Cladribine modifies functional properties of microglia

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April 28, 2020

Abstract

Background: Cladribine (CdA), an oral prodrug approved for the treatment of relapsing multiple sclerosis, selectively depletes lymphocytes. CdA passes the blood-brain barrier suggesting a potential effect on CNS resident cells. Objective: We examined, if CdA modifies the phenotype and function of naïve and activated primary mouse microglia, when applied in different concentrations including 0.1-1 μM that putatively overlaps human CSF concentrations. Methods: Primary microglia cultures without stimulation or in the presence of proinflammatory lipopolysaccharide (LPS) or anti-inflammatory IL-4 were co-treated with different concentrations of CdA for 24 hours. Viability was assessed by MTT assay. Phagocytotic ability and morphology were examined by flow cytometry, and random migration by IncuCyte Zoom and TrackMate. Change in gene expression was examined by qPCR, and protein secretion by Meso Scale Discovery. Results: LPS and IL-4 upregulated deoxycytidine kinase (DCK) expression. Only activated microglia were affected by CdA, and this was unrelated to viability. CdA 0.1-1 μM significantly reduced granularity, phagocytotic ability and random migration of activated microglia. CdA 10 μM increased the IL-4-induced gene expression of Arg1 and LPS-induced expression of IL-1beta, TNF, iNOS, and Arg1, but protein secretion remained unaffected. CdA 10 μM potentiated the increased expression of anti-inflammatory TNFR2 but not TNFR1 induced by LPS. Conclusion: Microglia acquire a less activated phenotype when treated with 0.1–1 μM CdA that putatively overlaps human CSF concentrations. This may be related to the upregulated gene expression of DCK upon activation and suggests a potential alternative mechanism of CdA with direct effect on CNS resident cells.

Introduction

Cladribine (CdA), also known as 2-chlorodeoxyadenosine, is a synthetic deoxyadenosine analogue that selectively depletes lymphocytes (1-3). CdA is a prodrug, and accumulation of the active drug CdA triphosphate depends on the ratio of deoxycytidine kinase (DCK), that catalyzes the first of three phosphorylation steps, and 5'-nucleotidases (5'-NTases), that converts phosphorylated cladribine to cladribine nucleoside. In lymphocytes due to a high DCK to 5'-NTase ratio, accumulation of CdA triphosphate results in apoptosis by several mechanisms (2, 3).

Cladribine tablets has been approved for the treatment of relapsing MS, based in part on a placebo-controlled, double-blind, multicenter phase-III trial (CLARITY). 3.5 mg/kg oral CdA reduced the annual relapse rate by around 57% (4), and the relative reduction in the risk of 3-month sustained progression of disability was 33% compared to placebo. The mean number of active T2 lesions on MRI was reduced by 73.4% (4). The extension of the phase-III trial showed a durable effect on MRI and clinical outcomes (5, 6).

The dose of 3.5 mg/kg CdA applied orally in short treatment cycles causes a short-term lymphocyte depletion followed by immune-reconstitution (7). However, CdA passes the blood-brain barrier (BBB), and gives rise to 25% of the plasma concentration in CSF resulting in a maximum CSF concentration of 0.019-0.025 μM (2, 3), and this may suggest a potential effect on CNS resident cells as well.

Microglia comprise 10-15% of all glial cells in the CNS (8). Pro-inflammatory microglia secrete pro-inflammatory cytokines, chemokines, reactive oxygen and nitrogen species, which are toxic for oligodendrocytes and neurons (9, 10). Lipopolysaccharides (LPS) induce a pro-inflammatory phenotype of microglia characterized by the expression of interleukin-1β (IL-1β), IL-6, major histocompatibility complex II (MHC II), tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS) (11, 12). Microglia also exert neuroprotective and repairing functions, and can produce neurotrophic factors and anti-inflammatory cytokines (11, 13, 14). The anti-inflammatory cytokine IL-4 induces the anti-inflammatory phenotype characterized by arginase 1 (Arg1) and IL-10 expression among others (11, 12, 15).

Data about the effect of CdA on microglia are scarce (16). When investigating the effect of the drug in murine cells, higher CdA concentrations are required because the activity of DCK is ten times lower in mice than in humans (17, 18). Here, we examined the effect of CdA on the phenotype and function of naïve and activated mouse microglia in concentrations that putatively overlap with the levels in the human CSF.

Materials and Methods

Animals

C57BL/6 mice were obtained from Taconic Ltd. (Ry, Denmark). Mice were bred at the Biomedical Laboratory, University of Southern Denmark, according to protocols and guidelines approved by the Danish Animal Experiments Inspectorate (approval NO: 2014-15-00369). Animal experiments complied with the EU Directive 2010/63/EU for animal experiments.

Microglia cell culture

Mixed primary glial cell cultures were prepared by enzymatic dissociation of postnatal P0-P5 mouse brains using Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, 130-092-628). The cells were allowed to proliferate for 10 days prior to isolation of microglia by the "shake off" method (19). The isolated microglia were on average 95% pure, when characterized as $CD11b^{+}/CD45^{low}$ on the flow cytometer (BD^{TM} LSR II, BD biosciences).

In vitro ***stimulation of microglia***

CdA powder was provided by Merck. CdA was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2438). Microglia were treated with 0.2% DMSO, LPS (10 ng/ml) (Sigma-Aldrich, L2630), DMSO and LPS, one of four concentrations of CdA (0.01 μM, 0.1 μM, 1 μM and 10 μM) alone or in combination with LPS for 24 hours. For migration, the cells were stimulated immediately before placement in the IncuCyte. Microglia were stimulated with IL-4 (20 ng/ml) or LPS (10 ng/ml) alone or together with CdA for 24 hours for qPCR and Meso Scale.

MTT viability assay

MTT solution (0.5 mg/ml) (Sigma-Aldrich, M0283) was added to LPS- and CdA-pretreated microglia for 4 hours. The absorbance was measured by a microplate reader (Molecular Devices, 6465).

Phagocytosis assay, size and granularity

Microglia were incubated with fluorescent latex beads and analyzed by flow cytometry as described (20). Fluorescent latex beads (Polysciences, Inc., 17154-10) were added to LPS and CdA treated microglia for 40 min at 37°C (sample) and 4°C (control). Cytochalasin D (5 μg/ml) was applied prior to addition of the beads as negative control. Phagocytosis was stopped by placing the cells on ice. Cells were detached by using 0.2% Trypsin-EDTA and analyzed by flow cytometry. Microglia size and granularity were assessed by flow cytometry by measuring forward scatter (FSC) and side scatter (SSC), respectively.

Random migration assay

Microglia were seeded at a density of 12,000 cells/well in a PDL coated 96-well ImageLock Plate (Essen Bioscience, 4379) and stained with CellTracker Red CMTPX Fluorescent Probe (4.5 μM) (Life Technologies). DMSO (0.2%), LPS (10 ng/ml), and CdA in different concentrations alone (0.1-10 μM) or together with LPS were added, and cells were placed in the IncuCyte Zoom live cell imaging system (Essen BioScience, USA). Pictures were taken every 20 min for 24 hours. Single cell motility was quantified using the Fiji plugin TrackMate for semi-automated particle tracking (21). To detect individual cells in TrackMate, the Laplacian of Gaussian (LoG) detector with estimate spot diameter of 24.12 μm and a threshold of 0.3 was used. The simple linear assignment problem (LAP) tracker with a linking maximum distance of 60 μm, a gap-closing max distance of 15 μm and a gap closing max distance of 2 was used to track cell migration through the time course movies. The number of spots in track was set to 71.09 to exclude cells, which were not detectable throughout the movies from the analysis.

RNA extraction and quantitative PCR

RNA was extracted using RNeasy micro kit (Qiagen, Denmark) and reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4374966). Quantitative PCR (qPCR) was performed on Bio-Rad CFX Connect^{TM} Real-Time System (Software Bio-Rad CFX Manager 3.1) using SYBR green chemistry (Thermo Scientific) and corresponding primers (**Supplementary Table 1**). The expression levels are reported relative to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1).

Meso Scale Discovery (MSD) multiplex analysis

Cytokine levels in the microglia cell culture media were measured with the Meso Scale Discovery (MSD, USA) proinflammatory mouse V-Plex Plus kit (22).

Statistics

The statistical analyses were performed in Prism 6.01 (GraphPad) using one-way ANOVA followed by Sidak's or Dunnett's multiple comparisons tests or by unpaired T test with Welch's correction. Outliers were removed using the ROUT method (Q=1%). Differences among means with $p<0.05$ were accepted as statistically significant, while differences among means with $0.05<p<0.10$ were accepted as representing tendencies of difference.

Results

Cladribine (0.01-10 μM) does not influence the viability of naïve or LPS-stimulated microglia

Microglia were treated with DMSO, LPS, DMSO and LPS, different concentrations of CdA, and CdA together with LPS for 24 hours before incubation with MTT. DMSO, LPS, and their combination did not affect the viability of microglia compared to control (**Figure 1**). CdA in the applied concentration range (0.01-10 μM) did not affect the viability of naïve and LPS-stimulated microglia either compared to DMSO control (**Figure 1**).

Cladribine (0.1 μM) decreases the granularity of LPS-stimulated microglia

The size and granularity of naïve and LPS-stimulated microglia treated with CdA were investigated by flow cytometry (**Figure 2**). LPS stimulation increased the SSC mean fluorescent intensity (MFI) indicating increased cellular granularity (**Figure 2A and B**). We observed a trend towards reduced granularity of LPS-stimulated microglia treated with 0.1 μM CdA (p=0.07), while CdA did not influence the granularity of naïve microglia (**Figure 2A and B**). No apparent difference in cell size reflected by FSC MFI was observed (**Figure 2A and C**).

Cladribine (0.1-1 μM) decreases the phagocytosis ability of LPS-stimulated microglia

To investigate the effect of CdA on phagocytosis by naïve and LPS-stimulated microglia, the cells were incubated with fluorescent latex beads, and analyzed by flow cytometry. LPS stimulation significantly increased phagocytosis compared to control, while treatment with cytochalasin D, an inhibitor of actin polymerization, reduced phagocytosis (**Figure 3A**). CdA in the range of 0.1-1 μM significantly decreased phagocytosis by LPS-stimulated microglia, while treatment with CdA (0.01-10 μM) did not affect phagocytosis by naïve microglia compared to DMSO control (**Figure 3A**).

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Microglia motility after CdA treatment was measured by random migration: track displacement refers to the bee-line distance the cells migrate in 24 hours, while the total distance travelled is the actual distance covered by the cells. Microglia were stained with cell tracker and treated with DMSO, LPS, DMSO and LPS, different concentrations of CdA, or CdA together with LPS. Movies were recorded, and the track displacement and total distance travelled by the microglia were determined.

The microglia track displacement was significantly increased by LPS stimulation compared to untreated control (**Figure 3B**). Treatment with CdA 0.1 μM significantly decreased the track displacement of LPS-stimulated microglia, whereas CdA (0.1-10 μM) did not alter the track displacement of naïve microglia compared to DMSO control (**Figure 3B**).

The total distance travelled by microglia was not significantly increased by LPS stimulation, though a trend was observed (p=0.07) (**Figure 3C**). CdA in the range of 0.1-1 μM significantly decreased the total distance travelled by LPS-stimulated microglia (**Figure 3C**). CdA 0.1-10 μM did not have an effect on the total distance travelled by naïve microglia compared to DMSO control (**Figure 3C**).

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Next, we investigated the effect of CdA on the relative mRNA expression and secretion of selected pro- and anti-inflammatory markers after stimulation with LPS or IL-4 for 24 hours. The expression of IL-1β, TNF, IL-10, TNF-receptors, iNOS, and Arg1 were examined by qPCR. The secretion of IL-1β, TNF, and IL-10 were investigated by Meso Scale Discovery.

LPS stimulation resulted in an increased mRNA expression of IL-1β, TNF, and IL-10 compared to control (**Figure 4A**). Treatment with CdA 10 μM further increased the mRNA expression of IL-1β and TNF in LPS-stimulated cells (**Figure 4A**). CdA (0.01-10 μM) did not affect the expression of these cytokines in naïve microglia (data not shown).

The protein secretion of IL-1β, TNF, and IL-10 was increased by LPS stimulation (**Figure 4B**). In contrast, CdA (0.1-10 μM) did not affect the secretion of IL-1β, TNF, and IL-10 from LPS-stimulated or naïve microglia (**Figure 4B**).

LPS stimulation increased the mRNA expression of both TNFR1 and TNFR2 compared to control. CdA 10 μM further increased the expression of TNFR2 but not TNFR1 (**Figure 5**). CdA (0.01-10 μM) did not affect the expression of TNF receptors in naïve microglia (data not shown).

LPS stimulation resulted in a significantly increased expression of iNOS and Arg1 mRNA compared to the untreated control, but these were reduced by DMSO co-treatment (**Figure 6**). Co-treatment with 10 μM CdA significantly increased the expression of iNOS and Arg1 in LPS-stimulated microglia compared to DMSO control (**Figure 6**). CdA did not affect the mRNA expression of iNOS or Arg1 in naïve microglia (data not shown).

IL-4 stimulation for 24 hours only increased Arg1 expression, whereas no changes were observed in mRNA expression of IL-1β, TNF, IL-10, TNFR1, and TNFR2. Furthermore, the expression of these genes was not affected by CdA co-treatment (**Figure 7 and Supplementary Figure 1-2**). IL-4 did not induce the expression of iNOS (data not shown). Similarly, no difference was observed in the secretion of IL-1β, TNF, and IL-10 from IL-4-stimulated and CdA co-treated microglia (**Supplementary Figure 1)** .

LPS and IL-4 increases the mRNA expression of the enzyme DCK

We examined the mRNA expression of DCK in naïve and activated microglia treated with CdA. LPS stimulation increased the expression of DCK, while IL-4 stimulation resulted in a similar trend compared to DMSO control (p=0.08) (**Figure 8A and B**). CdA (0.01-10 μM) did not change the increased expression induced by LPS and IL-4 (**Figure 8A and B**). The expression of DCK was not affected by CdA (0.01-10 μM) in naïve microglia either (data not shown).

Discussion

To determine the effect of CdA on murine microglia, we examined phenotypic and functional properties of naïve, LPS-, and IL-4-stimulated microglia treated with different concentrations of CdA for 24 hours. We found that CdA only affects stimulated and not naïve microglia. Furthermore, different concentrations of CdA influenced different aspects of microglia function. CdA concentrations putatively overlapping with CSF concentrations in humans (0.1-1 μM) significantly reduced the granularity, phagocytotic ability, and motility of LPS-stimulated microglia, while higher CdA concentration (10 μM) increased the mRNA expression of pro- and anti-inflammatory molecules, but not protein secretion. In addition, we found that activation of microglia increased the expression of DCK responsible for the activation of the prodrug.

CdA can cross the BBB and thereby potentially exerts an effect on CNS resident microglia (2, 3). The maximum plasma concentration of CdA after administration of a 10 mg tablet is in the range of 0.07-0.1 μM (23). As the CSF concentration is 25% of the plasma concentration (2, 3), the maximum CSF concentration attainable is approximately 0.019-0.025 μM. The active form of CdA is CdA triphosphate, which is the phosphorylated form of the drug (2, 3). The first step of this process is catalyzed by deoxycytidine kinase (DCK), the activity of which is 10 times lower in mice than humans (17, 18). The maximum CdA concentration detected in the CSF of patients thus may correspond to murine concentrations in the range of 0.1-1 μM. Therefore, we applied CdA concentrations of 0.01-10 μM in this study of murine microglia.

We first investigated whether the applied CdA concentrations were toxic to microglia. We found that the viability of microglia was not affected during the short culture conditions, similar to a previous study that applied the intravenous compound to rat microglia cultures (16). Therefore, we considered that using these concentrations for short time stimulation would not have a depleting effect on microglia.

LPS stimulation resulted in a morphological change of microglia characterized by an increase in SSC and thus a more granular morphology, as expected. Co-treatment with CdA 0.1 μM showed a tendency towards decreased granularity suggesting that CdA may have effect on the functional properties of microglia.

Therefore, we examined the phagocytotic ability and motility of microglia. We found that LPS stimulation significantly increased the phagocytotic ability of microglia, which correlated with the pro-inflammatory phenotype induced by LPS. Such increased phagocytotic ability of LPS-stimulated microglia was decreased by CdA in the range of 0.1-1 μM. Furthermore, the decrease in phagocytosis by CdA corresponds to the observed decreased granularity of microglia. CdA had no effect on naive microglia, which is consistent with findings of former studies performed on rat microglia (16), and murine dendritic cells (24).

Microglia motility is important for maintaining CNS homeostasis via interaction with other cells and scanning of the CNS environment (8, 11, 25, 26). We measured the random migration by (i) track displacement, i.e. the distance from start to end point, and (ii) total distance travelled that includes twists and turns and describes the patrolling of migrating cells. LPS stimulation significantly increased track displacement and showed a tendency towards increased total distance travelled. This is consistent with the acquisition of effector functions induced by LPS and a tendency towards increased patrolling of the surroundings. In contrast to random migration, LPS decreased chemotactic migration of rat microglia (27). We also found that CdA (0.1-1 μM) did not affect the motility of resting microglia.

Observing changes in morphology, phagocytotic ability and migration of LPS-stimulated microglia, we next examined how CdA alters gene expression and protein secretion of microglia stimulated with LPS and with anti-inflammatory IL-4. LPS increased the expression of pro- and anti-inflammatory markers consistent with

a recently published study (27). Co-treatment with CdA in the high concentration (10 μM) increased the gene expression of both pro- and anti-inflammatory markers, such as IL-1β, TNF, iNOS, Arg1, and TNFR2 suggesting a potentiating effect on activated microglia. Interestingly, only the gene expression of anti-inflammatory TNFR2, but not the pro-inflammatory TNFR1 (28) was potentiated by CdA. The increased protein secretion of IL-1β, TNF, and IL-10 induced by LPS was not further increased by CdA (0.1-10 μM) consistent with a previous study on rat microglia (16). This suggests that CdA only affects gene expression but not protein secretion. We may speculate that accumulation of CdA in microglia may interfere with RNA processing besides interference with DNA replication and this may alter translational mechanisms.

We also found that IL-4 stimulation resulted in an increased gene expression only of Arg1 but it was not affected by CdA co-treatment. This could indicate that IL-4 is a less potent stimulus in the short-term, and microglia may not be sufficiently activated for the potentiating effect of CdA. In summary, CdA in the applied concentration did not have effect on cytokine secretion of activated or resting microglia in short-term primary cultures. The observed effect on gene expression was achieved by higher concentrations of CdA than may be expected in the human CSF during treatment.

Activation of microglia by LPS and IL-4 also increased the gene expression of DCK that phosphorylates CdA to its active form. This may suggest that activated microglia may increase its ability to respond to CdA. In lymphocytes, CdA interferes with DNA synthesis and repair resulting in depletion of both proliferating and resting cells (2, 3). The mechanism by which CdA affects microglia functions remains to be determined, and our data do not indicate microglia depletion in the short term. A previous study also suggested that an additional mechanism beside depletion may be responsible for the effects on microglia, such as reduced proliferation (16). In dendritic cells, the function of CdA is partially independent from its phosphorylation (24); but it may not be true in microglia, as the effect of CdA on proliferation was lost upon addition of a competitive substrate of DCK (16).

In summary, we found that CdA used in a concentration that may be reached in the human CSF during approved treatment of MS, affected activated but not naïve microglia; this may be related to the upregulated gene expression of DCK upon activation. Co-treatment with CdA reduced the phagocytotic ability and random migration of activated microglia, while viability was not affected among the applied conditions. Although gene expression of both pro- and anti-inflammatory molecules was potentiated by CdA, it was induced only by higher concentration than should be expected in the CSF, and protein secretion was not altered. CdA also potentiated the gene expression of anti-inflammatory TNFR2. These data suggest that beside depletion of peripheral lymphocytes, CdA may induce additional effects particularly in CNS resident cells that contribute to its beneficial effects in MS.

Acknowledgements

We thank Ulla Damgaard Munk for expert technical assistance.

Funding: Merck A/S, Denmark; an affiliate of Merck KGaA, Darmstadt, Germany.

Conflicts of interest: LØJ received support for congress participation from Merck. MLE received a speaker fee from Merck. AEP was affiliated with Merck during conductance of the study, and AEP is now an employee with Almirall, but the work is unrelated to this employment. AEP also host a guest affiliation with University of Copenhagen. ZI has served on scientific advisory boards, served as a consultant, received support for congress participation, received speaker honoraria, and received research support among others from Biogen, Merck-Serono, Sanofi-Genzyme, Novartis, Roche, Lundbeckfonden. ÅFS, ABW, and KHH have nothing to declare.

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Figure legends

Figure 1

The effect of cladribine on the viability of naïve and LPS-stimulated microglia

Microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, different concentrations of CdA (0.01-10 μM), and different concentrations of CdA together with LPS for 24 hours, followed by an MTT assay. The results (mean±SD) are shown relative to DMSO control.

n=4-5 in each group, one-way ANOVA followed by multiple comparisons tests.

Figure 2

The effect of cladribine on microglia granularity and size

Microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, different concentrations of CdA (0.01-10 μM), and different concentrations of CdA together with LPS for 24 hours. **A.** A representative example of size (FSC) and granularity (SSC) of microglia by flow cytometry. **B.** The effect of CdA on the granularity (SSC) of naïve and LPS-stimulated microglia relative to DMSO control. **C.** The effect of CdA on the size (FSC) of naïve and LPS-stimulated microglia relative to DMSO control.

***p[?]0.001, n=3-4 in each group, one-way ANOVA followed by multiple comparisons tests, mean+-SD.

Figure 3

The effect of cladribine on the phagocytotic ability and random migration of microglia

A. Phagocytosis was quantified after microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, different concentrations of CdA (0.01-10 μM), and different concentrations of CdA together with LPS for 24 hours. Fluorescent latex beads were added to the cell media for 40 minutes. As negative control, microglia were treated with cytochalasin D (5 μg/ml) for 30 minutes before addition of latex beads. The cells were analyzed by flow cytometry, and the phagocytotic ability was quantified by mean fluorescent intensity (MFI) and presented relative to DMSO control. **B.** Track displacement was quantified by analysis of timelapse videos using TrackMate after microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml),

DMSO and LPS, different concentrations of CdA (0.1-10 μM), and different concentrations of CdA together with LPS for 24 hours. The results are shown relative to DMSO control. **C.** Total distance travelled was quantified by analysis of timelapse videos using TrackMate after microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, different concentrations of CdA (0.1-10 μM), and different concentrations of CdA together with LPS for 24 hours. The results are shown relative to DMSO control.

*p[?]0.05, ****p[?]0.0001, n=3-5 in each group, one-way ANOVA followed by multiple comparisons tests, mean±SD.

Figure 4

The effect of cladribine on the mRNA expression and protein secretion of cytokines by LPS-stimulated microglia

A. mRNA expression of IL-1β, TNF and IL-10 was quantified by qPCR after microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, and different concentrations of CdA (0.01-10 μM) together with LPS for 24 hours. The expression is shown relative to DMSO control.**B.** The protein concentration of IL-1β, TNF and IL-10 in the microglia cell culture supernatant was quantified by Meso Scale. The microglia were treated with DMSO (28.2 mM), DMSO and LPS, and different concentrations of CdA (0.1-10 μM) together with LPS for 24 hours.

*p[?]0.05, **p[?]0.01, ***p[?] 0.001, ****p[?]0.0001, n=4 in each group, unpaired t test with Welch's correction and one-way ANOVA followed by Dunnett's multiple comparisons test, mean±SD.

Figure 5

The effect of cladribine on the mRNA expression of TNF receptors in LPS-stimulated microglia

mRNA expression of TNFR1 and TNFR2 was quantified by qPCR after microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, and different concentrations of CdA (0.01-10 μM) together with LPS for 24 hours. The expression is shown relative to DMSO control.

*p[?]0.05, **p[?]0.01, ***p[?] 0.001, n=4 in each group, one-way ANOVA followed by multiple comparisons tests, mean±SD.

Figure 6

The effect of cladribine on the mRNA expression of arginase 1 and iNOS in LPS-stimulated microglia

mRNA expression of arginase 1 (Arg1) and iNOS was quantified by qPCR after microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, and different concentrations of CdA (0.01-10 μM) together with LPS for 24 hours. The expression is shown relative to DMSO control.

**p[?]0.01, ***p[?] 0.001, n=4 in each group, one-way ANOVA followed by multiple comparisons tests, mean±SD.

Figure 7

The effect of cladribine on the mRNA expression of arginase 1 in IL-4-stimulated microglia

mRNA expression of arginase 1 (Arg1) was quantified by qPCR after microglia were treated with DMSO (28.2 mM), IL-4 (20 ng/ml), DMSO and IL-4, and different concentrations of CdA (0.01-10 μM) together with IL-4 for 24 hours. The expression is shown relative to DMSO control.

*p[?]0.05, n=3-4 in each group, one-way ANOVA followed by multiple comparisons tests, mean±SD.

Figure 8

The effect of cladribine on the mRNA expression of DCK in LPS- and IL-4-stimulated microglia

mRNA expression of deoxycytidine kinase (DCK) was quantified by qPCR and is shown relative to DMSO control. **A.** Microglia were treated with DMSO (28.2 mM), LPS (10 ng/ml), DMSO and LPS, and different concentrations of CdA (0.01-10 μM) together with LPS for 24 hours.**B.** Microglia were treated with DMSO (28.2 mM), IL-4 (20 ng/ml), DMSO and IL-4, and different concentrations of CdA (0.01-10 μM) together with IL-4 for 24 hours.

**p[?]0.01, ***p[?] 0.001, n=3-4 in each group, one-way ANOVA followed by multiple comparisons tests, mean±SD.

Supplementary Figure 1

The effect of cladribine on the mRNA expression and protein secretion of cytokines by IL-4-stimulated microglia

A. mRNA expression of IL-1β, TNF and IL-10 was quantified by qPCR after microglia were treated with DMSO (28.2 mM), IL-4 (20 ng/ml), DMSO and IL-4, and different concentrations of CdA (0.01-10 μM) together with IL-4 for 24 hours. The expression is shown relative to DMSO control. **B.** The protein concentration of IL-1β, TNF and IL-10 in the microglia cell culture supernatant was quantified by Meso Scale. Microglia were treated with DMSO (28.2 mM), DMSO and IL-4 (20 ng/ml), and different concentrations of CdA (0.1-10 μM) together with IL-4 for 24 hours.

n=3-4 in each group, unpaired t test with Welch's correction and one-way ANOVA followed by Dunnett's multiple comparisons test, mean±SD.

Supplementary Figure 2

The effect of cladribine on the mRNA expression of TNF receptors in IL-4-stimulated microglia

mRNA expression of TNFR1 and TNFR2 was quantified by qPCR after microglia were treated with DMSO (28.2 mM), IL-4 (20 ng/ml), DMSO and IL-4, and different concentrations of CdA (0.01-10 μM) together with IL-4 for 24 hours. The expression is shown relative to DMSO control.

n=3-4 in each group, one-way ANOVA followed by multiple comparisons tests, mean±SD.

Figure 1

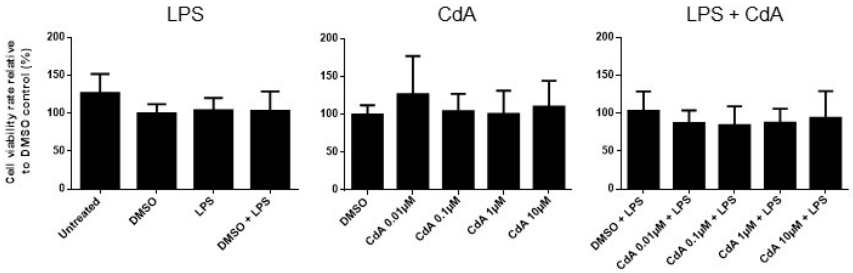
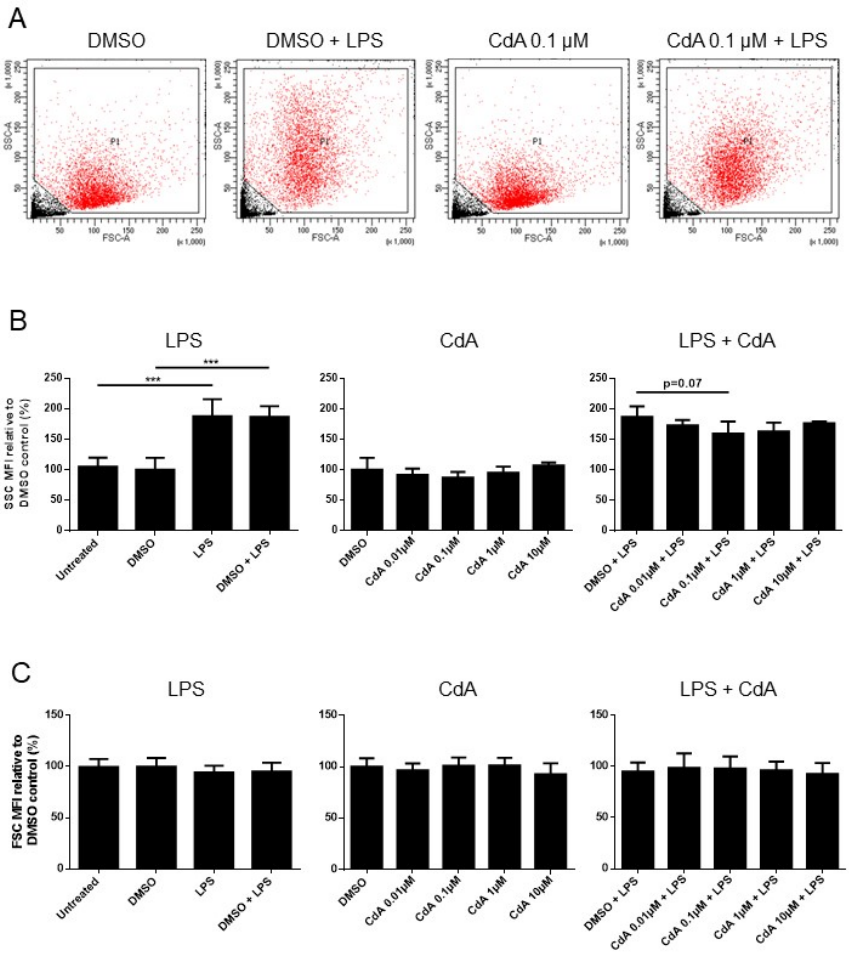


Figure 2



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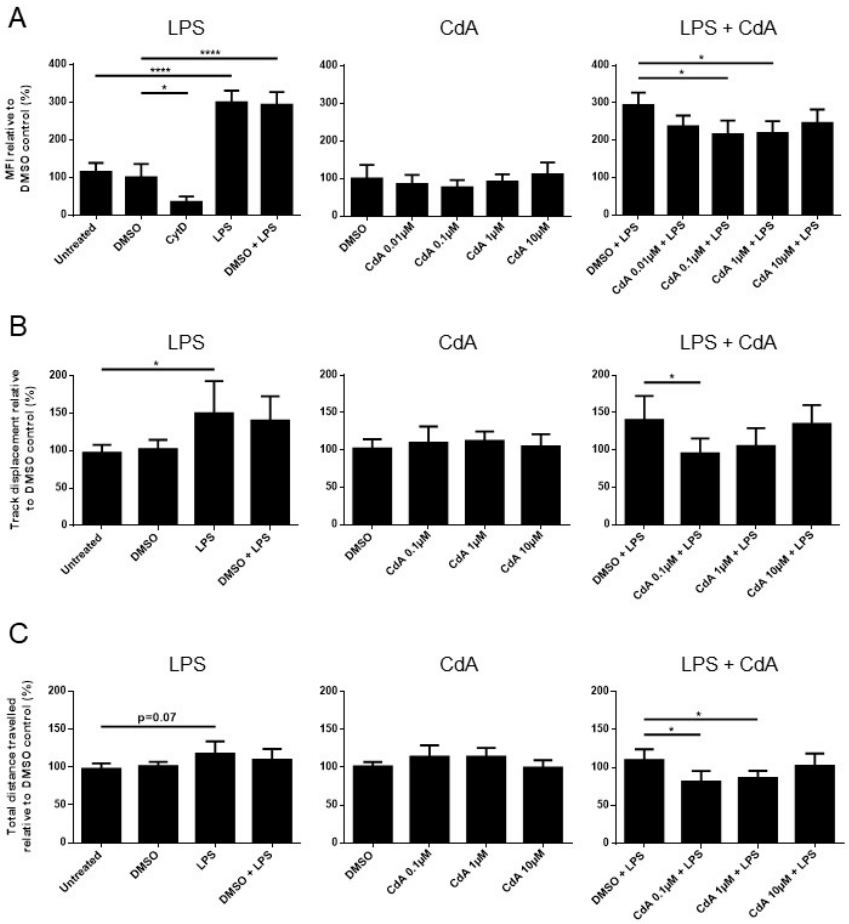

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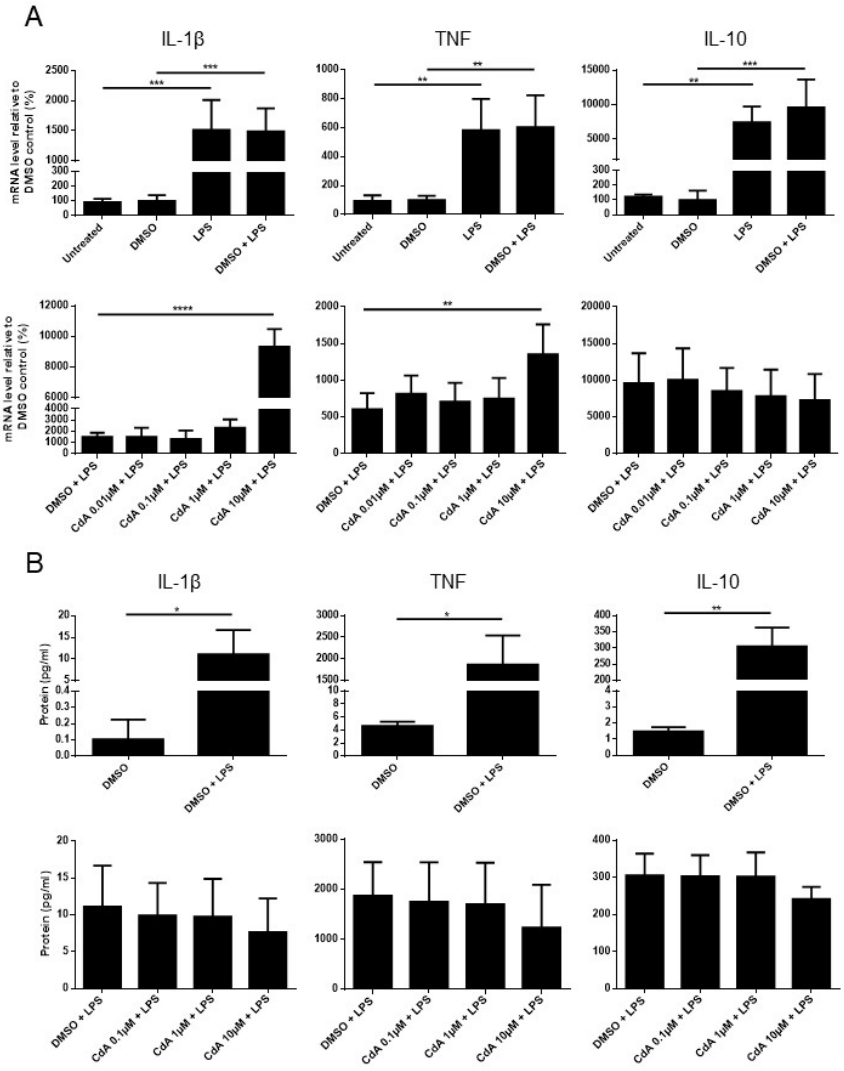

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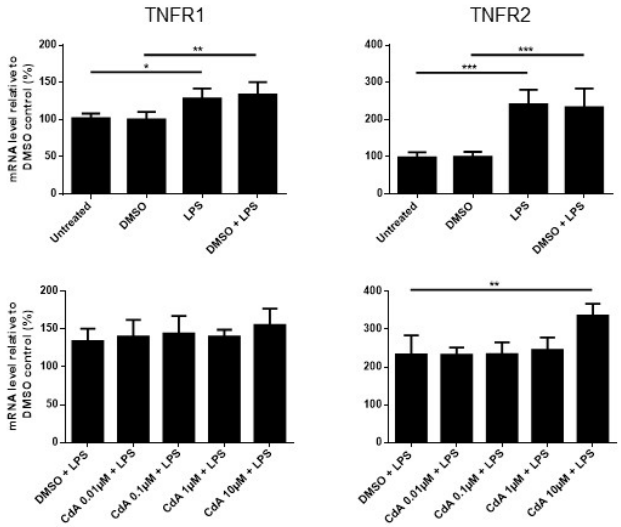

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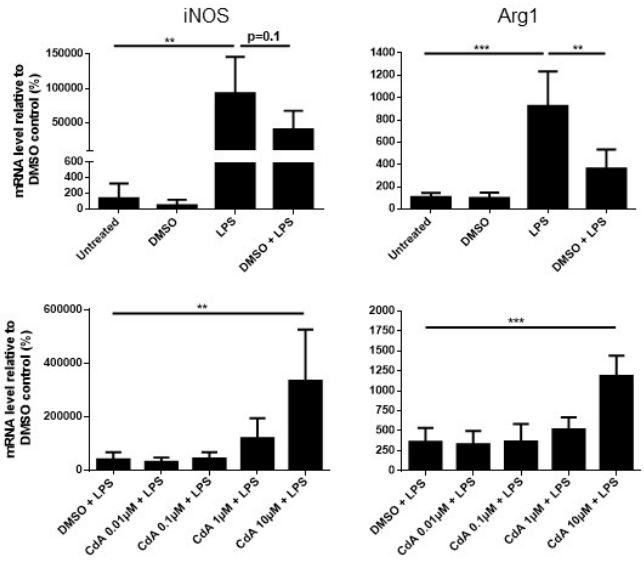

Figure 7

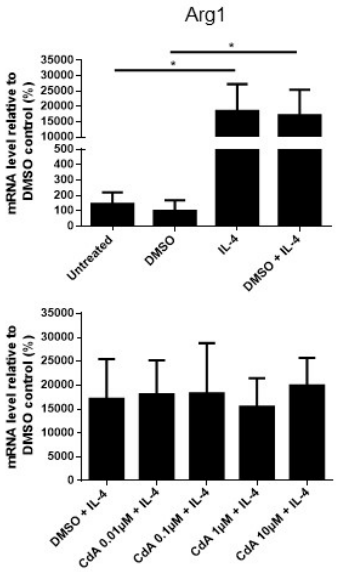

Figure 8