



BASIC SCIENCE ARTICLE

Modulatory activity of adenosine on the immune response in cord and adult blood

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BACKGROUND: Neonatal sepsis is a leading cause of neonatal morbidity and mortality, associated with immunosuppression. Myeloid-derived suppressor cells (MDSCs) are cells with immunosuppressive activity, present in high amounts in cord blood. Mechanisms regulating MDSC expansion are incompletely understood. Adenosine is a metabolite with immunoregulatory effects that are elevated in cord blood.

METHODS: Impact of adenosine on peripheral and cord blood mononuclear cells (PBMCs and CBMCs) was analysed by quantification of ectonucleotidases and adenosine receptor expression, MDSC induction from PBMCs and CBMCs, their suppressive capacity on T cell proliferation and effector enzyme expression by flow cytometry.

RESULTS: Cord blood monocytes mainly expressed CD39, while cord blood T cells expressed CD73. Adenosine-induced MDSCs from PBMCs induced indoleamine-2,3-dioxygenase (IDO) expression and enhanced arginase I expression in monocytes. Concerted action of IDO and ArgI led to effective inhibition of T cell proliferation. In addition, adenosine upregulated inhibitory A₃ receptors on monocytes.

CONCLUSION: Adenosine acts by inducing MDSCs and upregulating inhibitory A₃ receptors, probably as a mode of autoregulation. Thus, adenosine contributes to immunosuppressive status and may be a target for immunomodulation during pre- and postnatal development.

Pediatric Research (2021) 90:989–997; <https://doi.org/10.1038/s41390-020-01275-8>

IMPACT:

- Immune effector cells, that is, monocytes, T cells and MDSCs from cord blood express ectonucleotidases CD39 and CD73 and may thus serve as a source for adenosine as an immunomodulatory metabolite.
- Adenosine mediates its immunomodulatory properties in cord blood by inducing MDSCs, and by modulating the inhibitory adenosine A₃ receptor on monocytes.
- Adenosine upregulates expression of IDO in MDSCs and monocytes potentially contributing to their suppressive activity.

INTRODUCTION

Postnatal immunosuppression is a key feature of the neonatal immune system and is considered a remnant of materno-foetal tolerance. Consequences such as susceptibility to infection have been well documented. Recently, however, a more physiological role of impaired inflammation in neonates has been postulated, chaperoning neonatal microbiome development¹ and potentially reducing oxidative stress. Mechanisms that regulate perinatal inflammation are only incompletely understood.

Adenosine is an endogenous metabolite with immunomodulatory properties. It is produced by the placenta acting as a vasodilator.² Adenosine can cross the placenta and enter the foetal circulation.^{2,3} Extracellular adenosine concentration lies within the nanomolar range in healthy adults, while it can be elevated up to 100-fold under pathological conditions such as rheumatoid arthritis.^{4,5} Intriguingly, in cord blood, adenosine is physiologically elevated up to 3-fold over adult levels and may have anti-inflammatory effects such as enhanced interleukin-6 (IL-6) production at the expense of tumour necrosis factor- α suppression.²

Adenosine is formed as a metabolite by the degradation of extracellular ATP originating, for example, from activated polymorphonuclear cells⁶ as well as from T cells⁷ released via pannexin-1 channel upon activation. First, extracellular ATP is cleaved to AMP by bifunctional ecto-apyrase CD39. This enzyme is capable of cleaving both ATP and ADP to AMP. The latter acts as a substrate for the 5'-ectonucleotidase CD73 cleaving AMP to adenosine.^{8–12} It has a negative effect on the activation, proliferation and expansion of various T cell subsets,³ such as regulatory T cells, which highly express CD73, while CD73 deficiency may result in attenuated immunosuppressive functions.¹³

Adenosine binds to corresponding adenosine receptors belonging to the group of G-protein-coupled receptors. Adenosine binding results in attenuation (A₁, and A₃) or elevation (A_{2A} and A_{2B}) of adenylate cyclase activity,¹⁴ and its receptor engagement may lead to different effects. For example, A₁ may influence heart rate,¹⁵ A_{2A} may affect inflammatory processes and vascular tone, A_{2B} predominantly affects cellular adaptation to hypoxia and A₃ is involved in allergic reactions.¹⁴

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Received: 27 June 2020 Revised: 29 September 2020 Accepted: 23 October 2020
Published online: 9 February 2021

Myeloid-derived suppressor cells (MDSCs) are cells with immunosuppressive activity, present in higher amounts in peripheral blood of pregnant women, placenta and cord blood and play a pivotal role in materno-foetal tolerance.^{16–18} They can be subdivided into granulocytic and monocytic MDSCs (Gr- and Mo-MDSCs). Gr-MDSCs present in cord blood induce a tolerogenic state mainly by hampering T cell proliferation.^{19,20} Mechanisms regulating MDSC expansion are incompletely understood.

In the present study, we aimed to further determine the role of adenosine in neonatal immunosuppression, especially whether immune cells from cord blood are able to express ectonucleotidases and, thereby, to produce adenosine. Further, we focused on the role of adenosine for MDSC induction and activation and analysed adenosine receptor expression.

We found that adenosine production occurs through an interplay of at least two immune cell lines, CD39⁺ monocytes and CD73⁺ T cells, and that CD73 expression on monocytes could be induced by treatment with distinct pathogen-associated molecular patterns (PAMPs), for example, Pam3Cys. Adenosine induced the formation of CD33⁺ MDSCs by exerting inhibitory capacity against T cells, enhanced the production of indoleamine-2,3-dioxygenase (IDO) by both monocytes and MDSCs and of arginase I (ArgI) by cord blood monocytes, and upregulated inhibitory A₃ receptors on monocytes.

Thus, adenosine may contribute to an immunosuppressive status during pre- and postnatal development.

METHODS

Blood collecting and mononuclear cell isolation

Blood samples from healthy adult individuals and cord blood samples from caesarean sections were collected in tubes pre-filled with sterile isotonic saline (Fresenius Kabi, Bad Homburg, Germany) and heparin [100 U/ml] (Ratiopharm, Ulm, Germany). Mononuclear cells from peripheral blood of adults (PBMCs) or cord blood (CBMCs) were isolated by Pancoll density gradient centrifugation (Pancoll human 1.077 g/ml, PAN Biotech, Aidenbach, Germany) according to Boyum.^{21,22} Cells were washed in Dulbecco's phosphate-buffered saline (DPBS, PAN Biotech) to remove the Pancoll residues, counted by an automated haemocytometer (Sysmex KX-21N, Sysmex, Norderstedt, Germany) and diluted in complete medium containing Roswell Park Memorial Institute-1640 medium (RPMI-1640 medium, PAN Biotech), 10% foetal calf serum (Biocrom, Berlin, Germany), 1% penicillin/streptomycin (Biocrom), 1% L-glutamine (Biocrom) or in a sterile DPBS (PAN Biotech) up to 1×10^7 per ml.

Detection of CD39 and CD73

PBMCs or CBMCs were diluted in complete medium (see above) to 2×10^6 cells/ml and either analysed immediately or put in a cell culture (6-well plate, Corning Inc., Wiesbaden, Germany) and incubated at 37 °C in the presence of lipopolysaccharides (LPS, 1 µg/ml), lipoteichoic acid (LTA, 10 µg/ml) (both Sigma, Steinheim, Germany), ((S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys₄-OH, trihydrochloride, (Pam3Cys, 10–1–0.1 µg/ml, EMC Microcollections, Tuebingen, Germany) or left untreated, respectively, for 24 h. Cells were then harvested, stained for CD3 (FITC, UCHT-1, BD Biosciences, Heidelberg, Germany), CD14 (FITC, MφP9, BD Biosciences) and CD66b (FITC, G10F5, BD Biosciences), as well as for both ectonucleotidases CD73 (AD2, BD Biosciences) and CD39 (REA739, Miltenyi Biotec, Bergisch Gladbach, Germany) and analysed by flow cytometry (FACS Calibur, BD Biosciences).

Detection of adenosine receptors

PBMCs and CBMCs were diluted in complete medium to 2×10^6 cells/ml, put in a cell culture (6-well plate, Corning Inc.) and incubated at 37 °C in the presence of adenosine [100 µM] (Sigma), or left untreated. Cells were harvested after 24 h, stained for CD3

(FITC, UCHT-1, BD Biosciences), CD14 (FITC, MφP9, BD Biosciences), CD66b (FITC, G10F5, BD Biosciences) and for the adenosine receptors A_{2A} (Alexa Fluor 647, 7F6-G5-A2, Santa Cruz Biotechnology, Heidelberg, Germany) and A₃ (PE, polyclonal, Biozol, Eching, Germany), and analysed by flow cytometry.

MDSC induction and T cell inhibition assay

PBMCs and CBMCs were diluted in complete medium (see above) to 5×10^5 cells/ml. Cells were incubated in 6-well plate (5 ml cell suspension/well) (Corning Inc.) at 37 °C and 5% CO₂ for 7 days with medium change on day 4, according to established protocols.^{23,24} On day 7, cells were harvested applying Detachin™ (GenLantis, San Diego, CA, USA) and using a cell scraper (Corning Inc.) to detach the cells from the plastic dish bottom. Percentages of CD33⁺ cells were analysed by flow cytometry (WM53, BD Biosciences). For functional analysis, CD33⁺ cells were isolated by AutoMACS Separator (Miltenyi Biotec) using anti-CD33 microbeads (Miltenyi Biotec) according to the manufacturer's protocol, washed, counted by a binocular (Olympus, Hamburg, Germany) and diluted in sterile DPBS (PAN Biotech) up to 1×10^6 per ml. In the meantime, autologous PBMCs were freshly isolated by Pancoll (PAN Biotech) density gradient centrifugation, diluted in DPBS (PAN Biotech) to 1×10^7 cells/ml and stained with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen/Thermo Fisher Scientific Inc., Langenselbold, Germany) according to the manufacturer's instructions. Cells were counted by an automated hemocytometer (Sysmex KX-21N, Sysmex), resuspended and diluted to 2×10^6 cell/ml in modified medium containing autologous adult blood plasma instead of foetal calf serum, followed by stimulation with murine monoclonal antibody Muromonab-CD3 (OKT3, 10 µg/ml, Invitrogen/Thermo Fisher Scientific) and IL-2 (100 U/ml, R&D Systems). Stimulated cells were co-incubated with induced CD33⁺ MDSCs in distinct ratios (1:2, 1:4 and 1:8) at 37 °C and 5% CO₂ for 4 days in a 96-well round-bottom plate. Non-stimulated PBMCs served as a negative control, while stimulated PBMCs without induced CD33⁺ MDSCs were used as a positive control. On day 4, cells were harvested, washed and stained for CD4 (APC, RPA-T4, BD Biosciences). T cell proliferation was assessed as CFSE dye dilution by flow cytometry.

Detection of effector enzymes

PBMCs and CBMCs were diluted in complete medium (see above) to 2×10^6 cells/ml and stained for immediate effector enzyme detection or cultured with or without adenosine [100 µM] (Sigma) at 37 °C and 5% CO₂ for 24 h. After 24 h, cells were harvested by spinning down and intracellular staining of effector enzymes was done as follows: cell pellets were resuspended in DPBS (PAN Biotech) and aliquoted into two aliquots. The first aliquot remained unstained. The second aliquot was stained for surface marker (a) CD14 (APC, Mφ9, BD Biosciences) and (b) CD66b (FITC, G10F5, BD Biosciences), and then incubated at 4 °C for 10 min. Afterwards, the cell suspension was washed by adding FACS buffer (BD Biosciences) and spinning down (310 × g, 5 min) to remove the unbound antibodies. The supernatant was discarded and the pellet resuspended. To fix and permeabilize the cells of both unstained and stained samples, BD Cytofix/Cytoperm (BD Biosciences) was added (20 µl per tube for unstained sample, otherwise 200 µl) and incubated at 37 °C for 10 min. To stop the fixation and permeabilization process, BD Perm/Wash buffer (BD Biosciences) was added (ten times diluted with Ampuwa water, 1 ml for unstained samples, otherwise 4 ml) and samples were spun down (310 × g, 5 min). Afterwards, the supernatant was discarded, pellet resuspended in BD Perm/Wash buffer (BD Biosciences) and aliquoted in FACS tubes (Corning Inc.) containing corresponding antibodies—zeta-chain-associated protein kinase of 70 kDa (Zap70, R-PE, 1E7.2, Thermo Fisher), ArgI (PE, 658922, R&D Systems), inducible nitric oxide synthase (Alexa Fluor 647, C-11, Santa Cruz Biotechnology), IDO (PE, 700838, R&D Systems).

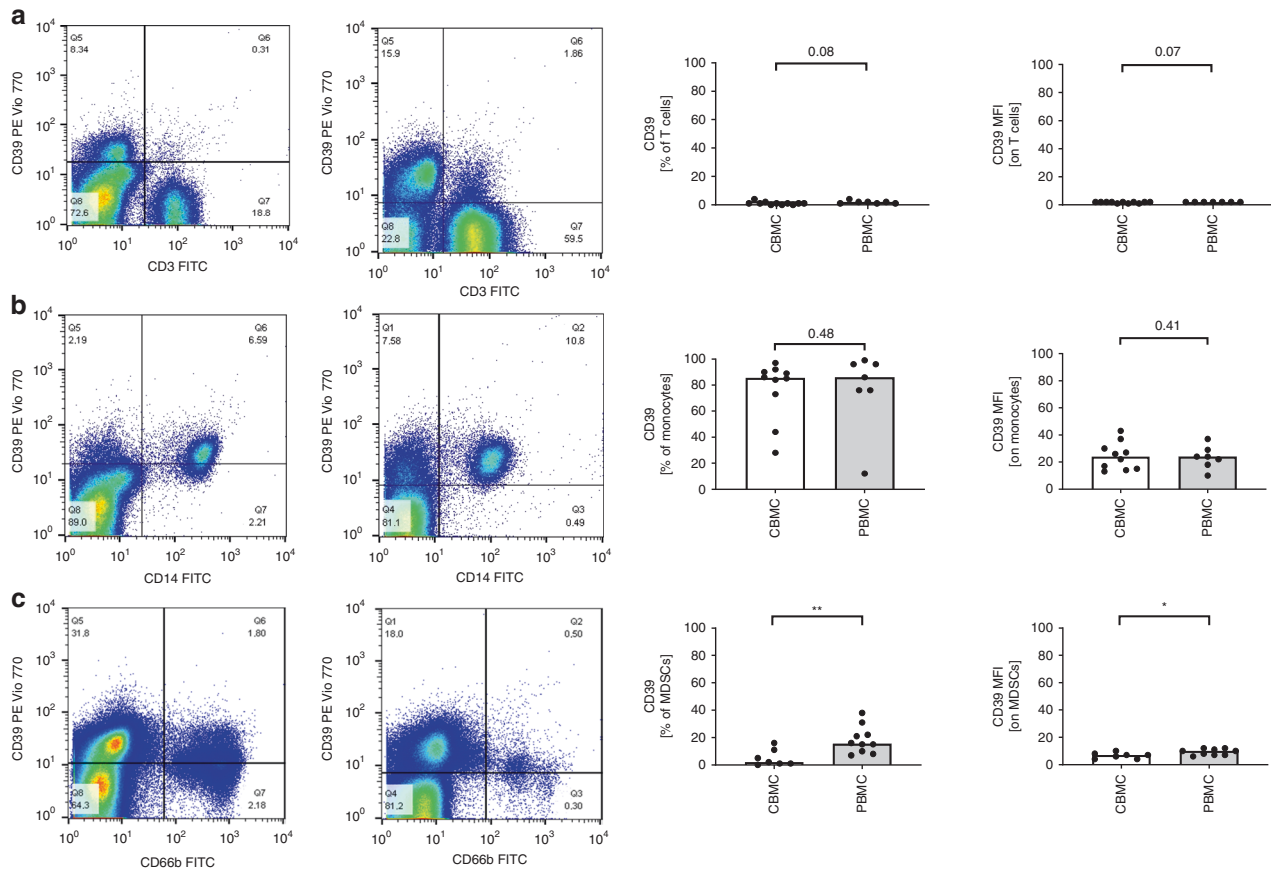


Fig. 1 CD39 expression on freshly isolated cord blood and peripheral blood immune effector cells. Cord blood (CBMCs, white bars) and peripheral blood mononuclear cells (PBMCs, grey bars) were isolated followed by staining for lineage markers for **a** T cells (upper diagrams), **b** monocytes (mid diagrams) and **c** MDSCs (lower diagrams) and CD39. Expression was quantified by flow cytometry. Given are (i) representative density plots, (ii) mean fluorescence intensities (MFI) and (iii) percentage of positive cells (%). CBMCs $n = 11$, PBMCs $n = 7$, * $p < 0.05$; ** $p < 0.01$.

Detection of Zap70 was used as a control of successful permeabilization as formerly described.²⁵ Zap70 was not the object of investigation. Samples were incubated at 37°C for 10 min and then washed by adding FACS buffer and spinning down (310×*g*, 5 min). The supernatant was discarded and the pellet was resuspended. The unstained samples were used as negative control. Effector enzyme expression was determined by flow cytometry.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Group comparisons were done by Student's *t* test. A *p* value of <0.05 was considered statistically significant.

RESULTS

CD39 is dominantly expressed by monocytes and CD73 by T cells. To evaluate the possibility that neonatal immune effector cells may autonomously produce adenosine, we first asked for their ectonucleotidase expression. CD39 expression on T cells from CBMCs and PBMCs was low (Fig. 1a). However, 85 ± 5% of cord blood and 86 ± 5% of adult monocytes expressed CD39 with moderate density (22 ± 10 vs. 23 ± 10 mean fluorescence intensity (MFI), $n = 7-11$, $p = 0.48$, Fig. 1b). Cord blood MDSCs expressed CD39 at low percentages (2 ± 0.5%) and at low levels (7 ± 2.5 MFI, Fig. 1c), while MDSCs from adults showed stronger expression (15 ± 5%, 10 ± 2.5 MFI, $n = 9$, $p < 0.01$, Fig. 1c). CD73 was expressed

mainly by T cells (Fig. 2). Cord blood T cells showed stronger expression (30 ± 10%; 7 ± 2.5 MFI) than adult T cells (22 ± 2.5%, 5 ± 1.0 MFI, $n = 7-11$, $p < 0.05$, Fig. 2a). Cord and adult blood monocytes both showed low CD73 expression (8 ± 5.0%, 3 ± 1 MFI and 2 ± 0.5%, 3 ± 0.5 MFI, respectively, $n = 7-10$, $p < 0.05$, Fig. 2b). The CD73 expression of cord blood MDSCs was weaker (2 ± 0.5%, 5 ± 0.5 MFI, $n = 7$) than that of adult MDSCs (10 ± 8%, 5 ± 1 MFI, $n = 10$, $p < 0.01$, Fig. 2c).

CD73 expression is modulated by Pam3Cys

To elucidate if the ectonucleotidase expression pattern would change in the presence of PAMPs, PBMCs and CBMCs were treated with Pam3Cys, LTA and LPS. The majority (81.6 ± 5%, $p < 0.001$) of cord blood monocytes, initially single positive for CD39 (Fig. 1b), upregulated CD73 expression upon treatment with Pam3Cys in a dose-dependent manner (74.3 ± 15 MFI, $p < 0.05$, $n = 3$, [10 µg/ml], Fig. 2d). Interestingly, LTA and LPS induced CD73 expression upregulation in adult monocytes only, but not in cord blood monocytes (Supplemental Figure S1).

Likewise, most (90.2 ± 5%, $p < 0.001$) adult monocytes upregulated CD73 expression upon treatment with Pam3Cys (115.3 ± 20 MFI, $p < 0.01$, $n = 3$, [10 µg/ml], Fig. 2d).

MDSCs are induced by adenosine

To evaluate the role of adenosine in MDSC induction, we incubated PBMCs and CBMCs with adenosine and quantified the generation of CD33⁺ MDSC. After 7 days of incubation, adenosine led to a strong induction of CD33⁺ MDSCs in PBMCs

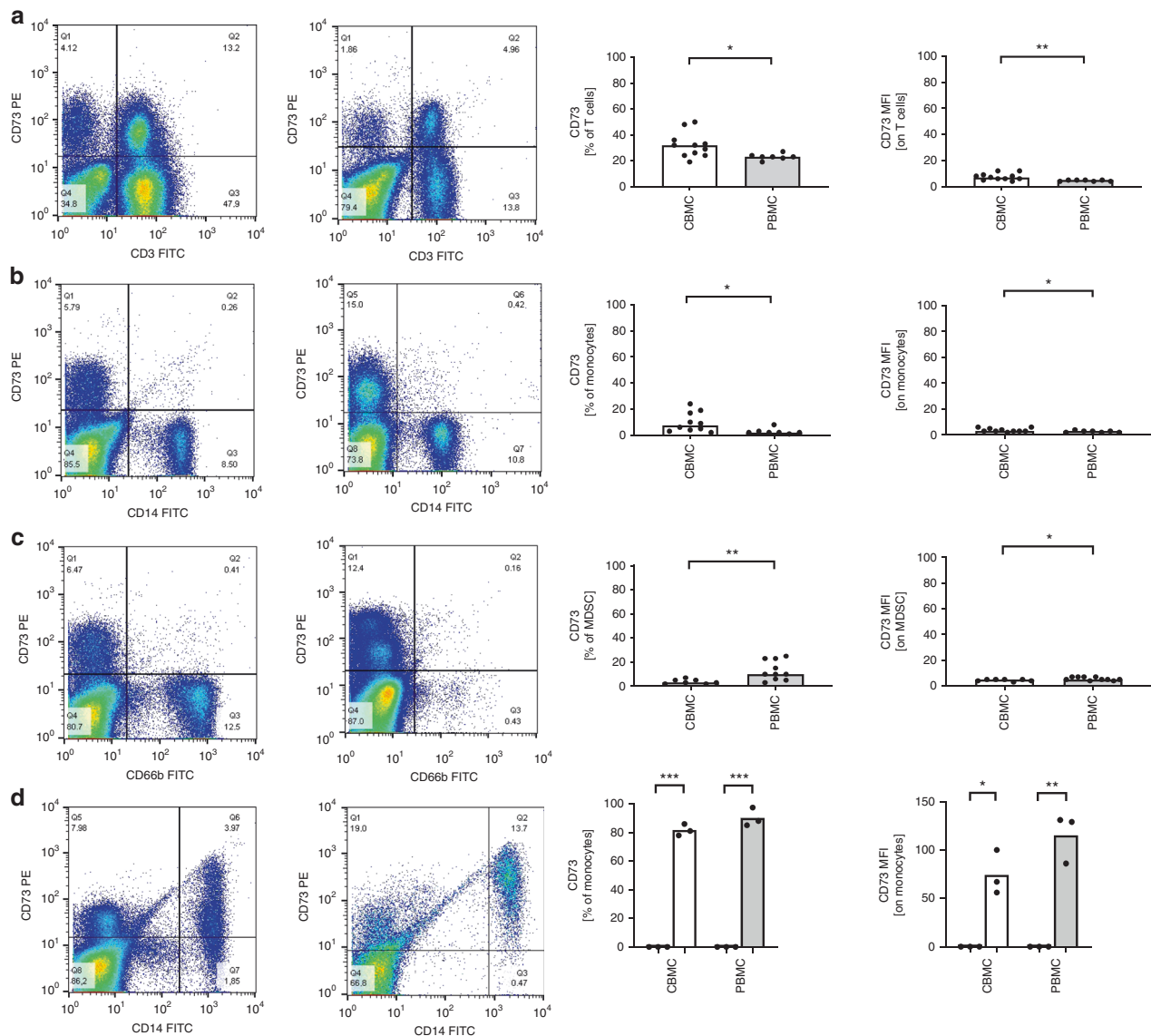


Fig. 2 CD73 expression on cord blood and peripheral blood immune effector cells. Cord blood (CBMCs, white bars) and peripheral blood mononuclear cells (PBMCs, grey bars) were isolated followed by staining for lineage markers for **a** T cells (upper diagrams), **b** monocytes (mid diagrams), **c** MDSCs (lower diagrams), **d** monocytes (incubated for 24 h in the absence (left bar) or presence (right bar) of Pam3Cys [10 µg/ml]) and CD73. Expression was quantified by flow cytometry. Given are (i) representative density plots, (ii) mean fluorescence intensities (MFI) and (iii) percentage of positive cells (%). **a–c** CBMCs $n = 7$ – 11 , PBMCs $n = 7$ – 10 , **d** CBMCs $n = 3$, PBMCs $n = 3$, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

compared to untreated control ($14.6 \pm 5\%$ vs. $8.7 \pm 5\%$, $p < 0.05$, $n = 7$, Fig. 3a and c). Adenosine-based induction was comparable to that of granulocyte–macrophage colony-stimulating factor (GM-CSF) ($14.7 \pm 5\%$, Fig. 3a and c). Our results thus show for the first time that adenosine is capable of inducing MDSCs in healthy adult individuals. Intriguingly, in CBMCs, the addition of adenosine did not induce $CD33^{+}$ MDSCs, while $CD33^{low}$ MDSCs were still detectable after 7 days of incubation (Fig. 3b and d)

Adenosine-induced MDSCs effectively suppressed T cell proliferation

Cocultivation of adenosine-induced MDSCs with freshly isolated autologous PBMCs in different ratios dose-dependently inhibited $CD4^{+}$ T cell proliferation to $31.6 \pm 10.0\%$ (control set to 100%, $n = 5$ – 9 , Fig. 4). The adenosine-induced MDSCs exerted inhibitory capacity comparable to that of GM-CSF-induced MDSCs used as a positive control (31.6 ± 8 vs. $27.7 \pm 8\%$, $p = 0.21$, $n = 5$ – 9 , Fig. 4).

Thus, adenosine may exert its immunomodulatory properties via induction of activated MDSCs.

Adenosine upregulates effector enzyme IDO in MDSCs and monocytes

To investigate the mechanism by which adenosine-induced MDSCs inhibit T cell proliferation, we next quantified expression of typical MDSC effector enzymes IDO, Arg1, inducible nitric oxide synthase after stimulation with adenosine. Freshly isolated MDSCs from cord and adult blood expressed IDO at the basal level within the range of 45–50 MFI (Fig. 5). Cultivation for 24 h did not change basal expression, while IDO expression was upregulated in cord blood MDSCs in the presence of adenosine (76.1 ± 25 vs. 53.6 ± 23 MFI, $p < 0.01$, $n = 7$, Fig. 5b, left). Incubation of adult MDSCs for 24 h in the presence of adenosine also led to increased IDO expression in comparison to untreated cells (79.3 ± 20 vs. 55.4 ± 30 MFI, $p < 0.01$, $n = 7$, Fig. 5a, left).

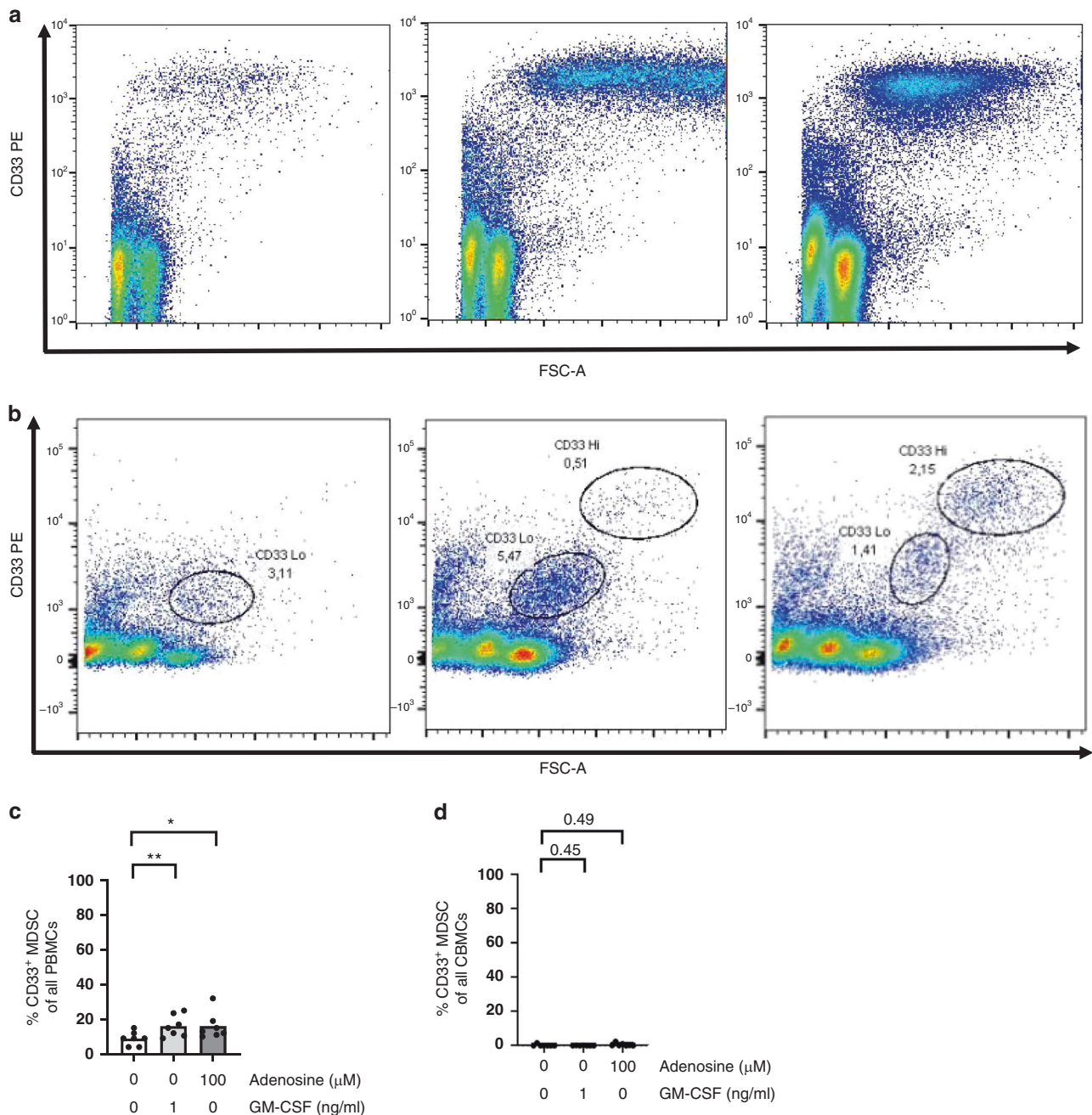


Fig. 3 CD33⁺ MDSC induction in PBMCs and CBMCs in the presence of adenosine and granulocyte-macrophage colony-stimulating factor (GM-CSF) after 7 days. **a** Peripheral blood mononuclear cells (PBMCs) and **b** cord blood mononuclear cells (CBMCs) were isolated by Biocoll density gradient centrifugation, resuspended in complete medium (RPMI-1640 medium + 10% foetal calf serum + 1% penicillin/streptomycin + 1% L-glutamine), diluted up to 5×10^5 cells/ml and left to incubate in the presence of GM-CSF [1 ng/ml], adenosine [100 μM], or left untreated for 7 days at 37 °C, 5% CO₂ with a medium change on day 4. Given are the representative density plots (left: negative control; middle: GM-CSF; right: adenosine) percentage of CD33⁺ MDSCs and the mean **c** PBMCs and **d** CBMCs, $n = 7$, $*p < 0.05$; $**p < 0.01$.

The IDO expression in freshly isolated cord and adult blood monocytes was within the range of 18–20 MFI, throughout (Fig. 5a and b, middle). Similar to MDSCs, cultivation of cord blood monocytes for 24 h did not change basal IDO expression (23.7 ± 10 MFI), while it was upregulated in the presence of adenosine (33.4 ± 5 vs. 23.7 ± 8 MFI, $p < 0.01$, $n = 7$, Fig. 5b, middle). Incubation of adult monocytes for 24 h in the presence of adenosine also led to increased IDO expression in comparison to untreated cells (34.3 ± 5 vs. 28.3 ± 7 MFI, $p < 0.05$, $n = 7$, Fig. 5a, middle).

In addition, Arg1 was significantly increased in cord blood monocytes after treatment with adenosine (338.7 ± 300 vs. 258.5

± 140 MFI, $p < 0.05$, $n = 7$, Fig. 5b, right). Contrary, Arg1 production in adult monocytes was not significantly affected by adenosine (Fig. 5a, right).

The remaining effector enzymes did not show significant changes upon treatment with adenosine (Supplemental Figure S2).

Adenosine upregulates inhibitory A₃ adenosine receptor

To further elucidate the differential effects of adenosine on neonatal and adult immune effector cells, we next quantified adenosine receptors. Treatment with adenosine led to slightly

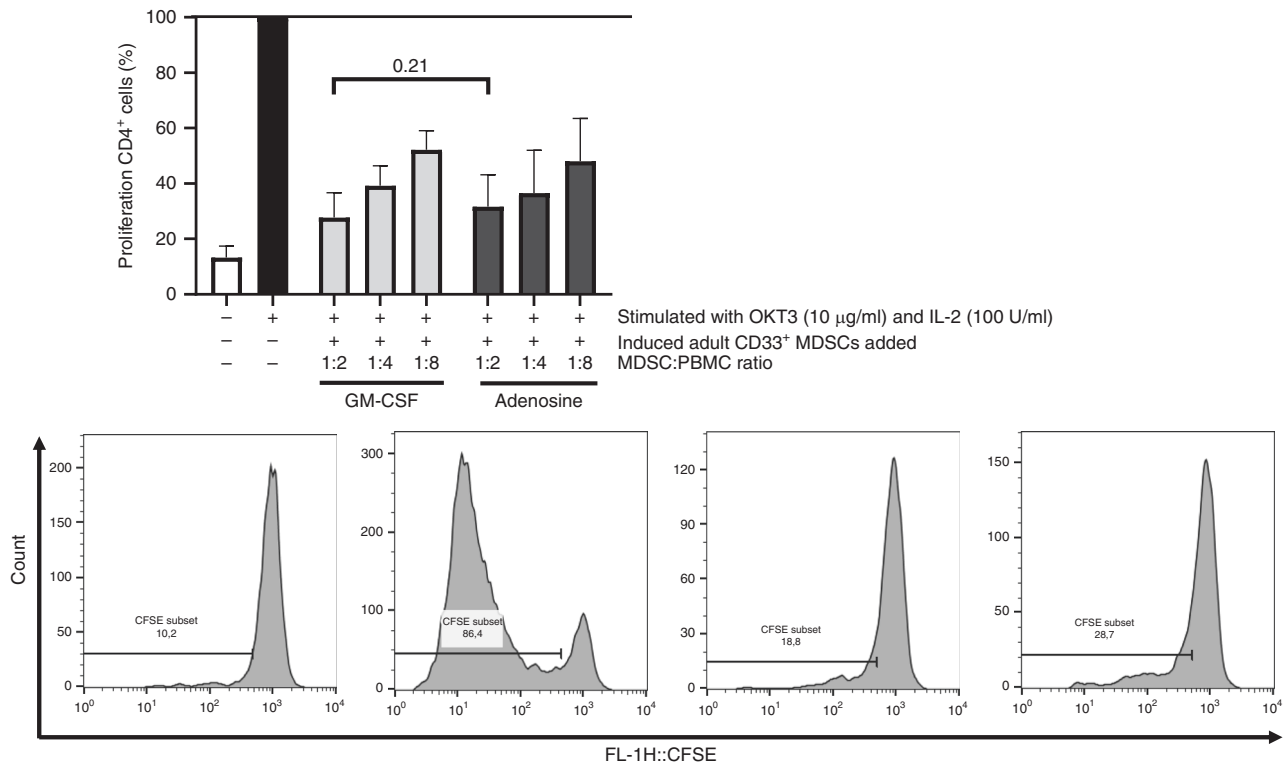


Fig. 4 T cell proliferation inhibition by induced MDSCs. Peripheral blood mononuclear cells (PBMCs) were isolated, resuspended in complete medium (RPMI-1640 medium + 10% foetal calf serum + 1% penicillin/streptomycin + 1% L-glutamine) and left to incubate in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) [1 ng/ml] or adenosine [100 µM] for 7 days at 37 °C, 5% CO₂ with medium change on day 4. On day 7, cells were harvested by applying Detachin™ and using cell scraper to detach the cells from the plastic dish bottom and investigated on CD33 expression by flow cytometry. The CD33⁺ cells were isolated by AutoMACS Separator using anti-CD33 microbeads, counted under the binocular, resuspended in a sterile 1× phosphate-buffered saline and diluted to 1 × 10⁶ cells/ml. In the meantime, autologous PBMCs were freshly isolated using Bicololl density gradient centrifugation, diluted with a sterile 1× phosphate-buffered saline to 1 × 10⁷ cells/ml and stained with CFSE according to manufacturer's instructions. Stained cells were counted on the Sysmex, resuspended and diluted to 2 × 10⁶ cells/ml in complete medium containing autologous blood plasma (10%), RPMI-1640 medium, penicillin/streptomycin (1%) and L-glutamine (1%) and split into two aliquots. One aliquot remained unstimulated as a negative control, the second aliquot was stimulated with IL-2 (100 U/ml) and OKT3 (*c*_{end} = 10 µg/ml, stock 1 mg/ml). The stimulated cells were cocultured with CD33⁺ cells in different ratios and left to incubate for 4 days at 37 °C, 5% CO₂. On day 4, cells were harvested, stained for CD4 and the proliferation rate was examined by flow cytometry. Given is the proliferation rate in percentage (%) ± SD and histograms (from left to right: negative control, positive control, 2:1 PBMCs: GM-CSF-induced MDSC, 2:1 PBMCs: adenosine-induced MDSCs), *n* = 5–9.

decreased expression levels of adenosine A_{2A} receptor in both cord blood T cells (11.5 vs. 9.8 MFI) and adult T cells (11.4 vs. 9.2 MFI, Fig. 6a). In contrast, A₃ receptor expression was strongly enhanced upon adenosine treatment in both cord and adult blood monocytes (cord blood monocytes 26 vs. 52 MFI, adult monocytes 27.9 vs. 53.6 MFI, Fig. 6b).

DISCUSSION

Adenosine, an immunomodulatory metabolite, is elevated in cord blood and exerts anti-inflammatory effects in the neonate. Here, we analysed mechanisms how adenosine may act on neonatal immune effector cells.

We found that (i) CD39 was mainly expressed on monocytes, while CD73 was mainly expressed on T cells, (ii) Pam3Cys induced CD73 expression in monocytes, (iii) adenosine effectively induced MDSCs, (iv) adenosine-induced MDSCs were capable of inhibiting T cell proliferation and (v) adenosine-induced IDO expression in MDSCs and monocytes as well as Arg1 expression in cord blood monocytes. Finally, (vi) adenosine upregulated A₃ adenosine receptors on monocytes.

Suppression of pro-inflammatory activation and T cell responses are key features of neonatal immune reactions. The adenosine system has been shown to act as a physiologic brake to

inflammation and has already been linked to neonatal immunosuppression. Interestingly, neonatal plasma contains ~3-fold greater concentrations of adenosine than adult plasma.² Former studies showed that adenosine reduced tumour necrosis factor-α and enhanced IL-6 production in cord blood and skewed the neonatal immune reaction towards anti-inflammation.²

Our data show that expression patterns of adenosine-producing ectonucleotidases CD39 and CD73 in newborns did not differ from those in healthy adults. In peripheral adult blood, CD39 is constitutively expressed on >90% of monocytes, 20–30% of CD4⁺ T cells and 5% of CD8⁺ T cells.²⁶ CD73 is expressed on ~50% of CD8⁺ T cells, 10% of CD4⁺ T cells and MDSCs can co-express CD39 and CD73. Thus, our results on the expression pattern of CD39 and CD73 on neonatal immune effector cells resemble those from adults. Similar to adults, at least two immune cell types, that is, monocytes and T cells (Figs. 1 and 2), may take part in neonatal adenosine production, occurring as a two-step degradation of extracellular ATP by CD39 and CD73. Taken together, our results show a differential expression of ectonucleotidases in neonatal T cells and monocytes rendering them capable of producing adenosine in a cooperative way.

It remains unclear, however, if other cell types, such as human umbilical vein endothelial cells or B cells, may contribute to the adenosine production in fetuses and neonates.

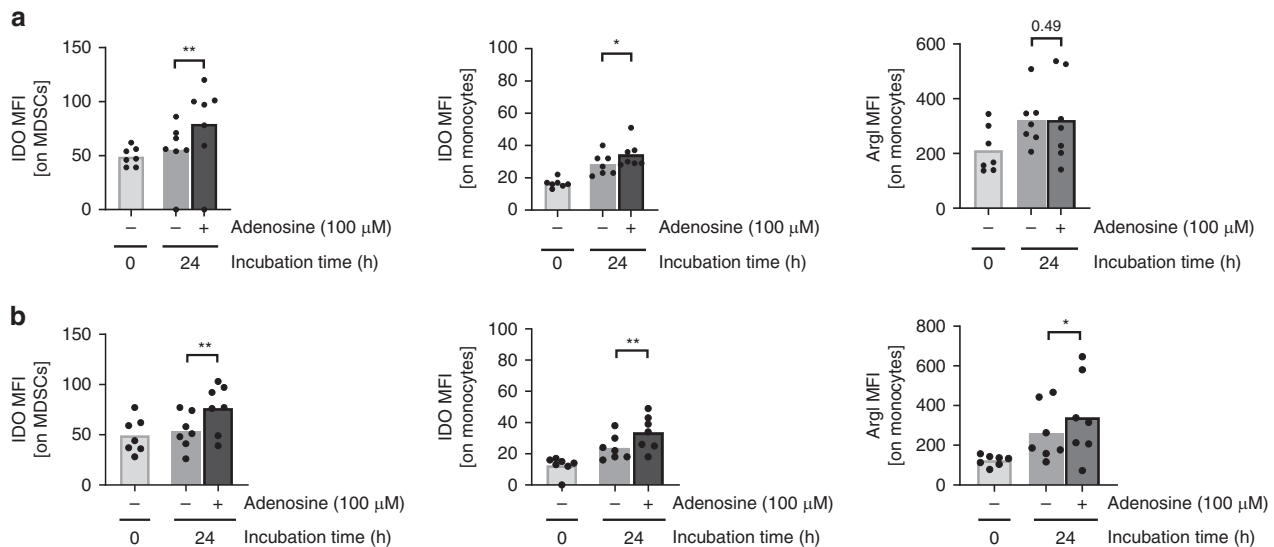


Fig. 5 Expression of IDO (indoleamine-2,3-dioxygenase) and Arg1 (arginase I) in the presence and absence of adenosine. **a** Peripheral blood mononuclear cells (PBMCs) and **b** cord blood mononuclear cells (CBMCs) were isolated, resuspended in complete medium (RPMI-1640 medium + 10% foetal calf serum + 1% penicillin / streptomycin + 1% L-glutamine) and stained for immediate detection ($t = 0$ h) as well as incubated in the presence (+) or absence (–) of adenosine [100 μ M] for 24 h. After incubation, aliquots were stained for IDO, Arg1, MDSC lineage marker (CD66b), monocyte lineage marker (CD14) and examined by flow cytometry. Mean fluorescence intensities (MFIs) of **a** PBMCs and **b** CBMCs, $n = 7$, * $p < 0.05$; ** $p < 0.01$.

We further showed that Pam3Cys induced CD73 expression in neonatal and adult monocytes (Fig. 2d). Ectonucleotidase induction upon distinct PAMPs such as LPS has been shown for different cell types and may attenuate inflammation.²⁷

We show here for the first time that adenosine may induce MDSCs under physiologic conditions. MDSC accumulation is an outstanding feature of the immune system during the foetal and neonatal period. Thus, mechanisms leading to MDSC accumulation are of special interest. We reported previously that soluble HLA-G, a non-classical major histocompatibility complex with immunoregulatory properties belonging to the HLA-Ib family, efficiently induced MDSCs from PBMCs.²⁴ Similar to HLA-G, adenosine is a soluble metabolite present in neonatal plasma in higher concentrations than in adults.² Adenosine-based MDSC induction was comparable to that of GM-CSF (Fig. 3). These results point to the ability of adenosine to induce MDSCs in healthy adults under physiological conditions.

Interestingly, CBMC-based induction of MDSCs failed in our settings. The potential to induce MDSCs in CBMCs may be exhausted. Adenosine concentrations in cord blood have been shown to be enhanced compared to those detected in adult blood;² thus, it is possible that the addition of further external adenosine has no effect. Another explanation may be that the potential to generate MDSCs is also exhausted due to high concentrations of GM-CSF in neonatal blood plasma, which is a strong MDSC inducer.^{28–30}

The question remains whether the adenosine-based induction occurs via a direct action of adenosine or indirectly via other mechanisms. Adenosine may directly promote the MDSC function via the A_{2B} receptor, leading to MDSC expansion and immune tolerance.³¹ Moreover, it may mediate a shift in cytokine production towards tumour growth factor- β .³² Enhanced tumour growth factor- β production may then promote CD73 expression, thus contributing to the conversion of AMP to adenosine,³³ which in turn leads to stimulation of A_{2A} and A_{2B} receptor.

Here, we focused on the capability of induced MDSCs to inhibit the proliferation of freshly isolated autologous adult T lymphocytes, after showing that MDSCs descending from adult blood as well as from cord blood may effectively inhibit proliferation of cord blood T cells.¹⁶

Adenosine-induced MDSCs were potent inhibitors of polyclonal T cell proliferation (Fig. 4) and upregulated IDO expression (Fig. 5). Different suppressive effects of MDSCs may be mediated by different effector enzymes. We have previously shown that activation of MDSCs by HLA-G also was mediated by increased IDO expression.²⁴

Our data also showed enhanced IDO expression by monocytes upon adenosine treatment (Fig. 5), which may contribute to T cell inhibition as well. In addition, upregulation of Arg1 by cord blood monocytes in the presence of adenosine (Fig. 5) may contribute to temporary suppression of maternal T cell responses.³⁴

T cells, monocytes and MDSCs (supplemental Figure S3) from both cord and adult blood expressed A_{2A} and A_3 receptors. There was a slight downregulation of A_{2A} receptor on T cells upon incubation with adenosine. Contrarily, A_3 receptors on monocytes were upregulated (Fig. 6).

Upregulation of inhibitory A_3 receptors may be a possible cause of the immunosuppressive effect of adenosine due to adenylyl cyclase inhibition.^{35,2} Besides A_{2A} and A_3 receptors, several other adenosine receptors may affect immune responses.¹⁴ For example, A_{2B} attenuated LPS-induced inflammation and vascular injury in hypoxia-elicited tissue adaptation in lung³⁶ and myocardial injury.³⁷

Summarising, adenosine may be produced by immune cells of cord blood and may exert its immunomodulatory functions in neonates as a mode of autoregulation acting on several levels, for example, (a) induction of MDSCs, (b) MDSC-mediated inhibition of T cell proliferation, (c) enhanced IDO production and (d) enhanced expression of inhibitory A_3 receptor by monocytes.

Immunosuppressive effects of adenosine are known from pathological conditions such as malignancies, where they have been linked to tumour escape.³⁸ They may have a physiological role in neonates. Intriguingly, substances with opposite effects on the main intracellular signal transduction pathways, that is, cyclic AMP phosphodiesterase inhibitors such as caffeine or pentoxifylline, also exhibit immunosuppressive properties.^{39,40} These data demonstrate that signal transduction pathways other than cAMP, such as STAT1 signalling, may be involved in adenosine effects.

Therefore, adenosine metabolism may be an interesting target to modulate immunosuppression in neonates.

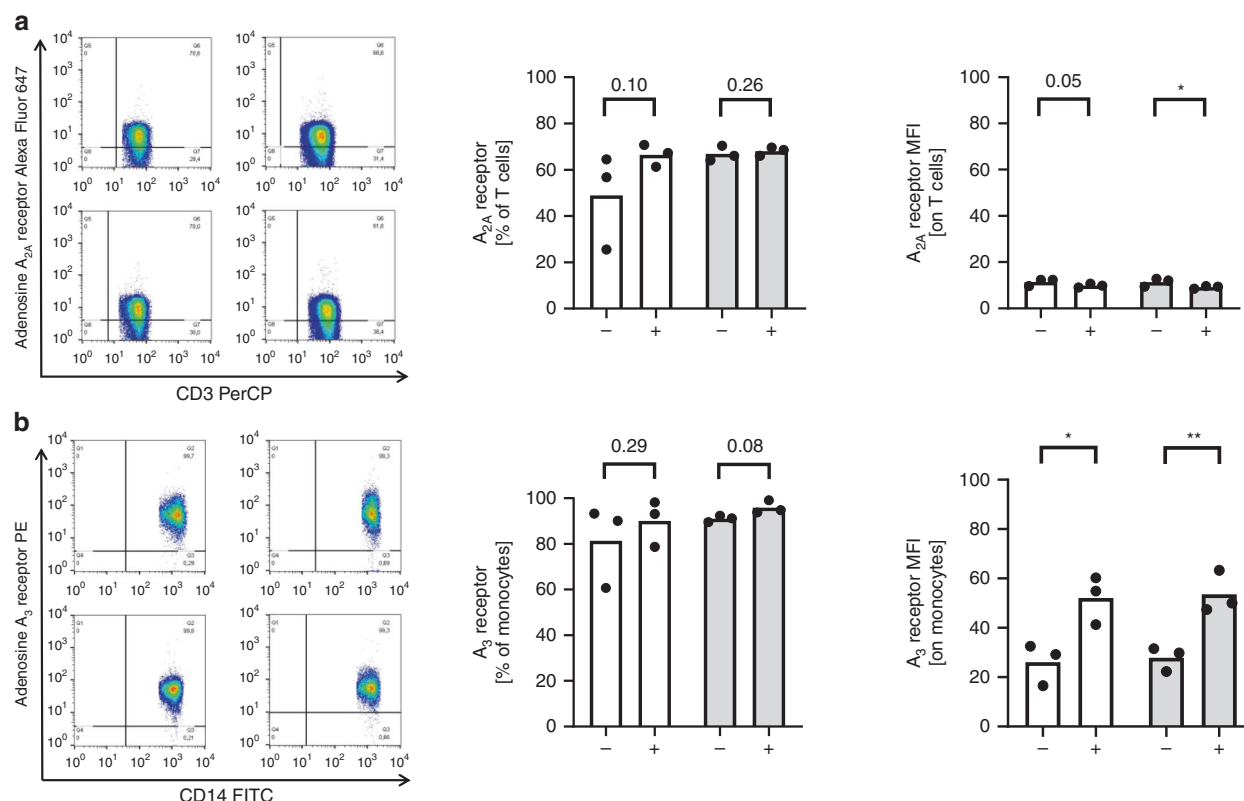


Fig. 6 Expression of adenosine receptors A_{2A} and A_3 on immune effector cells. Cord blood (CBMCs, white bars) and peripheral blood mononuclear cells (PBMCs, grey bars) were isolated, resuspended in complete medium (RPMI-1640 medium + 10% foetal calf serum + 1% penicillin/streptomycin + 1% L-glutamine). The cell suspension was incubated for 24 h in the presence (+) or absence (–) of adenosine [100 μ M]. After 24 h, cells were harvested, stained and examined on the expression of adenosine receptors. The cells were stained for **a** T cell (CD3), **b** monocyte (CD14) lineage marker and adenosine receptors (A_{2A} and A_3). Expression was quantified by flow cytometry. Given are mean fluorescence intensities (MFIs), percentage of positive cells (%), representative density plots (**a** T cells, **b** monocytes, left: CBMCs, right: PBMCs, upper part: with adenosine, lower part: without adenosine). CBMCs $n = 3$, PBMCs $n = 3$, * $p < 0.05$; ** $p < 0.01$.

ACKNOWLEDGEMENTS

This project was funded by The Federal Ministry of Education and Research (BMBF) grant no GFGL1165817-01 GL1746F to C.G. and The Ministry of Science, Research and Art Baden-Württemberg and The European Social Fund to N.K.-G.

AUTHOR CONTRIBUTIONS

F.Ď., N.K.-G. and C.G.: substantial contributions to conception and design; F.Ď.: acquisition of data, or analysis and interpretation of data; F.Ď., C.G.: drafting the article or revising it critically for important intellectual content; C.G., C.F.P.: revising the manuscript and final approval of the version to be published.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41390-020-01275-8>.

Competing interests: The authors declare no competing interests.

Statement of consent: All parents gave written informed consent. The study protocol was approved by ethics committee of the Medical Faculty of Tuebingen University, application number 248/2005A.

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