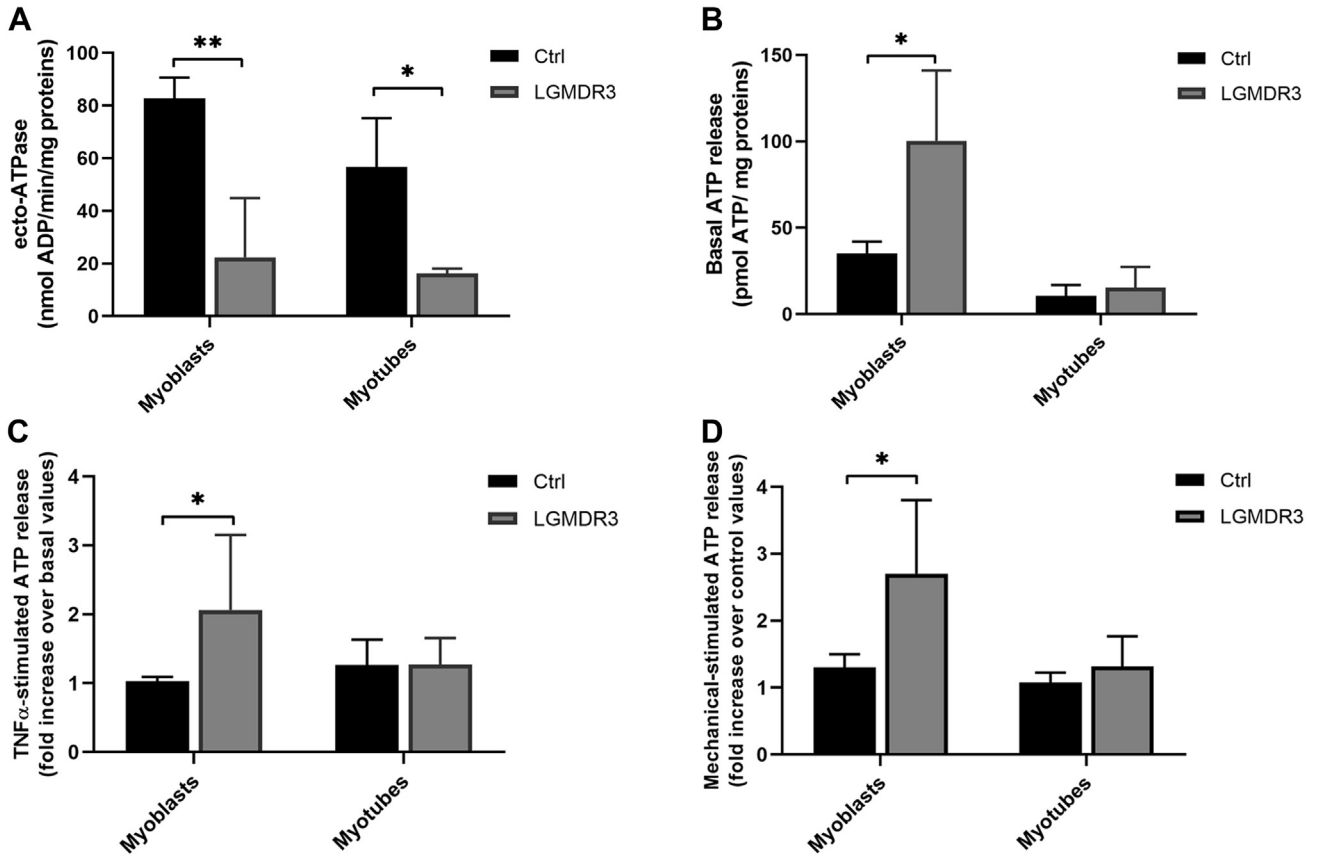

Figure 1.

Ectocellular ATPase and eATP release in myoblasts and myotubes of control subjects and patients with LGMDR3. (A) Ectocellular ATPase activity was evaluated by adding 0.3 mM ATP to myoblasts and myotubes and the formation of ADP by high-performance liquid chromatographic analysis. B-D, Cells were rinsed and the basal ATP release was evaluated after 30 min incubation in Hanks balanced salt solution (B), on (or not) a further 5-minute stimulation with TNF-α (C), or with a mechanical stress (D). *$P < .05$, ** $P < .01$ vs control subjects (controls, n = 4; patients, n = 4).

(~50% confluence for myoblasts) were washed once with 1 mL Hanks balanced salt solution (HBSS), and 0.35 mL HBSS containing 0.3 mM ATP were added. At different time points (0, 2, 5, and 10 minutes), aliquots of the incubations were withdrawn, and trichloroacetic acid was added (5% vol/vol final concentration). ATP degradation was determined by high-performance liquid chromatography (phosphate analysis).[28] Cells were lysed, and protein content in each well was determined by Bradford assay.

Release of Extracellular ATP

Myoblasts and myotubes were grown in 24-well plates. Cells (~50% confluence for myoblasts) were rinsed with HBSS and let sit for 30 minutes in 0.25 mL HBSS before cell stimulation with (or without) 100 ng/mL tumor necrosis factor (TNF)-α for 5 minutes or with mechanical stress by placing the cell plate on a horizontal shaker (speed 120; Phoenix Instrument) for 2 or 5 minutes. After collection and centrifugation, supernatants were assayed for evaluation of eATP levels by a luciferin-luciferase assay (ATP Bioluminescence Assay Kit CLS II; Roche) using a luminometer plate reader (FLUOstar Optima; BMG Labtechnologies). Cell death was determined through evaluation of lactate dehydrogenase: aliquots of the supernatants were incubated in the presence of 0.4 mM lactate and 0.4 mM NAD^+, and absorbance at 340 nm was registered. Protein determination was performed on cell lysates with the Bradford assay.

Fluorimetric Determination of Intracellular Calcium Levels

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes were evaluated as previously described.[29] In brief, myoblasts (~50% confluence) and myotubes, cultured in 96-well plates, were loaded with 10.0 μM FLUO-3AM for 45 minutes at 37 °C in the complete medium and washed with Ca^{2+}-containing HBSS or with Ca^{2+}-free HBSS. Fluorescence (excitation, 485 nm; emission, 520 nm) was measured every 3 seconds with a fluorescence plate reader (FLUOstar Optima). The intensity of emitted light was plotted as a function of time. Calcium changes (Δ = difference between intensity at peak on addition and basal intensity) were calculated for each trace using the formula Δ/basal × 100.

Real-time PCR Analyses

RNA was isolated from the patient and control myoblasts and myotubes and purified by NucleoSpin RNAII Kit (Macherey-Nagel, Dueren Germany). cDNA was prepared with High-Capacity cDNA Archive Kit (Applied Biosystems) and used as a template for real-time PCR analysis; reactions were performed in an iQ5 real-time PCR detection system (Bio-Rad) following the experimental conditions described in a previous study.[30] PCR primers were designed through Beacon Designer 2.0 Software (Bio-Rad): (NM_002562) P2RX7_F:5'-GAAGTTGTTGCACAGTGTCT-3'; P2RX7_R:5'-GCTGCT CTTGGCCTTCTGTT-3'; (NM_176072) P2RY2_F:5'-CTCTGCTTCCTGC

CATTCCA-3'; P2RY2_R:5'-ATGTTGATGGCGTTGAGGGT-3'; (XM_005 249818) Actb_F:5'-AATGAGCTGCGTGTGGCTCC-3'; Actb_R:5'-CAAT GGTGATGACCTGCCG-3'; (NM_000194) Hprt1_F:5'-GGTCAGGCAGT ATAATCCAAAG-3'; Hprt1_R:5'-TTCATTATAGTCAAGGGCATATCC-3'. A statistical analysis of the qPCR was performed using the IQ 5Optical System Software version 1.0 (Bio-Rad) based on the $2^{-\Delta\Delta Ct}$ method.[31] The dissociation curve for each amplification was analyzed to confirm the absence of unspecific PCR products.

Western Blot Analyses

Control- and patient-derived immortalized human myoblasts and primary myoblasts from one patient with LGMDR3 and one healthy control were lysed in 25 mM Tris-HCl, pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, and 1% Triton X-100, with the addition of protease inhibitor (cOmplete Mini; Roche) and phosphatase inhibitor (bimake.com). Lysates (30 μg proteins) were loaded on a 10% for polyacrylamide gel, and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Detection was performed with the primary antibodies against P2Y2 (Genetex) or antivinculin (Cell Signaling Technology), both used at 1:1000 dilution. After incubation with the appropriate secondary antibodies and enhanced chemiluminescence detection (GE Healthcare), band intensity was quantified with the ChemiDoc imaging system (Bio-Rad).

Immune Cell Chemotaxis

Control- and patient-derived immortalized human myoblasts were seeded into 24-well plates (80% confluence). Preemptively, the extracellular UTP-degrading activity of immortalized human myoblasts (from the control subject and patient) was measured by incubating cells in chemotaxis buffer (HBSS, phosphate-buffered saline, and 5% bovine serum albumin, 39:16:1), in the presence of exogenous 10.0 μM UTP for 3 hours. The remaining UTP concentration in the buffer was measured by high-performance liquid chromatography (see the "Ectocellular ATPase activity" section) and was estimated to be 3.0 ± 0.5 μM in both cell types (determinations from 3 different wells).

Complete culture media of control and patient cells, cultured in 24-well plates, were removed and replaced with the chemotaxis buffer, in the presence or absence of 10.0 μM UTP, with or without 10.0 μM AR-C 118925XX. After 3 hours of incubation at 37 °C, the buffer from the different conditions was recovered, centrifuged to remove cell debris, and frozen for chemotaxis experiments with immune cells.

Polymorphonuclear leukocytes (PMNLs) and peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn blood of healthy subjects by density centrifugation through Lympholyte cell separation media (Cedarlane) and hypotonic lysis of erythrocytes.[32]

Granulocytes and PBMCs were resuspended at 10^7/mL in chemotaxis buffer. Chemotaxis assays were performed using 96-well ChemoTx system microplates (Neuro Probe) with a 3.0-μm and 5.0-μm pore size polycarbonate filter for granulocytes and PBMCs, respectively. Media collected from the different conditions (control or patient cells, with or without UTP stimulation) or chemotaxis buffer alone or with 3.0 μM UTP were added to the bottom wells. Cell suspensions (25.0 μL) were placed directly on top of the filter, and the plates were incubated for 60 minutes at 37 °C. The transmigrated cells were collected following ChemoTx system instructions, transferred into a 96-well plate, and quantified by adding 60 μL of a solution composed of 0.2% Nonidet P-40 and 1.0 mM SYTOX Green. After 20 minutes of incubation at 37 °C, fluorescence was recorded (excitation, 485 nm; emission, 520 nm). A standard curve was obtained by placing a serial dilution of the cell suspension in the bottom wells. The results were expressed as chemotaxis index. Chemotaxis index=number of cells migrated toward the buffer from each different condition/number of cells migrated toward the chemotaxis buffer alone).

Release of IL-8 and MCP-1

LGMDR5 myoblasts (~50% confluence) were grown in 24-well plates. Cell culture media were removed, and cells were rinsed with HBSS. Cells were incubated in HBSS for 3 and 6 hours at 37 °C, in the presence or absence of ATP (final concentration 100.0 μM), with or without 10.0 μM AR-C 118925XX. Interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 release in the supernatant were evaluated using commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits (Biolegend).

Results

Myoblasts of Patients With LGMDR3 Are Exposed to Higher Concentrations of eATP Compared With Those of Controls

Proliferating $CD56^+$ myoblasts from healthy controls and patients with LGMDR3 (hereafter defined as control and LGMDR3 myoblasts) were grown in Full Aneural medium and, at confluence, switched to No-Factor medium for 7 days to differentiate into myotubes.

The ecto-ATPase activity was compared by adding eATP and evaluating the amount of produced adenosine diphosphate. As shown in Figure 1A, the ecto-ATPase activity decreased significantly in LGMDR3 myoblasts compared with that in control cells, in line with results obtained on myoblasts from Sgca mice.[26] The difference in ecto-ATPase activity between LGMDR3 and control cells was maintained in differentiated myotubes too (Fig. 1A).

Next, we quantified the level of eATP in myoblast and myotube medium. After 30 minutes of incubation, the eATP detected in the HBSS buffer was 3-fold higher in LGMDR3 myoblasts than in control cells (Fig. 1B). Conversely, the basal release of ATP in culture media was not significantly different in LGMDR3 and control myotubes (Fig. 1B).

Biopsies of dystrophic muscles overexpress the proinflammatory cytokines, TNF-α, IL-1, and IL-6.[33] These cytokines may activate the release of DAMPS from tissue-target cells, forming an "amplification loop," increasing the production of further inflammatory mediators.[34] On stimulation with TNF-α for 5 minutes, eATP increased by 2-folds in the medium of LGMDR3 myoblasts (Fig. 1C).

Myoblasts and myotubes from patients are characterized by weaker plasma membranes, mechanically damaged during contraction.[13] Hence, control and LGMDR3 cells were challenged with mechanical stress for 5 minutes: eATP was more than doubled in the media from LGMDR3 myoblasts, compared with that in controls (Fig. 1D). Notably, TNF-α and the mechanical stress failed to activate ATP release from control myotubes (Fig. 1C, D).

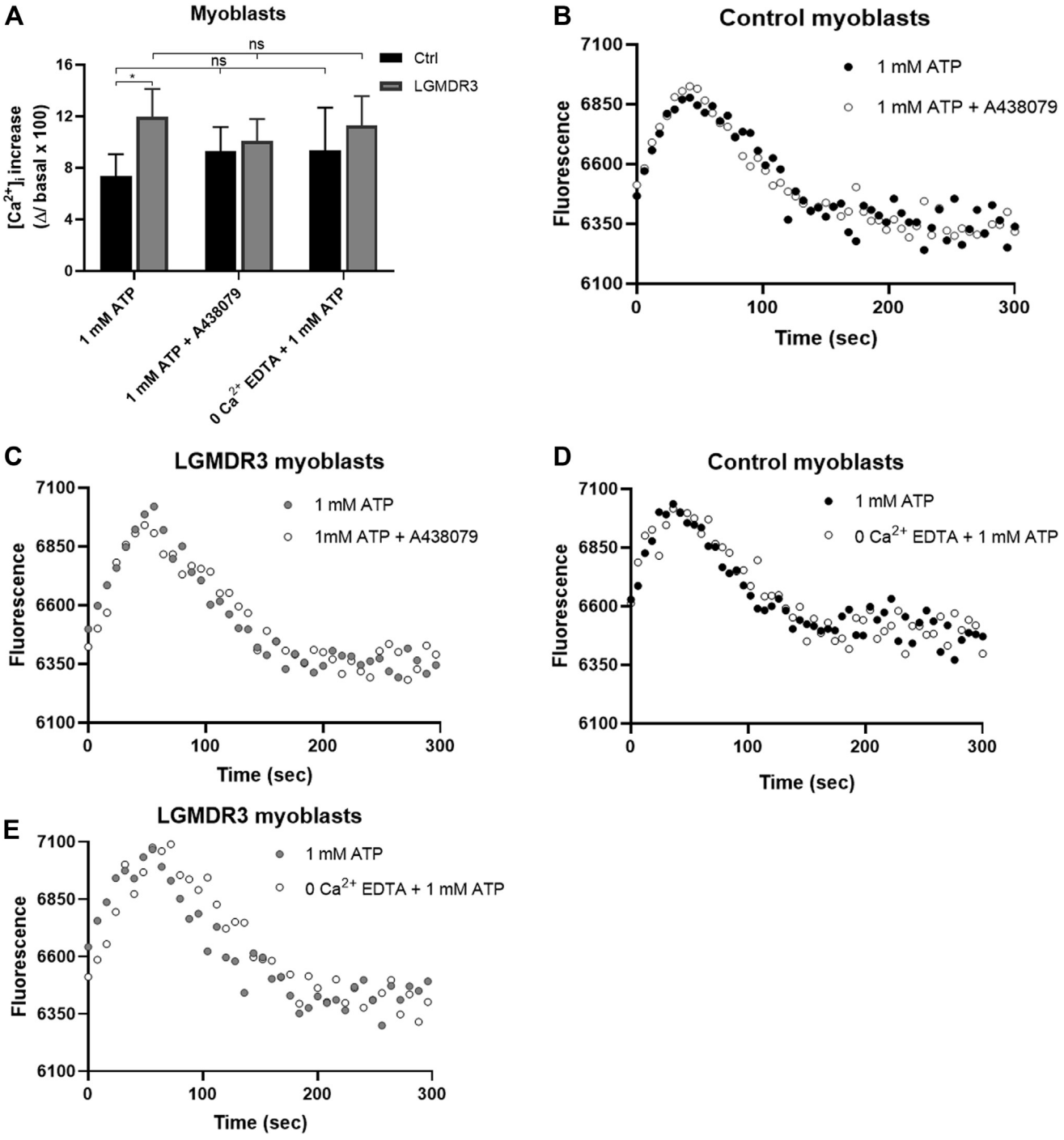

Figure 2.

Intracellular Ca^{2+} increase in response to 1 mM ATP in myoblasts of control subjects and patients with LGMDR3. Myoblasts were loaded with FLUO-3, and fluorescence changes were recorded with a fluorescence plate reader. (A) ATP (1 mM) was added to cells, preincubated (or not) with 10 μM A438079, or cells incubated in Ca^{2+}-free Hanks balanced salt solution. Results (calculated as the ΔCa^{2+} increase, normalized to the basal value) are presented as mean ± SD of at least 6 determinations for each control (n = 4) and patient (n = 4). (B-E) Representative traces of myoblasts from control subjects (B, D) or patients with LGMDR3 (C, E). *P < .05 vs control subjects; ns, no statistically significant difference.

Altogether, these data indicate that, as a consequence of the impaired ability to hydrolyze eATP and/or of an enhanced response to proinflammatory molecules and an increased membrane fragility to mechanical stimuli, myoblasts of patients with LGMDR3 are exposed to higher concentrations of eATP, compared with those of controls.

ATP-Induced Intracellular Ca^{2+} Increase Is Mediated by the Purinergic Receptor P2Y2

LGMDR3 and control myoblasts and myotubes were loaded with FLUO-3. ATP (1.0 mM) was added, and the $[Ca^{2+}]_i$ changes were recorded with a plate fluorometer. ATP induced a significantly

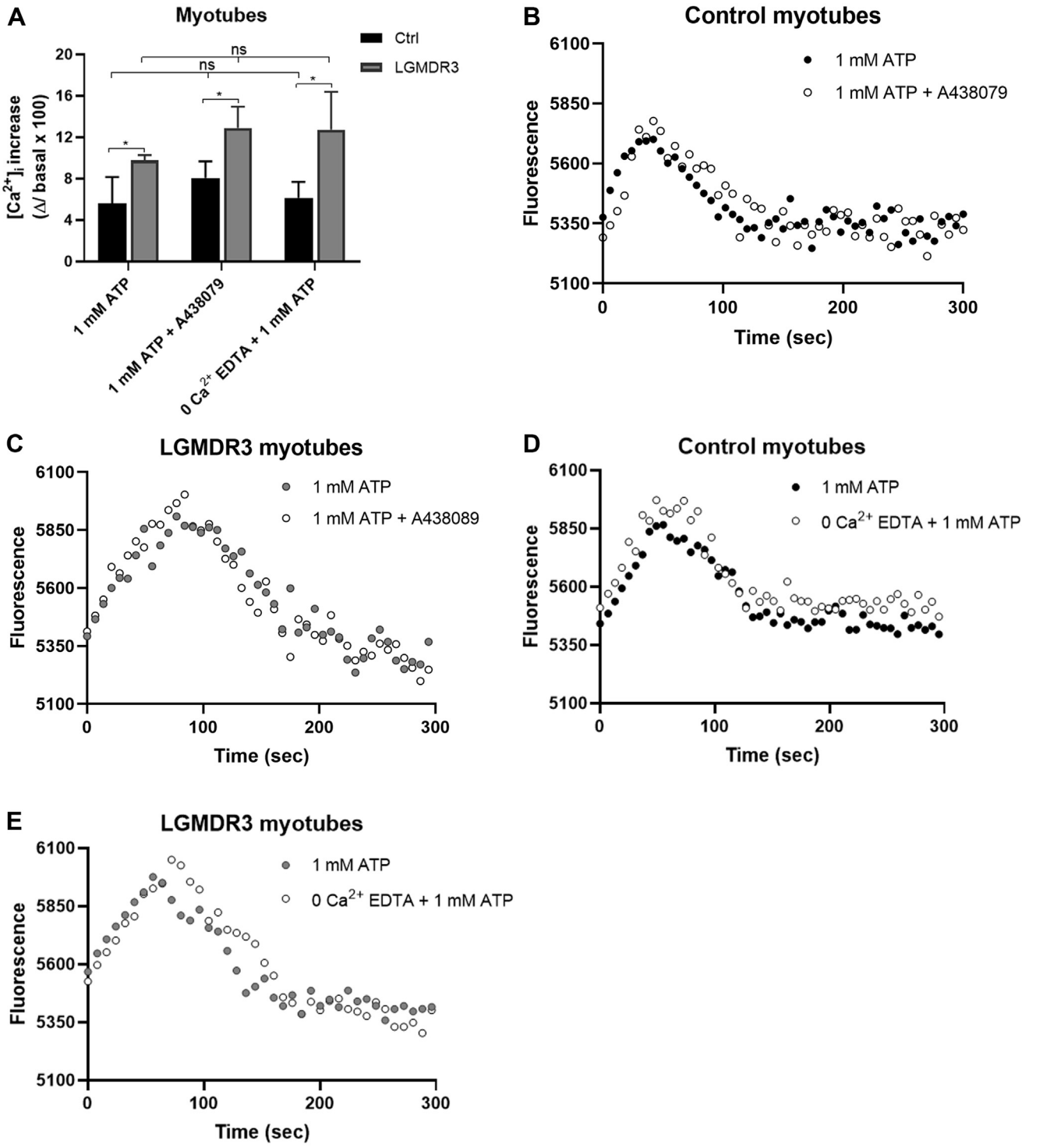

Figure 3.

ATP-induced Ca^{2+} increase in myotubes. Myotubes were loaded with FLUO-3, and fluorescence changes were recorded with a fluorescence plate reader. (A) ATP (1 mM) was added to cells, preincubated (or not) with 10 μM A438079, or to cells incubated in Ca^{2+}-free Hanks balanced salt solution (HBSS). Results (calculated as the ΔCa^{2+} increase, normalized to the basal value) are presented as mean ± SD of at least 6 determinations for each control (n = 4) and patient (n = 4). (B-E) Representative traces of myotubes from control subjects (B, D) or patients with LGMDR3 (C, E): ATP (1 mM) was added to cells, preincubated (or not) with 10 μM A438079 (B, D), or to cells incubated in in Ca^{2+}-free HBSS (D, E). *$P < .05$ vs control subjects; ns, no statistically significant difference.

higher response in LGMDR3 myoblasts than in control cells (Fig. 2A). Preincubation with 10.0 μM of the P2X7 inhibitor A438079 did not modify the $[Ca^{2+}]_i$ increase induced by 1.0 mM ATP, nor in control (Fig. 2A, B) or LGMDR3 myoblasts (Fig. 2A, C), indicating that the purinoceptor P2X7 is not the main purinergic receptor activated by eATP in LGMDR3 and control cells.

The addition of 1.0 mM ATP evoked a similar $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+}, in both control and LGMDR3

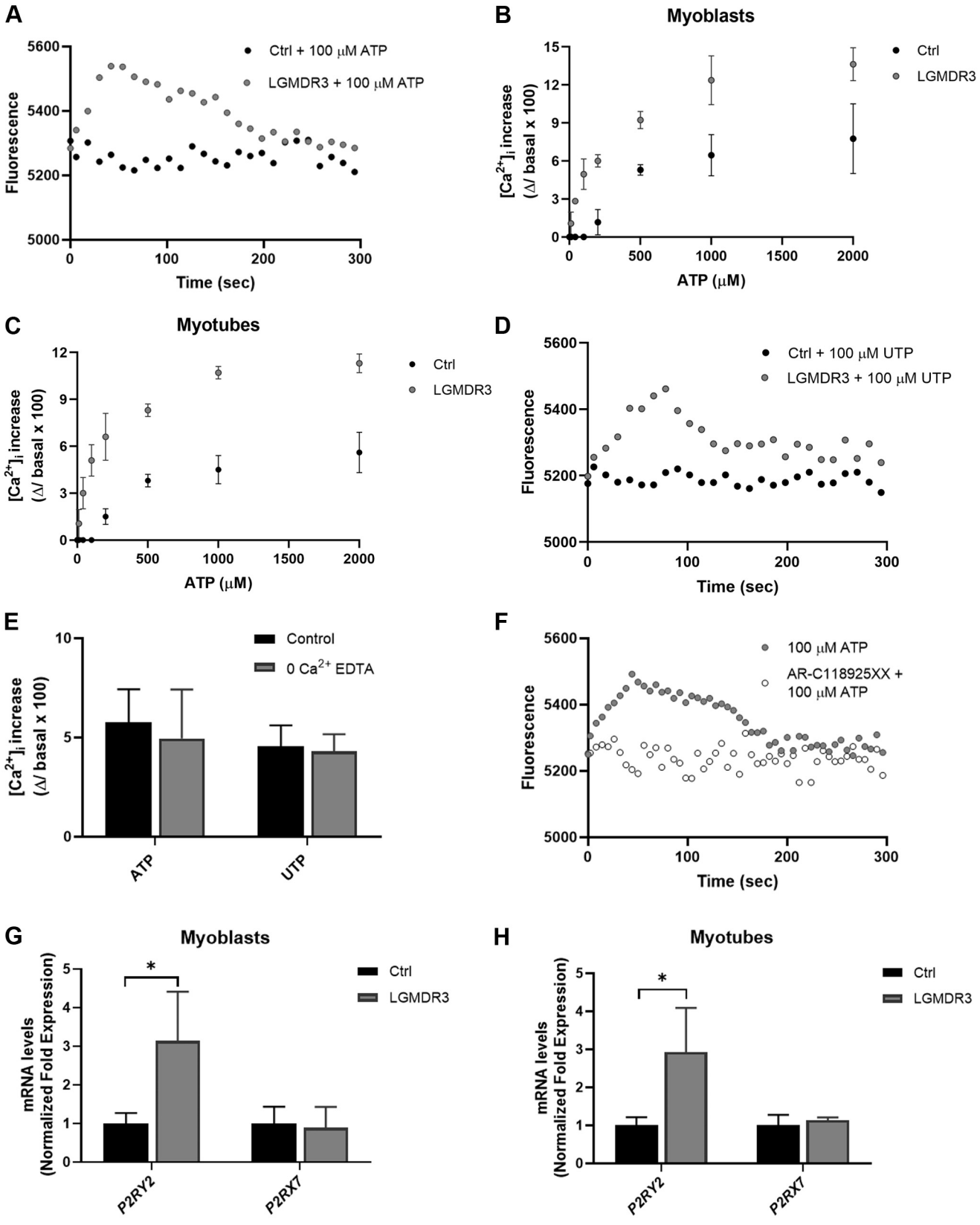

Figure 4.

P2RY2 mediates the intracellular Ca^{2+} increase in response to micromolar ATP in LGMDR3 cells. Myoblasts and myotubes were loaded with FLUO-3, and fluorescence changes were recorded with a fluorescence plate reader. (A, D) Representative traces of control and LGMDR3 myoblasts, stimulated with 100 μM ATP (A) or UTP (D). Myoblasts (B) and myotubes (C) were challenged with increasing concentrations of ATP. Results (calculated as the ΔCa^{2+} increase, normalized to the basal value) are presented as mean ± SD of least 4 determinations for each control (n = 4) and patient (n = 4). (E) ATP or UTP (100 μM) were added to cells in Hanks balanced salt solution (HBSS) or in Ca^{2+}-free HBSS.

myoblasts (Fig. 2A, D, E). Therefore, the main contribution in the ATP-induced $[Ca^{2+}]_i$ increase is not due to a Ca^{2+} influx from the extracellular space through a purinergic receptor of the P2X family but is due to the activation of purinergic receptor(s) of the P2Y subfamily. P2YRs are coupled to G proteins and trigger downstream signaling pathways evoking Ca^{2+} release from intracellular stores and/or synthesis of cAMP.[35] The use of the P2X7 receptor antagonist and the absence of extracellular Ca^{2+} did not significantly modify the response to 1.0 mM ATP in LGMDR3 myotubes (Fig. 3A-E), as observed for myoblasts. The Ca^{2+} increase induced by 1.0 mM ATP was significantly higher in LGMDR3 myotubes (Fig. 3A), indicating that α-SG–deficient myotubes were more responsive to eATP and that P2X7 was not the main purinoceptor mediating this effect.

Furthermore, myoblasts and myotubes were challenged with lower concentrations of ATP, in the micromolar range, to exclude the activation of P2X7, which requires concentrations of ATP greater than 100.0 μM to be gated,[36] whereas P2YRs have, in general, high affinity for their ligands (in the nanomolar/micromolar range). On addition of 100.0 μM ATP, a $[Ca^{2+}]_i$ increase was registered only in LGMDR3 myoblasts (Fig. 4A) and myotubes (not shown). In α-SG–deficient primary muscle cells, ATP showed a higher efficacy in stimulating a Ca^{2+} increase, being already active in the low micromolar range, as revealed in dose-response measurements (Fig. 4B). Similar dose-response curves were obtained on myotubes (Fig. 4C).

To identify the P2Y receptor mediating the response to eATP, myoblasts were challenged with UTP, a nucleotide exerting a different selectivity on the P2 receptor family members. As already observed with ATP, UTP induced a Ca^{2+} increase in LGMDR3 cells (Fig. 4D, E) but not in control cells (Fig. 4D). The UTP-induced Ca^{2+} increase was not modified in the absence of extracellular Ca^{2+} (Fig. 4E), confirming the stimulation of a member of the P2Y family. Because the P2Y2 receptor being equally activated by ATP or UTP,[37] ATP was added in the presence (or absence) of a selective P2Y2 antagonist (AR-C 118925XX). As shown in Figure 4F, the P2Y2 antagonist (5.0 μM final concentration) abrogated the ATP-induced Ca^{2+} increase in LGMDR3 cells. Accordingly, as detected by qPCR, *P2RY2* gene expression was significantly higher in LGMDR3 myoblasts and myotubes (Fig. 4G, H). Instead, *P2RX7* was expressed at comparable levels in LGMDR3 and control cells (Fig. 4G, H).

Altogether, our data demonstrated that myoblasts and myotubes from patients with LGMDR3 are exposed to higher eATP levels on proinflammatory and mechanical stresses, possibly due to a reduced ecto-ATPase activity, in addition to inflammasome activation and increased membrane leakage. In addition, we demonstrated that cells from patients with LGMDR3 are more sensitive to eATP, which induces P2RY2-mediated $[Ca^{2+}]_i$ changes.

An Immortalized Human Cell Line Recapitulates the Main ATP-Related Aberrations of Primary Human Myoblasts

We evaluated whether the main ATP-related aberrations found in primary myoblasts were present in immortalized human cell lines affected by the loss-of-function gene mutations in the *SGCG* gene (Supplementary Fig. S1). For this purpose, we used immortalized human cells isolated from a control subject and from a patient with LGMDR5.[2] The ecto-ATPase activity was not significantly decreased in LGMDR5 myoblasts, being 23.0 ± 4.8 and 20.2 ± 2.1 nmol adenosine diphosphate/min/mg in control and patient cells (n = 4), respectively. These data may be in line with the α subunit, but not with the γ subunit, being endowed with ecto-ATPase activity. Nevertheless, eATP concentration was greatly increased in culture media from the patient than that from the control patient (Fig. 5A), suggesting that a basal ATP "leakage" occurs from patient cells.

The addition of exogenous ATP, and of UTP, added in the micromolar range, elicited a $[Ca^{2+}]_i$ increase only in patient cells (Fig. 5B, C), suggesting that P2Y2 is the purinergic receptor mediating this response. Accordingly, qPCR analyses confirmed that *P2RY2* expression was markedly higher in the patient cells, whereas *P2RX7* expression was reduced in comparison with that in control cells (Fig. 5D). The western blot analysis confirmed an enhanced P2Y2 expression in both LGMDR5 and LGMDR3 patient cells (Fig. 5E).

Supernatants From LGMDR5 Myoblasts Trigger Chemotaxis in Immune Cells

Recently, we demonstrated that limb muscles of Sgca mice were characterized by infiltration of $CD45^+$ immune cells, not detected in wild-type mice.[25] Thus, PMNLs and PBMCs, freshly isolated from healthy subjects, were challenged to migrate toward cell supernatants collected from immortalized myoblasts (control and patient samples), stimulated for 3 hours with exogenous UTP (10.0 μM), to selectively activate P2Y receptors. The CI was calculated as the ratio between the number of migrated cells toward a specific condition and the number of migrated cells toward the chemotaxis buffer, not incubated in the presence of myoblasts. Notably, both PMNLs and PBMCs exhibited an enhanced migration toward the supernatant from patient myoblasts stimulated with UTP (Fig. 5F). As a control, immune cells were challenged to migrate toward 3 μM UTP, that is, at the final concentration extracellularly present in cell cultures incubated for 3 hours in the presence of 10.0 μM UTP. This comparison ruled out the possibility that immune cells were merely attracted by UTP itself (Fig. 5F). Finally, the addition of the specific P2Y2 antagonist to UTP stimulated patient myoblasts, abrogating immune cell migration toward the supernatants (Fig. 5G).

P2Y2 mediates the release of IL-8,[38] a chemokine known to attract neutrophils and PBMCs: exogenous ATP significantly increased IL-8 release in the patient but not in control cells (Fig. 5H). MCP-1, a key chemokine regulating the migration of monocytes/macrophages, was found in high levels in the supernatant of untreated patient cells. The stimulation with ATP did not enhance its release (Fig. 5I). The P2Y2 inhibitor significantly reduced the basal IL-8 release from both control and patient cells and abrogated the ATP-induced release (Fig. 5H). Conversely, MCP-1 levels were not affected by the presence of AR-C118925XX, neither in untreated nor in ATP-stimulated cells (Fig. 5I).

Results (calculated as the ΔCa^{2+} increase, normalized to the basal value) are presented as mean ± SD of least 4 determinations. (F) Myotubes were challenged with increasing concentrations of ATP. Results (calculated as the ΔCa^{2+} increase, normalized to the basal value) are presented as mean ± SD of least 3 determinations for each control (n = 4) and patient (n =4). Representative traces of LGMDR3 myoblasts, stimulated with ATP (100 μM), in the presence or absence of 10 μM AR-C 118925XX. (G, H) The relative expression of *P2RY2* and *P2RX7* in myoblasts (G) and myotubes (H), normalized toward the housekeeping genes *ACTB* and *HPRT1*, was analyzed by the qPCR analysis (n = 3). *$P < .05$ vs control subjects.

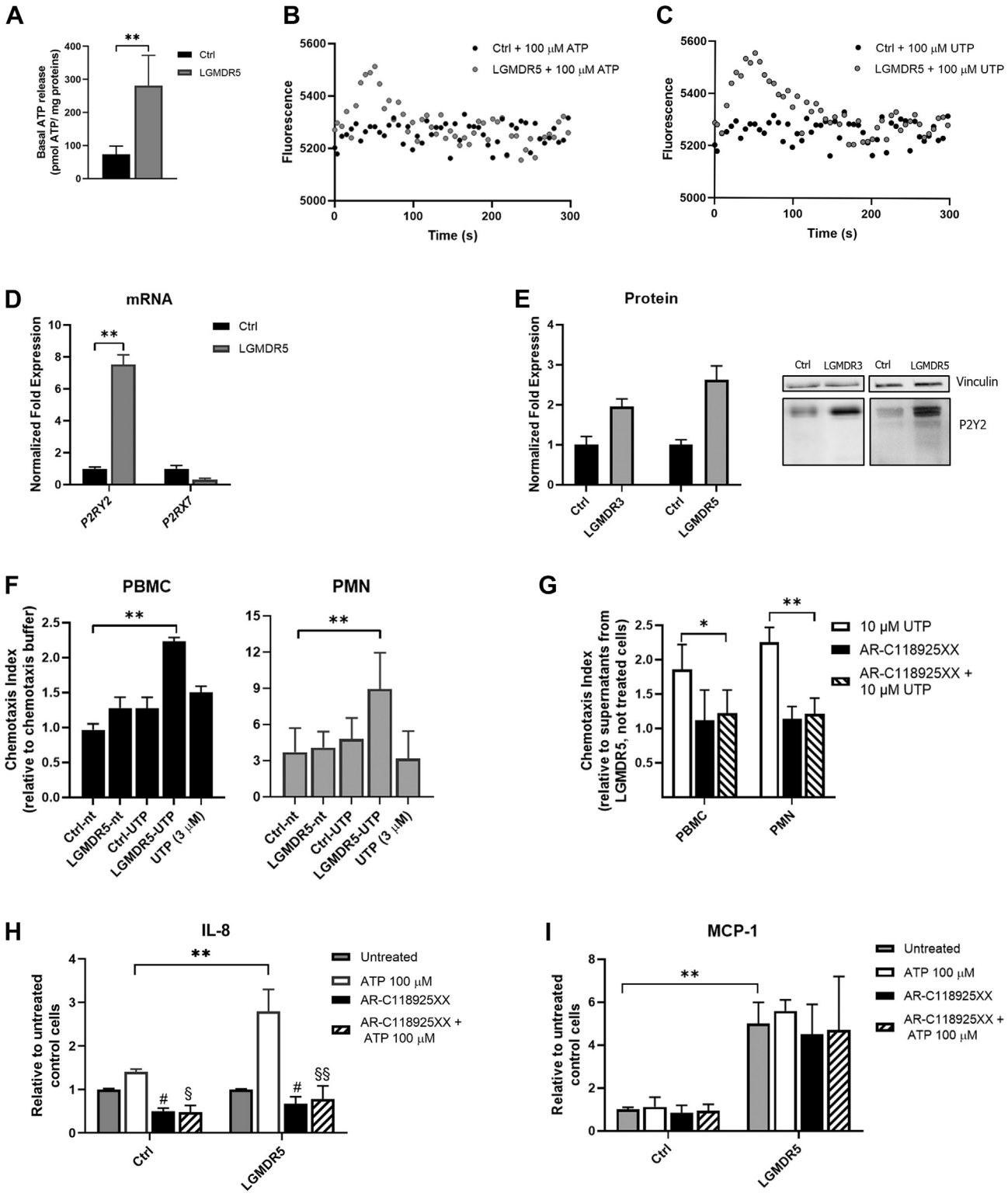

Figure 5.

ATP-related aberration in immortalized LGMDR5 myoblasts. (A) Control and LGMDR5 myoblasts were rinsed, and the basal ATP release was evaluated after a 30-minute incubation in Hanks balanced salt solution. (B, C) Representative traces of control and LGMDR5 myoblasts, stimulated with 100 μM ATP (B) or UTP (C). (D) The relative expression of *P2RY2* and *P2RX7*, normalized toward the housekeeping genes *TBP* and *HPRT1*, was analyzed by the qPCR analysis (n = 3). (E) Western blot analysis to evaluate P2Y2 expression in control and LGMDR5 immortalized myoblasts and in primary myoblasts from 1 healthy subject and 1 patient with LGMD3 (1 representative blot and quantifications, normalized to vinculin expression, are shown). (F) PBMC (black bars) and PMNLs (gray bars) were challenged to migrate toward the chemotaxis buffer alone or with 3 μM UTP or toward the same buffer preincubated with control or patient cells, stimulated (or not, Ctrl-nt and LGMD-nt) with 10 μM UTP for 3 hours (Ctrl-UTP and LGMD-UTP). Results are expressed as chemotaxis index (mean ± SD) of 4 different determinations. (G) PBMC and PMNLs were challenged to migrate toward the chemotaxis buffer preincubated with patient cells, stimulated (or not) with 10 μM UTP, in the presence or absence of 10 μM AR-C118925XX, a P2Y2 inhibitor. Results are expressed as chemotaxis index (mean ± SD) of 3 different determinations. (H, I) Control and LGMDR5 myoblasts were stimulated with 100 μM ATP for 6 hours, in the presence or absence of 10 μM AR-C118925XX. IL-8 (H) and MCP-1 (I)

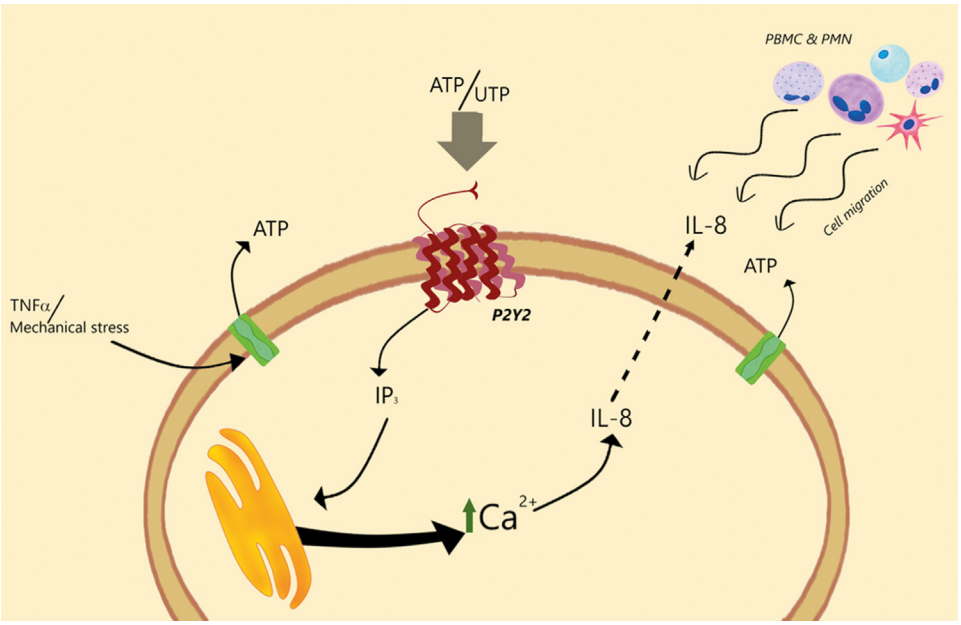

Figure 6.
Graphical representation of the eATP role in myoblasts of patients with LGMDR3 and LGMDR5. ATP is released in the extracellular environment by different stimuli. eATP activates the P2Y2 receptors, causing the increase in $[Ca^{2+}]_i$ and the release of IL-8 which, in turn, may be responsible for immune cell migration.

Discussion

In healthy conditions, skeletal muscle hosts few immune cells. In muscular dystrophies, reactive inflammatory cells, including a subpopulation of innate and adaptive immune responses, infiltrate the tissue and surround necrotic myotubes.[39-43] Muscle cells play a central direct role in the recruitment and activation of such immune response: the fragile muscle releases the so-called DAMPs, such as ATP, which, through the purinergic receptors, initiates and enhances the immune response. Different studies focused on the eATP/P2X7 receptor signaling cascade highlighted the potential of this purinoreceptor as a therapeutic target in muscular dystrophies.[25,44] The administration of a selective P2X7 antagonist (A438079) to Sgca mice proved that P2X7 inhibition improved muscle force and morphology by dampening the extent of muscle fibrosis and local inflammation.[25] Indeed, the main beneficial effect of A438079 in Sgca animals was on the modulation of muscle inflammation.[42,45] In particular, A438079 caused a significant reduction of innate immune cells, such as neutrophils, monocytes, and dendritic cells, infiltrating the limb muscles of Sgca-mutant mice. Similar results were obtained in *mdx* and Sgca mice treated with other P2X7 antagonists, such as oATP or zidovudine.[23,26,46]

Nevertheless, the mechanism by which P2X7 inhibition results in the improvement of muscular dystrophy is not completely understood. P2X7 antagonists could exert not only an indirect effect by blocking the activity of P2X7 expressed on inflammatory immune cells but also a direct effect on muscle cells.

In this study, we showed that the role of P2X7 activation in mediating the ATP-induced Ca^{2+} response is not predominant in human primary myoblasts and myotubes isolated from patients carrying different loss of mutations in the *SGCA* or *SGGC* genes. Instead, our data indicated that P2Y2 is the main receptor activated by eATP in muscle cells.

Evidence for the role of P2X7 in dystrophic myoblasts and myotubes was reported in cells from *mdx* mice: the exposure to eATP triggered an increase in $[Ca^{2+}]_i$ caused primarily by activation of P2X7 receptors and increased store-operated calcium entry. Specifically, in a dystrophin-negative muscle cell line established from the *mdx* mouse model, eATP-induced influx of extracellular Ca^{2+} was inhibited by zinc, Coomassie brilliant blue-G, and KN-62, suggesting activation of P2X7 receptors. Moreover, expression of P2X4 and P2X7 proteins were immunodetected in dystrophic myoblasts.[21]

In a most recent publication (excluding P2X7), a contribution of a metabotropic response to extracellular ATP/UTP was revealed in immortalized *mdx* mouse myoblasts, with dystrophin-positive cells used as controls. P2Y2 was identified as the receptor triggering a calcium release from intracellular stores in *mdx* myoblasts based on several pieces of evidence.[22]

In this study, we identified P2Y2 as a pivotal receptor responsible for the ATP- or UTP-induced Ca^{2+} increase in human myoblasts obtained from patients with α-sarcoglycanopathies. As summarized in Figure 6, we showed that (1) the ATP-induced Ca^{2+} increase was not reduced in the absence of extracellular Ca^{2+}, indicating the involvement of receptors of the P2Y subfamily (Figs. 2A and 3A); (2) UTP and low (micromolar) concentrations of ATP induced a Ca^{2+} increase in LGMDR3 cells; (3) a specific P2Y2R antagonist decreased the ATP-induced Ca^{2+} increase (Fig. 4F); (4) *P2RY2* mRNA and protein levels were increased in myoblasts and myotubes (Fig. 4G, H); and (5) stimulation of P2Y2 in myoblasts induced chemotaxis of immune cells and release of IL-8, both

levels in the supernatants were evaluated with ELISA assays. Results shown are referred to cytokine levels in the supernatant of untreated control cells and represent the mean ± SD of at least 3 determinations for each condition. $^{*}P < .05$, $^{**}P < .01$ vs control subjects, comparing conditions as indicated by the bars. In panel H, $^{\#}P < .5$ vs the untreated cells; $^{\S}P < .05$ and $^{\S\S}P < .01$ vs the ATP-treated cells. Only relevant comparisons are shown. *IL* = interleukin; *MCP* = monocyte chemotactic protein; *PBMC* = peripheral blood mononuclear cells; *PMNL* = polymorphonuclear leukocyte

effects being abrogated by the P2Y2 antagonist (Fig. 5). In addition, P2Y2 was confirmed as the responsible receptor in the immortalized cell line lacking γ-SG (Fig. 5). The concordance of P2Y2 biological effects in cell models of 2 different sarcoglycanopathies is in line with the secondary reduction of all elements of the SG complex, occurring in the presence of mutations in one of them.

Notwithstanding the similar involvement for P2Y2, there are important differences between *mdx* mouse myoblasts and human SG-deficient myoblasts. First, the absence of extracellular Ca^{2+} in our conditions did not alter the ATP-induced Ca^{2+} increase triggered by millimolar ATP concentrations (Figs. 2A and 3A). Moreover, the specific P2X7 antagonist A438079 failed to modify the ATP-induced Ca^{2+} increase (Figs. 2A and 3A). Thus, the P2X7 receptor does not seem to mediate the response to eATP in human primary cells affected by a loss of the SG complex.

Primary human muscle cells carrying α-SG loss-of-function mutations were sensitive to very low concentrations of ATP: in cells from 1 patient, as low as 10.0 μM eATP triggered a Ca^{2+} increase. Thus, the result of a higher eATP in LGMDR3 cells and a higher sensitivity to eATP indicate that α-SG cells respond to eATP by triggering Ca^{2+} movements, which, in turn, determines the release of factors that could attract inflammatory cells. Indeed, human neutrophils and PBMCs migrated toward the supernatant of UTP-treated LGMDR5 cells. Admittedly, the exact factors/cytokines released by human α-SG–deficient myoblasts responsible for attracting inflammatory cells need to be identified. However, this experiment clearly shows that the presence of ATP in the muscle extracellular milieu can act as an attractor for inflammatory cells and trigger the production of further chemoattractants from muscle cells. Among the factors released by myoblasts that may concur in triggering immune cell migration, we identified IL-8 and MCP-1 releases. Notably, P2Y2 activation seems to mediate IL-8, but not MCP-1, release (Fig. 5H, I). The presence of MCP-1 in the patient cell supernatant may be responsible for the slight increase in basal migration of PBMC (Fig. 5F, LGMDR5-nt vs Ctrl-nt).

The data from this study indicated the following: (1) P2X7 does not have a predominant role in eliciting ATP responses in human myoblasts/myotubes and (2) in Sgca null mice, the selective P2X7 antagonist A438079 is effective in counteracting the progression of the dystrophic phenotype and in reducing the inflammatory response. Hence, we suggest that P2X7 inhibition (expressed by inflammatory cells) reduces infiltration of inflammatory cells in the muscle, rather than acting on muscular P2X7 receptors.

P2Y2 receptors are expressed in physiologic conditions at the neuromuscular junction, where, in differentiated myotubes, their activation induced the expression of acetylcholinesterase and different acetylcholine receptor subunits.[47] Notably, in accordance with the data of this study and of the study by Rog et al,[22] P2Y2 receptor levels were upregulated in dystrophic hearts from *mdx* mice,[48] suggesting the possibility that P2Y2 selective drugs may ameliorate cardiomyopathy in dystrophinopathies. In addition, P2Y2 was demonstrated to represent a promoter of skeletal muscle atrophy and fibrosis after muscle injury, with P2Y2-triggered signaling through protein kinase B, extracellular signal-regulated kinases, and protein kinase C.[49] Thus, P2Y2 was proposed to be a potential therapeutic target after muscle injury.

Novel gene editing and gene therapy strategies are changing the landscape of treatment options for sarcoglycanopathies.[5,8,50] However, the innate immune–mediated damage associated with the dystrophic process represents a significant challenge because it may limit the efficient engraftment of such genetic therapeutic tools and may enhance the adaptive immune response against the transgene or the newly edited protein. This study presented the relevance of dissecting the basic players triggering inflammations in muscular dystrophies. The molecular mechanisms leading to increased transcription of the *P2RY2* gene in skeletal and cardiac dystrophic muscle are unknown and will be the focus of future studies.

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Author Contributions

S.Bruzzone, E.G., and C.B. conceived and designed the study. A.B., S.Baratto, and S.Bruzzone performed the experiments, data interpretation, and statistical analysis. C.A., L.S., C.P., and L.R. performed the experiments. K.M. provided technical and material support. S.Bruzzone and E.G. wrote and revised the paper. All authors read and approved the final paper.

Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supplementary information file.

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Declaration of Competing Interest

The authors declare no competing financial interests in relation to the work described.

Ethics Approval and Consent to Participate

For the isolation of primary cell culture, the ethics committee that approved the study is CER Liguria and the committee's reference number is 164/2018. For the generation of immortalized cell, human biopsies were obtained from MyoBank, affiliated to EuroBioBank, holding the agreement from the French Ministry of Research and Higher Education to distribute such human material after informed consent from patients (reference for the authorization AC-2019-3502). The study was performed in accordance with the tenets of the Declaration of Helsinki.

Supplementary Material

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References

1. Angelini C, Fanin M, Freda MP, Duggan DJ, Siciliano G, Hoffman EP. The clinical spectrum of sarcoglycanopathies. *Neurology*. 1999;52:176–179.
2. Alonso-Pérez J, González-Quereda L, Bello L, et al. New genotype-phenotype correlations in a large European cohort of patients with sarcoglycanopathy. *Brain*. 2020;143:2696–2708.
3. Mendell JR, Rodino-Klapac LR, Rosales XQ, et al. Sustained alpha-sarcoglycan gene expression after gene transfer in limb-girdle muscular dystrophy, type 2D. *Ann Neurol*. 2010;68:629–638.
4. Tedesco FS, Gerli MF, Perani L, et al. Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystrophy. *Sci Transl Med*. 2012;4:140ra89.
5. Escobar H, Krause A, Keiper S, et al. Base editing repairs an SGCA mutation in human primary muscle stem cells. *JCI Insight*. 2021;6:e145994.
6. Taheri F, Taghizadeh E, Pour MJR, et al. Limb-girdle muscular dystrophy and therapy: insights into cell and gene-based approaches. *Curr Gene Ther*. 2020;19:386–394.
7. Griffin DA, Pozsgai ER, Heller KN, Potter RA, Peterson EL, Rodino-Klapac LR. Preclinical systemic delivery of adeno-associated α-sarcoglycan gene transfer for limb-girdle muscular dystrophy. *Hum Gene Ther*. 2021;32:390–404.
8. Pozsgai E, Griffin D, Potter R, et al. Unmet needs and evolving treatment for limb girdle muscular dystrophies. *Neurodegener Dis Manag*. 2021;11:411–429.
9. Bartoli M, Gicquel E, Barrault L, et al. Mannosidase I inhibition rescues the human alpha-sarcoglycan R77C recurrent mutation. *Hum Mol Genet*. 2008;17:1214–1221.
10. Gastaldello S, D'Angelo S, Franzoso S, et al. Inhibition of proteasome activity promotes the correct localization of disease-causing alpha-sarcoglycan mutants in HEK-293 cells constitutively expressing beta-, gamma-, and delta-sarcoglycan. *Am J Pathol*. 2008;173:170–181.
11. Bianchini E, Fanin M, Mamchaoui K, Betto R, Sandonà D. Unveiling the degradative route of theV247M α-sarcoglycan mutant responsible for LGMD-2D. *Hum Mol Genet*. 2014;23:3746–3758.
12. Scano M, Benetollo A, Nogara L, et al. CFTR corrector C17 is effective in muscular dystrophy, in vivo proof of concept in LGMDR3. *Hum Mol Genet*. 2022;31:499–509.
13. Sandonà D, Betto R. Sarcoglycanopathies: molecular pathogenesis and therapeutic prospects. *Expert Rev Mol Med*. 2009;11:e28.
14. Rubartelli A, Lotze MT. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol*. 2007;28:429–436.
15. Garcia-Martinez I, Shaker ME, Mehal WZ. Therapeutic opportunities in damage-associated molecular pattern-driven metabolic diseases. *Antioxid Redox Signal*. 2015;23:1305–1315.
16. Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. *Nature*. 2014;509:310–317.
17. Burnstock G. Purinergic signalling: therapeutic developments. *Front Pharmacol*. 2017;8:661.
18. Rawat R, Cohen TV, Ampong B, et al. Inflammasome up-regulation and activation in dysferlin-deficient skeletal muscle. *Am J Pathol*. 2010;176:2891–2900.
19. Yeung D, Kharidia R, Brown SC, Górecki DC. Enhanced expression of the P2X4 receptor in Duchenne muscular dystrophy correlates with macrophage invasion. *Neurobiol Dis*. 2004;15:212–220.
20. Jiang T, Yeung D, Lien C-F, Górecki DC. Localized expression of specific P2X receptors in dystrophin deficient DMD and mdx muscle. *Neuromuscul Disord*. 2005;15:225–236.
21. Yeung D, Zablocki K, Lien CF, et al. Increased susceptibility to ATP via alteration of P2X receptor function in dystrophic mdx mouse muscle cells. *FASEB J*. 2006;20:610–620.
22. Róg J, Oksiejuk A, Gosselin MRF, et al. Dystrophic mdx mouse myoblasts exhibit elevated ATP/UTP-evoked metabotropic purinergic responses and alterations in calcium signalling. *Biochim Biophys Acta Mol Basis Dis*. 2019;1865:1138–1151.
23. Gazzerro E, Baldassari S, Assereto S, et al. Enhancement of muscle T regulatory cells and improvement of muscular dystrophic process in mdx mice by blockade of extracellular ATP/P2X axis. *Am J Pathol*. 2015;185:3349–3360.
24. Sinadinos A, Young CN, Al-Khalidi R, et al. P2RX7 purinoceptor: a therapeutic target for ameliorating the symptoms of Duchenne muscular dystrophy. *PLoS Med*. 2015;12:e1001888.
25. Raffaghello L, Principi E, Baratto S, et al. P2X7 receptor antagonist reduces fibrosis and inflammation in a mouse model of alpha-sarcoglycan muscular dystrophy. *Pharmaceuticals (Basel)*. 2012;15:89.
26. Gazzerro E, Baratto S, Assereto S, et al. The danger signal extracellular ATP is involved in the immunomediated damage of α-sarcoglycan-deficient muscular dystrophy. *Am J Pathol*. 2019;189:354–369.
27. Mamchaoui K, Trollet C, Bigot A, et al. Immortalized pathological human myoblasts: towards a universal tool for the study of neuromuscular disorders. *Skelet Muscle*. 2011;1:34.
28. Grozio A, Sociali G, Sturla L, et al. CD73 protein as a source of extracellular precursors for sustained NAD+ biosynthesis in FK866-treated tumor cells. *J Biol Chem*. 2013;288:25938–25949.
29. Magnone M, Bauer I, Poggi A, et al. NAD+ levels control Ca2+ store replenishment and mitogen-induced increase of cytosolic Ca2+ by cyclic ADP-ribose-dependent TRPM2 channel gating in human T lymphocytes. *J Biol Chem*. 2012;287:21067–21081.
30. Sturla L, Fresia C, Guida L, et al. LANCL2 is necessary for abscisic acid binding and signaling in human granulocytes and in rat insulinoma cells. *J Biol Chem*. 2009;284:28045–28057.
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCt method. *Methods*. 2001;25:402–408.
32. Bruzzone S, Moreschi I, Guida L, Usai C, Zocchi E, De Flora A. Extracellular NAD+ regulates intracellular calcium levels and induces activation of human granulocytes. *Biochem J*. 2006;393:697–704.
33. De Paepe B, De Bleecker JL. Cytokines and chemokines as regulators of skeletal muscle inflammation: presenting the case of Duchenne muscular dystrophy. *Mediators Inflamm*. 2013;2013:540370.
34. Anderton H, Ian P, Wicks IP, Silke J. Cell death in chronic inflammation: breaking the cycle to treat rheumatic disease. *Nat Rev Rheumatol*. 2020;16:496–513.
35. Giuliani AL, Sarti AC, Di Virgilio F. Extracellular nucleotides and nucleosides as signalling molecules. *Immunol Lett*. 2019;205:16–24.
36. North RA. Molecular physiology of P2X receptors. *Physiol Rev*. 2002;82:1013–1067.
37. Erb L, Weisman GA. Coupling of P2Y receptors to G proteins and other signaling pathways. *Wiley Interdiscip Rev Membr Transp Signal*. 2012;1:789–803.
38. Wu L, Oshima T, Fukui H, Watari J, Miwa H. Adenosine triphosphate induces P2Y2 activation and interleukin-8 release in human esophageal epithelial cells. *J Gastroenterol Hepatol*. 2017;32:1341–1347.
39. Fanin M, Angelini C. Regeneration in sarcoglycanopathies: expression studies of sarcoglycans and other muscle proteins. *J Neurol Sci*. 1999;165:170–177.
40. Kirschner J, Lochmüller H. Sarcoglycanopathies. *Handb Clin Neurol*. 2011;101:41–46.
41. Mercuri E, Bönnemann CG, Muntoni F. Muscular dystrophies. *Lancet*. 2019;394:2025–2038.
42. Rosenberg AS, Puig M, Nagaraju K, et al. Immune-mediated pathology in Duchenne muscular dystrophy. *Sci Transl Med*. 2015;7:299rv294.
43. Panicucci C, Baratto S, Raffaghello L, et al. Muscle inflammatory pattern in alpha- and gamma-sarcoglycanopathies. *Clin Neuropathol*. 2021;40:310–318.
44. Górecki DC. P2X7 purinoceptor as a therapeutic target in muscular dystrophies. *Curr Opin Pharmacol*. 2019;47:40–45.
45. Villalta SA, Rosenberg AS, Bluestone JA. The immune system in Duchenne muscular dystrophy: Friend or foe. *Rare Dis*. 2015;3:e1010966.
46. Al-Khalidi R, Panicucci C, Cox P, et al. Zidovudine ameliorates pathology in the mouse model of Duchenne muscular dystrophy via P2RX7 purinoceptor antagonism. *Acta Neuropathol Commun*. 2018;6:27.
47. Tung EK, Choi RC, Siow NL, et al. P2Y2 receptor activation regulates the expression of acetylcholinesterase and acetylcholine receptor genes at vertebrate neuromuscular junctions. *Mol Pharmacol*. 2004;66:794–806.
48. De Oliveira Moreira D, Santo Neto H, Marques MJ. $P2Y_2$ purinergic receptors are highly expressed in cardiac and diaphragm muscles of mdx mice, and their expression is decreased by suramin. *Muscle Nerve*. 2017;55:116–121.
49. Chen M, Chen H, Gu Y, et al. P2Y2 promotes fibroblasts activation and skeletal muscle fibrosis through AKT, ERK, and PKC. BMC. *Musculoskelet Disord*. 2021;22:680.
50. Stadelmann C, Di Francescantonio S, Marg A, Müthel S, Spuler S, Escobar H. mRNA-mediated delivery of gene editing tools to human primary muscle stem cells. *Mol Ther Nucleic Acids*. 2022;28:47–57.