MUSCULOSKELETAL PATHOLOGY

Enhancement of Muscle T Regulatory Cells and Improvement of Muscular Dystrophic Process in *mdx* Mice by Blockade of Extracellular ATP/P2X Axis

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Infiltration of immune cells and chronic inflammation substantially affect skeletal and cardiac muscle degeneration in Duchenne muscular dystrophy. In the immune system, extracellular adenosine triphosphate (ATP) released by dying cells is sensed as a danger associated molecular pattern through P2 purinergic receptors. Specifically, the P2X7 subtype has a prominent role in regulating immune system physiology and contributes to inflammasome activation also in muscle cells. Here, we show that *in vivo* blockade of the extracellular ATP/P2X purinergic signaling pathway by periodate-oxidized ATP delayed the progression of the dystrophic phenotype and dampened the local inflammatory response in *mdx* mice, a spontaneous mouse model of dystrophin deficiency. Reduced infiltration of leukocytes and macrophages and decreased expression of IL-6 were revealed in the muscles of periodate-oxidized ATP-treated *mdx* mice. Concomitantly, an increase in Foxp3⁺ immunosuppressive regulatory T cells was observed and correlated with enhanced myofiber regeneration. Moreover, we detected reduced concentrations of profibrotic cytokines, including transforming growth factor- β and connective tissue growth factor, in muscles of periodate-oxidized ATP-treated *mdx* mice. The improvement of inflammatory features was associated with increased strength and reduced necrosis, thus suggesting that pharmacologic purinergic antagonism altering the adaptive immune component in the muscle infiltrates might represent a promising therapeutic approach in Duchenne muscular dystrophy. (*Am J Pathol* 2015, 185: 3349–3360; <http://dx.doi.org/10.1016/j.ajpath.2015.08.010>)

Duchenne muscular dystrophy (DMD) is a devastating inherited disease of childhood, characterized by progressive muscle degeneration and weakness, leading to loss of ambulation in the early second decade of life and mortality in the thirties.¹ The primary cause of this disorder is mutations in the *DMD* gene, which encodes a 427-kDa protein (dystrophin) found throughout the cytoplasmic face of the plasma membrane in both skeletal and cardiac muscles.¹

Dystrophin binds distinct members of the dystrophin glycoprotein complex, forming a mechanical-signaling link from the extracellular matrix to the cytoskeleton.² Mutations in dystrophin result in a structurally weaker plasma membrane, which is more easily damaged during muscle contraction,

causing necrosis, massive infiltration of immune cells, chronic inflammation, necrosis, and muscle degeneration.^{2,3} The secondary processes that involve persistent inflammation and impaired regeneration aggravate the disease progression and ultimately lead to connective tissue replacement.

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Depletion of CD4⁺ and CD8⁺ T cells,⁴ macrophages,⁵ eosinophils, and neutrophils⁶ or treatment with immunosuppressive drugs^{7–11} have all been shown to improve pathogenic features of the disease. Despite these findings to indicate that the immune system critically contributes to the functional impairment of DMD patients, the molecular mechanisms that initiate and perpetuate inflammation in DMD are poorly defined.

An inflammatory response in the absence of infectious agents is initiated primarily by damage-associated molecular patterns (DAMPs).¹² DAMPs are endogenous molecules, which are virtually absent in the extracellular space in physiologic conditions; once released into the extracellular space by injured cells they display disparate functions. Extracellular adenosine triphosphate (eATP) and its derivatives are pleiotropic regulators of mammalian cell function,¹³ although they act as DAMPs when massively released by dying cells. eATP activates plasma membrane receptors for extracellular nucleotides termed P2 receptors. P2X1 to P2X7 receptors open nonselective ion channels, whereas P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 to P2Y14 are guanine nucleotide-binding protein (G protein)-coupled receptors, which bind also ADP, UDP, UTP, or UDP-glucose.¹³ Activation of P2 receptors regulates many cellular functions that range from survival and proliferation to apoptosis. The final effect of eATP on a given cell depends, therefore, on the composition of P2 receptors expressed on its surface.^{14,15}

In the immune system, P2X7 receptor was extensively investigated because its activation in mouse macrophages by exogenous ATP contributes to inflammasome activation and is required as a second signal for IL-1 β processing and secretion.¹⁶ Primary muscle cells express various P2X subtypes in physiologic conditions, and substantial up-regulation of P2X7 was immunodetected in skeletal muscle from *mdx* mice (a spontaneous mouse model of dystrophin deficiency) and in myoblasts isolated from patients affected by DMD or by dysferlin deficiency.^{17–21} Moreover, exposure to eATP of myoblasts from *mdx* or dysferlin knockout mice triggers an increase in cytosolic Ca²⁺ ions and release of IL-1 β , suggesting that muscle cells can also actively participate in the inflammatory process through purinergic signaling.²¹

We have previously shown that P2XR activity affects the immunosuppressive potential of regulatory T cells (Tregs).²² Interestingly, Tregs were recently shown to inhibit the progression of muscle damage and to improve regeneration in *mdx* mice.^{23,24} Thus, in the present study we addressed whether inhibition of P2X receptors could beneficially impinge on the *in vivo* inflammatory response associated with dystrophin deficiency and enhance Treg function.

We therefore treated *mdx* mice with periodate-oxidized ATP (oATP), a compound that irreversibly antagonizes P2X receptors through the selective modification of lysine residues in the vicinity of the ATP binding site.²⁵ Our results point to a role of eATP in contributing to the immunopathologic damage of dystrophic muscle and to a

possible beneficial effect of pharmacologic purinergic antagonism in DMD.

Material and Methods

Muscle Biopsies

All human samples were obtained after patients had signed informed consent forms in accordance with the guidelines of the Istituto Giannina Gaslini Ethical Committee, including experimental protocols for muscle biopsies.

Total RNA was isolated from muscle biopsies obtained for diagnostic purposes from seven and three patients with confirmed DMD and Becker muscular dystrophy, respectively. Hematoxylin and eosin (H&E)-stained sections were scored for the presence of fibrosis, degeneration/regeneration, and inflammatory infiltrates, according to arbitrary criteria, by two independent pathologists.

In Vivo Treatment with oATP

B6Ros.cG-*Dmd*^{*mdx-4Cv*}/J (*mdx-4Cv*, males, 4 weeks old; Charles River Laboratories, Wilmington, MA) and wild-type (WT) C57Bl/6J mice were treated with 100 μ L oATP at 6 mmol/L or phosphate-buffered saline (PBS) alone every day for 2 or 4 weeks by i.p. injection and were sacrificed by carbon dioxide inhalation ($n = 4$ to 8 mice for each experimental group). A group of WT age-matched C57Bl/6J male mice was used as internal control ($n = 5$). The *mdx-4Cv* mice were randomly assigned to experimental groups, and testing of samples collected from the studies was performed blindly (S.B. and E.G.).

The blood samples were collected by periorbital puncture at the end of the second and fourth week of treatment and centrifuged for 10 minutes at 7000 $\times g$. Immediately after centrifugation, the serum was isolated and stored at -20°C . Creatine kinase (CK) concentrations were measured with the CK-NAC quantitative system according to the manufacturer's instructions (BPC BioSED, Rome, Italy).

All animal experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and authorized by the Animal Studies Committee of Cantonal Veterinary.

Forced Treadmill Running

Mice were subjected to chronic treadmill running with the use of an Exer 3/6-treadmill system (Columbus, OH) according to standard operating procedures.^{26,27} Briefly, oATP-, PBS-treated *mdx-4Cv*, and WT control mice were acclimated to the treadmill for 3 days and then challenged with treadmill running twice a week for a total of 4 weeks. Each session lasted 40 minutes, including 10 minutes of warm-up at the speed of 8 miles per minute, followed by 30 minutes of running at a speed of 12 miles per minute on a horizontal treadmill (plus the extra time of the stops). At the

end of the second and fourth week of treatment, the number of stops for each experimental group was measured.

Hanging Wire Tests

At the conclusion of the second and fourth week of treatment, the muscle strength of oATP-, PBS-treated *mdx*-4Cv, and WT control mice was scored with the hanging wire analysis through the longest suspension time-method. In the experiment, all of the mice had to hang for three trials, and the average maximum hanging time of the three trials was measured.²⁸

Vital Staining with Evans Blue Dye

To detect damaged muscle fibers, 20 hours before the sacrifice Evans Blue Dye (EBD; stock solution; 10 mg/mL in PBS) was injected (0.1 mL/10 g body weight) into the peritoneal cavity of each mouse.²⁹ After sacrifice, the gastrocnemii (GN) were excised, immediately frozen in nitrogen-cooled isopentane, and stored for further analyses. Frozen sections, obtained by cutting the muscle on a cryostat, were observed under an Axioplan M2 Imager microscope (Zeiss, Göttingen, Germany) to detect the EBD red autofluorescence signal. EBD⁺ fibers were quantified in two sections for each muscle ($n = 5$ mice for each experimental group) and expressed as number per mm² of total area.

Reagents and Antibodies

oATP (Sigma-Aldrich, St. Louis, MO; A6779-25MG) was reconstituted at a final concentration of 100 mmol/L in PBS and stored at -80°C ; the reconstituted drug was thawed and diluted in PBS at 6 mmol/L immediately before use.

The antibodies and dilutions used in the study include the following: rabbit polyclonal antibody to IL-6 (dilution 1:1000 for Western blot analysis, WB) from Abcam (Cambridge, UK). Purified rat monoclonal antibodies to CD45 [dilution 1:10 for immunohistochemistry (IHC)] was from BD Pharmingen (San Jose, CA). Purified rat antibody to Foxp3 (dilution 1:20 for IHC) was from eBioscience (San Diego, CA). Rat monoclonal antibody to F4/80 (dilution 1:100 for IHC), mouse monoclonal antibody to embryonal myosin heavy chain (e-MYHC) 1/2/3 (dilution 1:100 for IHC), mouse monoclonal antibody to myogenin (dilution 1:100 for IHC), rabbit polyclonal antibody to glyceraldehyde phosphate dehydrogenase (GAPDH; dilution 1:500 for WB), and goat polyclonal antibody to connective tissue growth factor (CTGF; dilution 1:500 for WB) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to CD3 (dilution 1:20 for IHC) was from Dako (Glostrup, Denmark). Rabbit polyclonal antibody to transforming growth factor (TGF)- β (dilution 1:1000 for WB) was from Cell Signaling Technology (Danvers, MA). Rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated (dilution 1:700 for WB) was from Dako, and donkey anti-rabbit IgG HRP-conjugated (dilution 1:5000 for WB) was from GE Healthcare (Milan, Italy). EnVision Detection

System Peroxidase/3'-3'-diaminobenzidine, rabbit and mouse, were from Dako.

Histologic Studies

Sections (7 μm) from GN and anterior tibialis (A.Tib.) muscles isolated from PBS-, oATP-*mdx*-4Cv, and WT control mice were cut on a cryostat and later stained with H&E. Cross-sectional areas (CSAs) and central nucleation were quantified in a blinded manner (S.B. and E.G.) in three sections for each muscle ($n = 5$ mice for each experimental group, $n = 400$ to 600 myofibers) with ImageJ software version 1.45s (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) under an Axioplan M2 Imager microscope. Fiber size variability was calculated according to the formula: $\text{SD} \times 1000/\text{mean fiber diameter}$.²⁹

Paraffin-embedded diaphragms isolated from PBS-, oATP-*mdx*-4Cv, and WT control mice were cut in cross section at the mid-belly of the muscle (7- μm thickness). After deparafinization the slides were stained with H&E.²⁹

IHC

Five-micron thick cryosections of GN and A.Tib. muscles isolated from PBS-, oATP-*mdx*-4Cv, and WT control mice were fixed in acetone at -20°C for 10 minutes, washed two times in PBS, and then in bovine serum albumin (BSA) 4% in PBS for 5 minutes. Sections were incubated in 3% hydrogen peroxide in BSA 8% in PBS for 10 minutes at room temperature and washed for 5 minutes in BSA 8% in PBS. After two washes in BSA 4% in PBS (blocking solution), sections were permeabilized in 0.3% Triton X-100 in BSA 4% in PBS and incubated with primary antibodies (for CD45, F4/80, Foxp3, and CD3) diluted in blocking solution overnight at 4°C . The next day, sections were washed three times with BSA 4% in PBS and then incubated with Envision secondary antibody. After being rinsed three times with PBS (5 minutes each), the sections were stained with a diaminobenzidine substrate solution (freshly made just before use), counterstained with hematoxylin, washed with deionized water, dehydrated through five changes of alcohol (70%, 90%, 95%, 100%, and 100%) for 1 minute each, cleared in two changes of xylene (3 minutes each), and mounted with a Vectashield mounting medium (Vector Laboratories; Burlingame, CA). Slides were observed under a Leica Diaplan microscope (Leica, Wetzlar, Germany). The number of positive cells was blindly counted on the whole sections per mouse (S.B. and E.G.). The total area of the section was measured and expressed in mm².

qPCR

Total RNA was extracted from GN muscles (from 20 to 55 mg) isolated from PBS-, oATP-*mdx*-4Cv, and WT control mice or from fragments of human muscle biopsies with the use of the RNeasy Lipid Tissue Mini Kit (Qiagen, Milan,

Table 1 qPCR Human and Murine Probes

Probe	Catalog number
hP2RX1	Hs00175686_m1
hP2RX2	Hs04176268_g1
hP2RX3	Hs01125554_m1
hP2RX4	Hs00602442_m1
hP2RX5	Hs01112471_m1
hP2RX6	Hs01003997_m1
hP2RX7	Hs00175721_m1
hGAPDH	4333764F
hACTA1	Hs00559403_m1
mP2RX1	Mm00435460_m1
mP2RX2	Mm01202368_g1
mP2RX3	Mm00523699_m1
mP2RX4	Mm00501787_m1
mP2RX5	Mm00473677_m1
mP2RX6	Mm00440591_m1
mP2RX7	Mm00440578_m1
mGAPDH	4352932E
mACTA1	Mm00808218_g1

qPCR, quantitative real-time PCR.

Italy) and according to the manufacturer's protocol (DNase treatment). Quality and quantity of RNA were analyzed with a NanoDrop spectrophotometer.

The cDNA was synthesized from 350 ng of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Milan, Italy). Each RNA sample was controlled for genomic DNA contamination without reverse transcriptase addition into cDNA synthesis mixture. For *Foxp3* and *Amphyregulin* (*Areg*) transcript quantification, quantitative real-time PCR (qPCR) was performed in triplicate with the 2× Sso Fast EvaGreen Supermix (Bio-Rad Laboratories) in CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories).

Briefly, the 15-μL PCR mixture contained diluted cDNA that corresponded to 8.75 ng of total RNA and 0.2 mmol/L of each primer. Relative expression levels were normalized with the comparative Ct ($\Delta\Delta C_t$) method with the housekeeping gene (*Gapdh*) by the Bio-Rad CFX manager software version 3.1. For each specific primer set, the efficiency was >95%, and a single product was seen on the melting curve analysis. Specific primers for *Areg* (NM_009704), *Foxp3* (NM_001199347), and *Gapdh* (NM_008084) were designed through Beacon Designer 2.0 Software (Premier Biosoft, Palo Alto, CA). *Areg*: 5'-AA-GGAGGCTTCGACAAGAAA-3' (forward), 5'-TTTACCCT-GCATTTGCTCTCA-3' (reverse); *Foxp3*: 5'-CAGTCAAAG-AGCCCTCACAA-3' (forward), 5'-AAGGCAGGCTCTTC-ATGTTT-3' (reverse); *Gapdh*: 5'-AACTTTGGCATTGTGG-AAGG-3' (forward), 5'-CCATCCACAGTCTTCTGGGT-3' (reverse).

For human and mouse P2X receptor quantification, qPCR was performed in triplicate with the 2× Sso Fast Probes Supermix (Bio-Rad Laboratories) in CFX96 Real-time PCR Detection System (Bio-Rad Laboratories). The 15-μL PCR mixture contained diluted cDNA that corresponded to 12.5 ng of total RNA, and premixed primer/probe sets were

ordered from Applied Biosystems, Foster City, CA (Table 1). Relative expression levels were normalized with the $\Delta\Delta C_t$ method with the housekeeping gene *Gapdh* and *actin, $\alpha 1$* , skeletal muscle (*ACTA1*, a muscle-specific gene NM_001272041.1), by the Bio-Rad CFX manager software.

Western Blot Analysis

Tissue samples were prepared on ice as follows: five to eight 10-μm-thick cryosections of GN and A.Tib. muscles isolated from PBS-, oATP-*mdx*-4Cv, and WT control mice were quickly dissolved in 40 μL of RIPA lysis buffer (Sigma-Aldrich) with 1:200 protease inhibitor cocktail (Sigma-Aldrich). Tissue lysates were then sonicated for 10 seconds on ice and centrifuged at $10,621 \times g$ for 15 minutes at 4°C. Supernatant fluids were collected, and the protein concentration was determined with the Bradford protein assay method (Bio-Rad Laboratories). Equal amounts of protein (40 to 120 μg) were mixed with SDS gel-loading buffer (100 mmol/L Tris-HCl, pH 6.8, 400 mmol/L dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), resolved in 6% to 15% SDS-PAGE gel, and run at

Figure 1 P2X receptors 4 and 7 are up-regulated in muscle tissue from patients affected by BMD and DMD as well as in GN from *mdx*-4Cv mice. **A** and **B**: Total RNA isolated from the muscle biopsies of three BMD (B1 to B3) and seven DMD (D1 to D7) patients and three controls, and from the GN of five *mdx*-4Cv and four age- and sex-matched WT mice was reverse transcribed. P2X4 and P2X7 transcripts were quantified by real-time PCR. GAPDH and ACTA1 served as control. The data are expressed as folds versus the mean of controls. Statistical analysis was performed with the *U* test. **P* < 0.05, ***P* < 0.01. ACTA1, actin, $\alpha 1$; BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GN, gastrocnemii; WT, wild-type.

Figure 2 oATP ameliorates CK serum concentrations in resting and exercised *mdx*-4Cv mice. **A:** Serum CK concentrations were evaluated in the short-term (2 weeks) experiment. Blood samples were obtained from *mdx* oATP, *mdx* PBS, and age-matched WT mice at the end of the treatment. **B:** Serum CK concentrations were evaluated in the 4-week experiment when all of the experimental groups were subjected to chronic exercise. Blood samples were obtained from *mdx* oATP, *mdx* PBS, and age-matched WT mice at the end of the second and fourth week of treatment. Each box represents the median with first and third quartile. $n = 4$ to 8 mice (**A**); $n = 5$ mice (**B**). Statistical analysis was performed with the *U* test. $*P < 0.05$. CK, creatine kinase; *mdx* oATP, oATP-treated *mdx*-4Cv; *mdx* PBS, untreated *mdx*-4Cv; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; WT, wild-type.

80 V for 2 to 2.30 hours in electrophoresis buffer (25 mmol/L Tris, 250 mmol/L glycine, and 0.1% SDS). Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon PVDF; Millipore, Milan, Italy). Membranes were blocked with 5% BSA in PBS/0.1% Tween 20. Blots were then incubated for 2 hours at room temperature with primary antibodies all diluted in 1% BSA/PBS. HRP anti-IgG was used to visualize bound primary antibodies with the chemiluminescence system (Bio-Rad Laboratories). Band intensities were evaluated by densitometry with the use of the ImageQuant 350 system (Amersham Biosciences, Piscataway, NY).

Statistical Analysis

Most of the analyzed variables were not normally distributed as confirmed by the Shapiro-Wilk test; therefore, differences of quantitative parameters between two groups of mice were analyzed by the nonparametric *U* test. $P < 0.05$ was considered as statistically significant. Data are expressed as median with first and third quartiles (Qs) as described in [Results](#) and the figure legends.

For the distribution of CSAs, the number of categories and range between each class was calculated according to Sturges formula. Differences between the axonal diameters corresponding to each row of bin limits were evaluated by the χ^2 test with a further post hoc correction (Bonferroni) to avoid multiple comparison errors.

The analysis of homogeneity between variances in myofiber areas between PBS- and oATP-treated mice was calculated by Hartley's F_{Max} test.

All correlations were assessed with Spearman's rank order correlation coefficient (rs). Correlation coefficients were classified as follows: $rs < 0.4$ = poor, ≥ 0.4 to 0.80 = moderate, and ≥ 0.80 = good correlation.³⁰

Statistica software version 9.1 (StatSoft Co., Tulsa, OK) was used for all of the statistical analyses. Graphs

were generated with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

Results

Targeting P2X Signaling *in Vivo* Improves Biochemical and Functional Parameters of Disease Progression in *mdx* Mice

We initially characterized the pattern of expression of the different P2X receptors in patients with different degrees of dystrophin deficiency. P2X1 to P2X7 transcript levels were quantified in the muscle biopsies of three patients with Becker muscular dystrophy, the milder allelic variant of DMD, and of seven DMD cases. The severity of disease was scored according to arbitrary histologic criteria ([Supplemental Table S1](#)). In accordance with previous studies, P2X4 and P2X7 transcripts were significantly up-regulated in all patients, although at different levels: milder Becker muscular dystrophy cases displayed a 1.6- to 4.3-fold increase, whereas the most severe DMD patients showed up to a 10.8- to 13-fold induction ([Figure 1A](#) and [Supplemental Table S1](#)).^{18–20} Analogously, P2X4 and P2X7 were increased in GN isolated from *mdx*-4Cv mice and WT sex- and age-matched controls ([Figure 1B](#)).

To be able to achieve an antagonistic effect on both P2X receptors, we hence chose to treat *mdx*-4Cv mice with oATP, which was originally described as an irreversible P2X7 antagonist but was shown later to block also all other P2X receptor subtypes, resulting in the improvement of immunopathology in several inflammatory mouse models.^{31–34}

The efficacy of the compound was first tested at 6 mmol/L intraperitoneally in a short-term treatment (2 weeks) of 4-week-old *mdx*-4Cv male mice. For these experiments, we chose as an end point serum CK concentrations because they reflect and correlate with active muscle degeneration. Intriguingly, oATP treatment led to a 44% reduction in serum CK in PBS-*mdx*-4Cv

Figure 3 oATP improves muscle strength and fatigue resistance of *mdx*-4Cv mice. **A:** Hangwire test was performed on *mdx* oATP, *mdx* PBS, and age-matched WT control mice at the end of the second and fourth week of treatment. **B:** Treadmill test was performed on *mdx* oATP, *mdx* PBS, and age-matched WT control mice throughout the length of treatment. At the end of the second and fourth week the number of stops were measured by the experimenter. Each box represents the median with first and third quartile. $n = 5$ mice. Statistical analysis was performed with the *U* test. $*P < 0.05$. *mdx* oATP, oATP-treated *mdx*-4Cv; *mdx* PBS, untreated *mdx*-4Cv; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; WT, wild-type.

(median, 10,740 U/L; first to third Q, 7460 to 33,480 U/L; $n = 5$) and oATP-*mdx*-4Cv (median, 6030 μ M/L; first to third Q, 4465 to 11,195 U/L; $n = 10$; $P < 0.043$) (Figure 2A).

Hence, we treated exercised mice for four consecutive weeks at the same dose. *Mdx*-4Cv mice injected with vehicle alone and a group of age- and sex-matched WT C57Bl/6 mice injected with vehicle alone or oATP served as controls. According to the standard operating procedure of preclinical drug testing in the *mdx* mouse model, all of the experimental groups were subjected to chronic exercise by treadmill running throughout the treatment period.²⁶ The mice were weighed at the beginning (baseline) of the treatment and then on a weekly basis. We did not detect any substantial difference between the various groups (data not shown).

Serum CK concentrations were evaluated at midpoint and at the end of treatment; at 2 weeks, oATP decreased serum CK concentrations by 44% (PBS-*mdx*: median, 2530 U/L; first to third Q, 2225 to 3470 U/L; $n = 5$; oATP-*mdx*-4Cv: median, 1420 U/L; first to third Q, 945 to 1775 U/L; $n = 5$; $P < 0.030$). The effect was maintained at 4 weeks with a 42% reduction in the oATP-treated group (PBS-*mdx*: median, 3100 U/L; first to third Q, 2315 to 4920 U/L; $n = 5$; oATP-*mdx*-4Cv: median, 1800 U/L; first to third Q, 1610 to 1880 U/L; $n = 5$) (Figure 2B). We did not observe any effect of oATP on serum CK concentrations in the WT group

(PBS-WT: median, 545 U/L; first to third Q, 440 to 600 U/L; $n = 4$; oATP-WT: median, 655 U/L; first to third Q, 575 to 670 U/L; $n = 4$).

The evaluation of fatigue resistance and muscle strength at the end of the second and fourth week of treatment revealed that oATP resulted into an improved functional performance by hangwire testing and aerobic capacity (number of stops during running test) compared with PBS-treated mice (Figure 3, A and B).

Targeting P2X Signaling *in Vivo* Ameliorates Structural Features in *mdx* Mice

Histologic analyses by H&E staining were completed on GN, A.Tib., and diaphragms. A degenerated muscle status in PBS-treated *mdx*-4Cv mice was revealed, with different signs of myopathic damage, including muscle fiber degeneration with centralized nuclei, increased variability in fiber diameter, and presence of inflammation. P2X antagonism resulted in a decrease in the number and area of the inflammatory reactions surrounding the muscle cells in necrosis, which reached statistical significance in A.Tib. (Figure 4, A and B, and Supplemental Table S2). The beneficial effect of purinergic antagonism was associated with a reduction of centralized nuclei in all investigated muscular sites (Figure 5A and Supplemental Table S3). When the distribution of frequencies of myofiber CSAs was investigated, in oATP-treated mice we observed a decrease of the heterogeneity of CSAs that was observed in PBS-treated *mdx*-4Cv mice, in which the largest diameters were a sign of degeneration (pathologic hypertrophy) (Figure 5B). Indeed, in the oATP group, CSA values showed a shift toward those observed in WT mice (Figure 5B) and displayed a 12.8% (A.Tib.) to a 36.7% (diaphragms) decrease of fiber size variability, $P < 0.01$ (Supplemental Table S3).

To further analyze the extent of muscle membrane damage and myofiber degeneration, we used the EBD agent. This compound is a membrane-impermeant molecule that binds to serum albumin and is physically restricted from fibers unless the integrity of the skeletal muscle membrane is disrupted.²⁷ When analyzed under the microscope, EBD emits a red auto-fluorescent signal. Hence, we injected EBD intraperitoneally in PBS- and oATP-treated *mdx*-4Cv mice at 20 hours before the sacrifice. The strong red fluorescent signal detected in GN frozen sections was reduced in oATP-treated mice by a 48% (PBS-*mdx*-4Cv: median, 0.95 EBD⁺ myofibers/mm²; first to third Q, 0.80 to 1.61 EBD⁺ myofibers/mm²; $n = 5$; oATP-*mdx*-4Cv: median, 0.50 EBD⁺ myofibers/mm²; first to third Q, 0.21 to 1.08 EBD⁺ myofibers/mm²; $n = 5$) (Figure 6).

Targeting P2X Signaling *in Vivo* Reduces the Severity of the Inflammatory Innate Response

The effect of oATP on the composition of the immune cells of the reactive inflammatory infiltrates was evaluated by serial immunostainings of GN and A.Tib. muscles with anti CD45 (lympho/monocytic marker) and anti F4/80 (macrophage

Figure 4 oATP improves the extension of reactive inflammatory infiltrates in *mdx*-4Cv mice. **A:** Frozen GN, A.Tib., and Diaph. muscle tissue sections from *mdx* oATP and *mdx* PBS mice were collected at the fourth week of treatment and stained with standard H&E technique. **White arrows** indicate inflammatory infiltrates. Representative images are shown. **B:** Graphical output of the areas and number of inflammatory infiltrates/mm² of total section area of muscles represented in **A**. Each box represents the median with first and third quartile. *n* = 5 mice for each experimental group. Statistical analysis was performed with the *U* test. ***P* < 0.01. Scale bar = 50 µm. Original magnification, ×10. A.Tib., anterior tibialis; Diaph., paraffin-embedded diaphragm; GN, gastrocnemii; H&E, hematoxylin and eosin; *mdx* oATP, oATP-treated *mdx*-4Cv; *mdx* PBS, untreated *mdx*-4Cv; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; WT, wild-type.

marker) antibodies. Positive cells were manually counted in the whole area of the tissue section for each mouse and expressed as number of cells per square millimeter (mm²). Interestingly and consistently with previous observations in chronic inflammatory diseases, administration of oATP led to a 43% decrease in the number of muscle-infiltrating CD45⁺ cells and a 31% reduction of F4/80⁺ macrophages (Figure 7, A and B, and Supplemental Table S4).^{31–34} The beneficial effects of oATP on tissue inflammation were also reflected on IL-6 protein concentrations, which were significantly reduced by immunoblot in oATP-treated mice (Figure 7C).

Treg Expansion in oATP-Treated *mdx* Mice and Improvement of Muscle Regeneration

We observed a 24% reduction of muscle-infiltrating CD3⁺ T cells in oATP-treated mice (Figure 8, A and B, and

Supplemental Table S4). However, despite the muscle T-cell decrease, Foxp3⁺ cells were significantly increased by IHC in *mdx*-4Cv mice treated with oATP compared with *mdx*-4Cv mice treated with PBS, indicating that pharmacologic P2X antagonism resulted in Treg enrichment in *mdx* skeletal muscle (Figure 8, A and B, and Supplemental Table S4). The increase of Tregs among infiltrating T cells was further confirmed by the quantification of the transcript levels of Foxp3 and Areg, a growth factor that is expressed by a specialized population of Tregs detected in muscle tissue. Areg is up-regulated during inflammation and participates in defensive and repair mechanisms (the so-called immune type 2 responses) (Figure 8C).^{23,35,36}

Because Areg was shown to display a direct effect on myofiber regeneration, the number of regenerative cells in the two experimental groups was quantitated by immunostaining for e-MYHC. e-MYHC⁺ myofibers were manually

counted in the whole area of the sections for each mouse and expressed as number of regenerative cells per square millimeter (mm^2). Notably, oATP induced a 10- and 3.4-fold increase in the number of regenerative cells, respectively, in the GN and A.Tib. muscles (Figure 9,

A and B, and Supplemental Table S5), and the number of Foxp3⁺ and eMYHC⁺ cells significantly correlated ($r_s = 0.80$; $n = 4$ mice per experimental group).

In accordance to the e-MYHC results, diaphragms isolated from oATP-treated mice displayed a 2.6-fold increase of cells positive for myogenin (a marker of early differentiating myoblasts) (Figure 9, A and B).

Conversely to this pro-regenerative image, P2X blockade led to a reduction of fibrogenic factors, which ultimately stimulate endomysial fibrosis and connective replacement of muscle tissue. TGF- β and CTGF protein levels were quantified in total muscle lysates isolated from GN of WT, PBS-, and oATP-treated mice ($n = 3$ for each experimental group). As expected, TGF- β protein levels were increased in untreated *mdx*-4Cv mice compared with WT mice.³⁷ However, this effect was reduced by 40% in the oATP-treated group. A similar result was observed for CTGF, one downstream effector of TGF- β , which was reduced by 60% (Figure 9C). These results suggest that pharmacologic P2X antagonism can improve dystrophic muscle regeneration and correlates with enhanced muscle Treg function.

Discussion

Appreciation is growing that, in DMD, the muscle membrane instability caused by dystrophin–dystrophin glycoprotein complex alterations and the consequent necrotic cell death result in the release of intracellular DAMPs. These DAMPs in turn recruit inflammatory cells with consequent oxidation of extracellular debris, internalization and presentation of antigens by professional antigen-presenting cells, and finally adaptive immune system activation.³⁸ In muscle, ATP abundance inside cells and its virtual absence in the interstitial space render it an ideal harbinger of tissue damage. Here, we have provided evidence that pharmacologic inhibition of eATP signaling via P2X receptors improves muscular function and structure and enhances myofiber regeneration in *mdx* mice. The beneficial effect exerted by purinergic blockade is associated with inhibition of the innate immune response and shaping of the muscular T-cell pool composition with selective increase of the Treg subset.

Figure 5 oATP ameliorates dystrophic features in *mdx*-4Cv mice. Frozen GN, A.Tib., and Diaph. muscle tissue sections from *mdx* oATP and *mdx* PBS mice were collected at the fourth week of treatment and stained with standard H&E technique. **A:** The number of central nuclei was quantified in 600 fibers of three consecutive sections for each muscle in GN, A.Tib., and Diaph. and expressed as percentage of total nuclei. **B:** The distribution of CSA values was evaluated in three consecutive sections in GN, A.Tib., and Diaph. muscles from oATP-treated (green line), untreated (red line) *mdx*-4Cv, and WT mice (blue line). $n = 5$ mice for each experimental group. Statistical analysis was performed with the χ^2 test with Bonferroni correction. $*P < 0.05$, $**P < 0.01$. A.Tib., anterior tibialis; CSA, cross-sectional area; Diaph., paraffin-embedded diaphragm; GN, gastrocnemii; H&E, hematoxylin and eosin; *mdx* oATP, oATP-treated *mdx*-4Cv; *mdx* PBS, untreated *mdx*-4Cv; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; WT, wild-type.

Figure 6 oATP reduces muscle degeneration and necrotic features in *mdx*-4Cv mice. **A:** EBD agent was injected intraperitoneally in untreated (saline only) and oATP-treated *mdx* mice 20 hours before the sacrifice to evaluate muscle fiber damage. Frozen GN muscle sections were examined by fluorescence microscopy. Damaged myofibers were visualized by a strong red auto-fluorescent signal that was reduced in the oATP group. Representative images are shown. **B:** EBD⁺ muscle fibers were quantified in the whole area of two sections for each muscle and expressed as number per mm² of total section area. Each box represents the median with first and third quartile. *n* = 5 mice for each experimental group. Statistical analysis was performed with the *U* test. **P* < 0.05. Scale bar = 50 μ m. Original magnification, $\times 10$. EBD, Evans Blue Dye; GN, gastrocnemii; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; WT, wild-type.

In the immune system P2X7 activation contributes to inflammasome activation and determines the release of inflammatory cytokines, including IL-1 β and IL-18. Indeed, P2X antagonists reduce IL-1 β release by lipopolysaccharide-activated macrophages and inhibit reactive oxygen species production and phagocytosis.^{39–41}

Remarkably, in addition, muscle cells express key components of the inflammasome pathway, suggesting that they can exert a direct role in the development and maintenance of the inflammatory response. In primary myoblasts isolated from dysferlin-deficient patients and mice, eATP triggers P2X7-mediated activation of phosphatidylcholine-specific phospholipase C and A2 and secretion of IL-1 β .²¹ Our results indicate a pathogenetic potential of eATP in *mdx* mice as suggested by the improvement of muscular strength and structure by systemic oATP administration associated with a substantial reduction of inflammatory infiltrates and a decrease of IL-6 in muscles.

However, in addition to the well-documented role of P2X7 in inflammasome activation in cells of the innate immune system,

P2X receptors were shown to participate in a feed-forward loop, which contributes to productive T-cell activation. This signaling is inhibited by oATP, which dampens proinflammatory T-cell differentiation and ameliorates immunopathologic tissue damage in several experimental models of autoimmunity.^{29–32} In addition, oATP determines the functional polarization of naive CD4 cells to adaptive Tregs on T-cell receptor stimulation.^{22,42,43}

In *mdx* mice blockade of the eATP/P2X axis resulted in a 24% decrease of CD3⁺ cells and an 1.8-fold increase of Foxp3⁺ cells in muscle, indicating that purinergic antagonism affected the composition of the T-cell pool in dystrophic muscles and increased the abundance of proregenerative Tregs. In the context of the immune response, Foxp3-expressing Tregs are specialized CD4 cells, which crucially contribute to tissues integrity and whose dysfunction was linked to several immunopathologic conditions.⁴⁴ Tregs are also characterized by the combined expression of CD39 and CD73 ectonucleotidases,

Figure 7 Effects of oATP on innate immunity in *mdx*-4Cv mice. **A:** Frozen GN and A.Tib. muscle sections from *mdx* oATP and *mdx* PBS mice were immunostained with primary antibody directed toward CD45 and F4/80 (white arrows). Representative images of GN are shown. **B:** In GN, CD45⁺ and F4/80⁺ fibers were quantified in the whole area of the tissue section and expressed as number of positive cells/mm² of section area. **C:** GN skeletal muscle lysates from *mdx* oATP and *mdx* PBS were subjected to immunoblot analysis with specific antibodies against IL-6. Determination of GAPDH expression was used to normalize sample loading. Representative images are shown. Each column represents the mean percentage change in oATP-treated versus untreated *mdx*-4Cv mice. *n* = 5 mice for each experimental group (**B**); *n* = 3 mice for each experimental group (**C**). Statistical analysis was performed with the *U* test. Scale bar = 20 μ m. Original magnification, $\times 20$. A.Tib., anterior tibialis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GN, gastrocnemii; *mdx* oATP, oATP-treated *mdx*-4Cv; *mdx* PBS, untreated *mdx*-4Cv; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; WT, wild-type.

Figure 8 Effects of oATP on adaptive immunity in *mdx*-4Cv mice. **A:** Frozen GN and A.Tib. muscle sections from *mdx* oATP and *mdx* PBS were immunostained with primary antibody directed toward CD3 (black arrows) and Foxp3 (black arrowheads). Representative images of GN are shown. **B:** In GN, CD3⁺ and Foxp3⁺ lymphocytes were quantified in the whole area of the tissue section and expressed as number of positive cells/mm² of section area. Each column represents the mean percentage change in oATP-treated versus untreated *mdx*-4Cv mice. **C:** Total RNA was isolated from GN skeletal muscle lysates from *mdx* oATP and *mdx* PBS mice and reverse transcribed. Foxp3 and Areg transcripts were quantified by quantitative real-time PCR. Each box represents the median with first and third quartile. *n* = 5 mice for each experimental group. Statistical analysis was performed with the *U* test. **P* < 0.05. Scale bar = 20 μ m. Original magnification, $\times$ 20. Areg, amphiregulin; A.Tib., anterior tibialis; GN, gastrocnemii; *mdx* oATP, oATP-treated *mdx*-4Cv; *mdx* PBS, untreated *mdx*-4Cv; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; WT, wild-type.

which rapidly degrade eATP to adenosine.^{45–47} The exclusive immunosuppressive properties of Tregs have prompted a number of attempts to exploit them in cell therapy protocols both in murine models of autoimmunity and human graft-versus-host disease.^{48,49} The safety and feasibility of the adoptive transfer of Tregs is currently being tested in clinical trials focused on inflammation and rejection in liver and kidney transplantation and on type 1 diabetes (<http://www.clinicaltrials.gov>; accession numbers NCT02088931, NCT02145325, NCT01210664, NCT02188719, and NCT01624077).

In muscle, increased Foxp3 expression was observed in *mdx* mice concomitantly with increased strength and reduced diaphragm and cardiac fibrosis.⁵⁰ Analogously, Tregs were shown to accumulate in the muscle infiltrates of

Figure 9 oATP enhances myofiber regeneration and inhibits the expression of the profibrotic factors TGF- β and CTGF. **A:** Frozen GN and A.Tib. muscle sections from *mdx* oATP and *mdx* PBS mice were immunostained with primary antibody directed toward the native form of e-MyHC. Representative images of GN are shown. Paraffin-embedded Diaph. sections from *mdx* oATP and *mdx* PBS mice were immunostained with primary antibody directed toward myogenin. Representative images are shown. **B:** E-MYHC⁺ and myogenin⁺ fibers were quantified in the whole area of the tissue sections. **C:** GN skeletal muscle lysates from *mdx* oATP and *mdx* PBS mice were subjected to immunoblot analysis with specific antibodies against TGF- β and CTGF. Determination of GAPDH expression was used to normalize sample loading. Representative images are shown. Each box represents the median with first and third quartile (**B**). *n* = 5 mice for each experimental group (**B**); *n* = 3 mice per experimental group (**C**). Statistical analysis was performed with the *U* test. **P* < 0.05. Scale bars: 50 μ m (**A**, GN); 20 μ m (**A**, Diaph.). A.Tib., anterior tibialis; CTGF, connective tissue growth factor; Diaph., diaphragm; eMyHC, embryonal myosin heavy chain; GAPDH, glyceraldehyde phosphate dehydrogenase; GN, gastrocnemii; *mdx* oATP, oATP-treated *mdx*-4Cv; *mdx* PBS, untreated *mdx*-4Cv; oATP, periodate-oxidized ATP; PBS, phosphate-buffered saline; TGF, transforming growth factor; WT, wild-type.

mdx mice where they exerted a protective role on muscle pathology and promoted muscle repair.^{23,24} On one side, Tregs were shown to regulate the myeloid populations that infiltrate the degenerative tissue and to induce their shift from a pro- to an anti-inflammatory phenotype. On the other side, Tregs exerted a direct stimulatory effect on satellite cells by expressing *Areg*, which enhances satellite cell differentiation *in vivo* and *in vitro*.²³

Along this line, in *mdx* mice, improvement of muscle Tregs by oATP likely enhanced myoblast regeneration and resulted in decreased expression of profibrotic stimuli such as TGF- β and CTGF.

Altogether, our data indicate a pleiotropic role of eATP and P2X signaling in the pathogenesis of dystrophic muscle destruction and point to pharmacologic P2X antagonism as a possible therapeutic strategy to ameliorate the prognosis of this devastating condition.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.08.010>.

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