

Granulocytic myeloid-derived suppressor cells from human cord blood modulate T-helper cell response towards an anti-inflammatory phenotype

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Summary

Infections are a leading cause of perinatal morbidity and mortality. The outstandingly high susceptibility to infections early in life is mainly attributable to the compromised state of the neonatal immune system. One important difference to the adult immune system is a bias towards T helper type 2 (Th2) responses in newborns. However, mechanisms regulating neonatal T-cell responses are incompletely understood. Granulocytic myeloid-derived suppressor cells (GR-MDSC) are myeloid cells with a granulocytic phenotype that suppress various functions of other immune cells and accumulate under physiological conditions during pregnancy in maternal and fetal blood. Although it has been hypothesized that GR-MDSC accumulation during fetal life could be important for the maintenance of maternal–fetal tolerance, the influence of GR-MDSC on the immunological phenotype of neonates is still unclear. Here, we investigated the impact of GR-MDSC isolated from cord blood (CB-MDSC) on the polarization of Th cells. We demonstrate that CB-MDSC inhibit Th1 responses and induced Th2 responses and regulatory T (Treg) cells. Th1 inhibition was cell-contact dependent and occurred independent of other cell types, while Th2 induction was mediated independently of cell contact through expression of Arg1 and reactive oxygen species by CB-MDSC and partially needed the presence of monocytes. Treg cell induction by CB-MDSC also occurred cell-contact independently but was partially mediated through inducible nitric oxide synthase. These results point towards a role of MDSC in regulating neonatal immune responses. Targeting MDSC function in neonates could be a therapeutic opportunity to improve neonatal host defence.

Keywords: myeloid-derived suppressor cells; neonate; regulatory T cells; T helper cells.

Introduction

Infections are a leading cause of perinatal morbidity and mortality. About one in one thousand newborns suffer

from sepsis; the lower the gestational age at birth, the higher the incidence^{1–3} (up to 55%). Besides high mortality rates, sepsis can result in severe impairments in neonatal outcome by triggering pro-inflammatory post-infectious

Abbreviations: 1-MT, 1-methyl-DL-tryptophan; CB-MDSC, cord blood myeloid-derived suppressor cells; CBMC, cord blood mononuclear cells; GR-MDSC, granulocytic myeloid-derived suppressor cells; IDO, indolamine 2,3 dioxygenase; IFN- γ , interferon- γ ; IL-2, interleukin-2; iNOS, inducible nitric oxide synthase; L-NMMA, L-N^G-monomethyl arginine citrate; MACS, magnetic activated cell sorting; MDSC, myeloid-derived suppressor cells; MO-MDSC, monocytic myeloid-derived suppressor cells; Nor-NOHA, N^ω-hydroxy-nor-arginin; PBMC, peripheral blood mononuclear cells; PD-1, programmed death 1; PD-L1, programmed death ligand 1; ROS, reactive oxygen species; TGF- β , transforming growth factor β ; Th, T helper; Treg cells, regulatory T cells

diseases like bronchopulmonary dysplasia or periventricular leucomalacia.^{4,5}

The high susceptibility to infections early in life has mainly been attributed to the 'neonatal' state of the immune system.^{6,7} At birth, the neonatal immune system undergoes a dramatic transition from a largely sheltered environment *in utero* to the outside world, where it has to handle commensal and pathogenic microorganisms. Compared with adults, neonates exhibit distinct differences in both their innate and adaptive immune responses, including defects in antimicrobial activity in neonatal neutrophils,^{8–10} a limited primary antibody response of neonatal B cells,¹¹ a diminished co-stimulatory capacity of neonatal antigen-presenting cells^{12,13} and a bias of neonatal T-cell responses towards T helper type 2 (Th2) function.¹⁴ However, mechanisms regulating the neonatal immune system and its adaptation to the adult state are as yet poorly understood.

Myeloid-derived suppressor cells (MDSC) are myeloid cells with the ability to suppress immune responses. In humans, two MDSC subsets exist, a granulocytic subset (GR-MDSC), which expresses the granulocytic lineage markers CD15 and/or CD66b, and a monocytic subset (MO-MDSC), which expresses the monocytic antigen CD14. Both subsets express CD33, but lack expression of the human leucocyte antigen DR (HLA-DR).^{15,16} MDSC were primarily described under tumour conditions, where they are induced by the tumour microenvironment and inhibit anti-tumour immune responses.^{17–20} Mechanisms by which MDSC exert their suppressive activity include the depletion of arginine by expression of Arginase I (ArgI) and inducible nitric oxide synthetase (iNOS), the production of reactive oxygen species (ROS) and the degradation of tryptophan by expression of indoleamine-2,3-dioxygenase (IDO). Recently, we demonstrated that GR-MDSC also accumulate during pregnancy in maternal and fetal blood^{21,22} and in placenta,²³ leading to the hypothesis that MDSC play a role in mediating maternal–fetal tolerance. The contribution of MDSC to the specific neonatal immune response has not yet been elucidated.

In the present study, we investigated whether increased levels of GR-MDSC in cord blood contribute to the altered immune response in neonates. Therefore, we analysed the effects of cord blood GR-MDSC (CB-MDSC) on the polarization of T helper cells and found that CB-MDSC mediate the induction of Th2 cells and regulatory T (Treg) cells, but inhibit Th1 cells and may thereby impede neonatal host defence.

Materials and methods

Patients

Cord blood was collected from healthy term newborns immediately after caesarean section. All women gave

written informed consent and the study was approved by the local ethics committee. Peripheral blood from healthy adults was gained from adult volunteers.

Cell isolation and culture

Mononuclear cells from heparinized cord (CBMC) and peripheral blood (PBMC) were isolated by density gradient centrifugation (lymphocyte separation medium; Biochrom, Berlin, Germany). CB-MDSC were separated from the CBMC fraction by magnetically activated cell sorting (MACS) after labelling with anti-CD66b-FITC (BD Pharmingen, Heidelberg, Germany) and anti-FITC magnetic beads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD66b⁺ cells was between 93 and 97%, as determined by flow cytometry.

For separation of T cells and monocytes from the PBMC fraction, cells were labelled with anti-CD3-, Pan T-cell Biotin-Antibody-Cocktail and Pan T-cell microbead cocktail or CD14-MicroBeads and separated by MACS according to the manufacturer's protocol (Miltenyi Biotec).

For co-culture experiments PBMC, CD3⁺ T cells or CD3⁺ T cells and CD14⁺ monocytes (ratio 2 : 1) were incubated alone or with CB-MDSC at a ratio of 2 : 1 in RPMI-1640 with 10% fetal calf serum in 24-well plates either with direct cell contact or separated by 0.4- μ m transwells (Greiner Bio-One, Frickenhausen, Germany) at 37° and 5% CO₂. After 4 days of culture, cells were harvested and extracellular or intracellular staining was performed.

For inhibition of MDSC effector enzymes and ROS production, PBMC/CB-MDSC co-cultures were performed as described above and inhibitors for ArgI (*N*^ω-hydroxynor-arginin, nor-NOHA, 5 μ M; Cayman Chemical, Ann Arbor, MI), iNOS (L-*N*^G-monomethyl arginine citrate, L-NMMA, 500 μ M; Cayman Chemical), ROS (catalase, 500 mIU; Sigma-Aldrich, Weinheim, Germany) and IDO [1-methyl-DL-tryptophan (1-MT) 50 μ M; Sigma-Aldrich] were added throughout the culture period.

For neutralizing/blocking experiments antibodies against transforming growth factor β (TGF- β ; clone 1D11, 5 μ g/ml; R&D Systems, Minneapolis, MN), CD95L (clone 100 419, 10 μ g/ml; R&D Systems) and PD-1 (Nivolumab, 10 μ g/ml; Bristol-Myers Squibb, New York, NY) were added over the complete culture period.

T-cell suppression assays

The PBMC from healthy non-pregnant controls were isolated, stained with carboxyfluorescein-succinimidyl ester (CFSE; Invitrogen, Heidelberg, Germany) according to the manufacturer's instructions and stimulated with 100 U/ml interleukin-2 (IL-2; R&D Systems,

Wiesbaden-Nordenstadt, Germany) and 1 µg/ml OKT3 (Janssen-Cilag, Neuss, Germany). 60 000 PBMC per well in RPMI-1640 supplemented with 10% autologous serum were seeded in a 96-well microtitre plate (BD Biosciences, Heidelberg, Germany) and 30 000 (1 : 4) or 60 000 (1 : 2) CB-MDSCs in RPMI-1640, were added. As control, only RPMI-1640 was added to the PBMCs. After 96 hr of incubation, cells were harvested and stained with anti-CD4-allophycocyanin. CFSE fluorescence intensity was analysed by flow cytometry to determine proliferation of CD4⁺ T cells. Proliferation index, defined as the ratio of T-cell proliferation after addition of CB-MDSC and T-cell proliferation without CB-MDSC, was determined. T-cell proliferation without CB-MDSCs was set to a fixed value of 1.

Flow cytometry

Antibodies used for extracellular staining of Th cell subsets were purchased from R&D Systems (anti-CCR4) and Miltenyi Biotec (anti-CD4). All other antibodies for extracellular staining were purchased from BD Pharmingen. Intracellular staining of cytokines was performed as described previously.²³ Antibodies against IL-4 and interferon-γ (IFN-γ) were purchased from BD Biosciences. For quantification of Treg cells, the human regulatory T-cell staining kit #3 from eBioscience (San Diego, CA) was used according to the manufacturer's protocol. Data acquisition was performed with a FACScalibur flow cytometer (BD Bioscience) and data were analysed using CELLQUEST (BD Biosciences).

Statistics

Statistical analysis was carried out using GRAPHPAD PRISM version 5.0 (La Jolla, CA, USA). Values were tested for Gaussian distribution using D'Agostino and Pearson omnibus normality test. Differences between percentages of Th1, Th2 and Treg cells in co-culture experiments were determined using the paired *t*-test or the Wilcoxon matched-pairs signed rank test. A *P*-value < 0.05 was considered significant.

Results

CB-MDSC polarize CD4⁺ T cells towards a Th2 response and induce Treg cells

First, we investigated the impact of CB-MDSC on the polarization of Th cells by performing co-cultures between CB-MDSC and PBMC from healthy adult donors. CB-MDSC were enriched and assessed for their suppressive activity as described previously.²¹ Figure S1 (see Supplementary material) shows gating strategy, phenotype and suppressive activity of CB-MDSC. According

to previous protocols,^{23,24} we characterized Th cell subsets by the surface expression of the chemokine receptors CCR4 (CD194), CCR6 (CD196) and CXCR3 (CD183). Gating strategies for Th1 and Th2 cells are depicted in Fig. 1(a). After 5 days of co-culture with CB-MDSC, the proportion of CXCR3⁺ CCR4⁻ CCR6⁻ Th1 cells within the CD4⁺ cell population was significantly decreased compared to PBMC cultured alone (5.6% ± 4.3% versus 9.9% ± 3.0%, *n* = 10, *P* = 0.0003), whereas the proportion of CXCR3⁻ CCR4⁺ CCR6⁻ Th2 cells increased from 10.2 ± 3.8% in PBMC cultured alone to 15.4% ± 7.3% in PBMC cultured with CB-MDSC (*n* = 10, *P* < 0.01, Fig. 1b,c). Proliferation of CD4⁺ T cells was not affected under these experimental settings (not shown). Staining for the intracellular cytokines IFN-γ and IL-4 provided similar results with a decrease of IFN-γ⁺ Th1 cells from 3.8% ± 4.9% to 1.5% ± 2.9% and an increase in the percentage of IL-4⁺ Th2 cells from 10.8% ± 9.0% to 30.3% ± 13.5% in CD4⁺ cells when PBMC were co-cultured with CB-MDSC compared with PBMC cultured alone (*n* = 14, *P* = 0.0002 and *P* < 0.0001, respectively; Fig. 1d-f). Co-culture of mature granulocytes (polymorphonuclear neutrophils) with PBMC did not have such effects (see Supplementary material, Fig. S2). Regarding the proportion of CD4⁺ CD25^{high} Treg cells expressing Forkhead box protein 3 (FoxP3), we also found a strong induction by CB-MDSC (1.8% ± 0.5% versus 3.8% ± 0.5%, *n* = 10, *P* < 0.0001) (Fig. 1g,h).

Inhibition of Th1 cells by CB-MDSC requires direct cell-contact and is independent of other cell types

To gain hints on underlying mechanisms for Th1 suppression by CB-MDSC, we next performed transwell experiments. Separation of CB-MDSC and PBMC by a membrane completely abrogated the suppression of Th1 cells by CB-MDSC (7.5% ± 3.7% versus 7.8 ± 4.8%, *n* = 11) (Fig. 2a), suggesting that direct cell contact is required for this effect. We further tested whether Th1 suppression was dependent on cell types other than T cells by conducting co-culture experiments with CD3-enriched and CD4-enriched T cells instead of PBMC. As observed in the CB-MDSC/PBMC co-cultures, addition of CB-MDSC to CD3-enriched or CD4-enriched T cells led to a significant reduction in Th1 cells as well (12.1% ± 3.6% versus 6.4% ± 2.4%, *n* = 7, *P* = 0.01 and 12.6% ± 3.8% versus 1.7% ± 1.2%, *n* = 6, *P* = 0.02) (Fig. 2b; and see Supplementary material, Fig. S3a). To test whether any of the well-known effector mechanisms of MDSC could be responsible for Th1 suppression, we added inhibitors for Arg1 (nor-NOHA), iNOS (L-NMMA), ROS (catalase) and IDO (1-methyl-tryptophan) to the cultures. However, none of these inhibitors could abolish the Th1-suppressive effect of CB-MDSC (Fig. 2c,d). Besides, blockade of TGF-β had

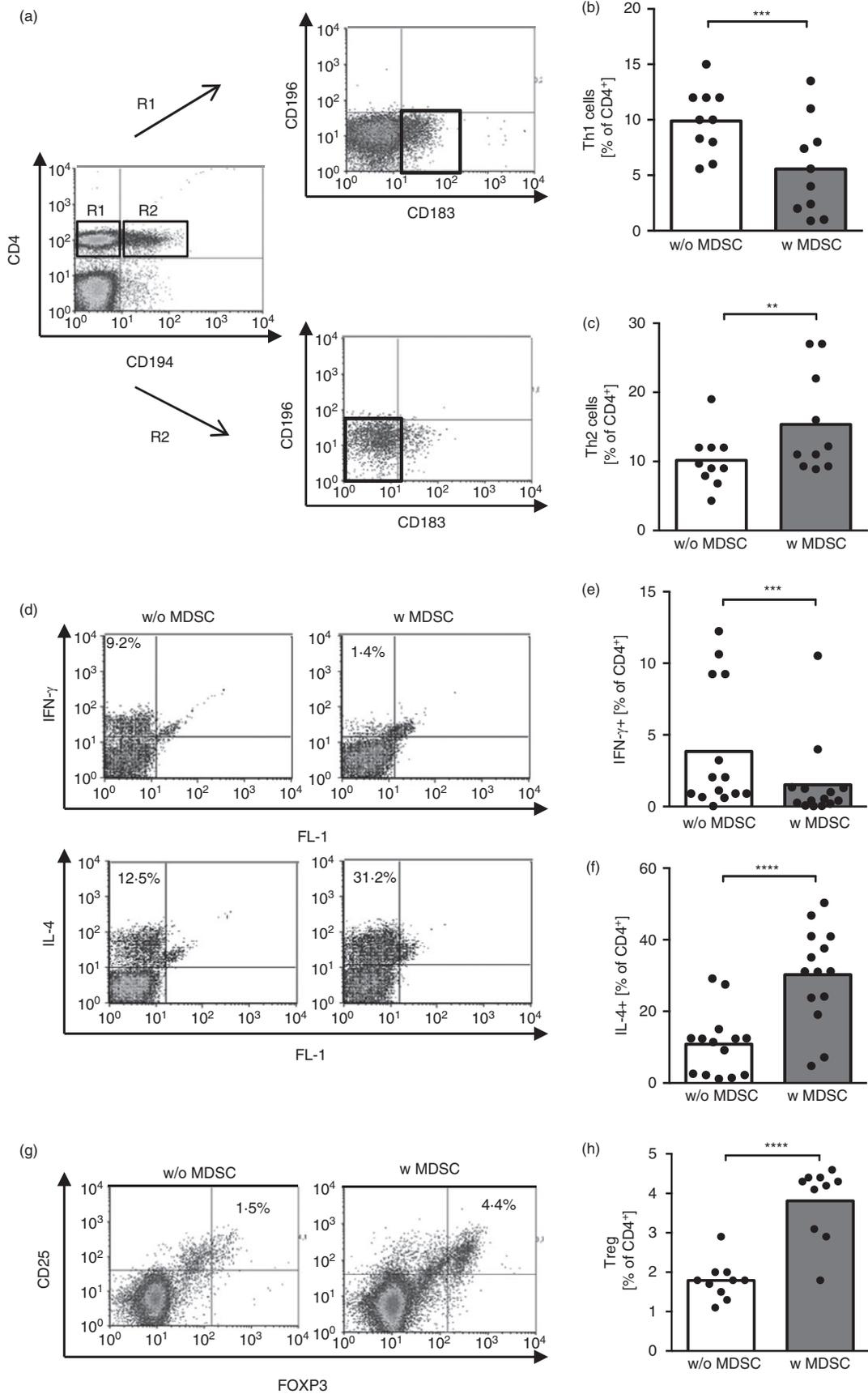


Figure 1. Polarization and cytokine production of CD4⁺ T cells under the influence of cord blood myeloid-derived suppressor cells (CB-MDSC). CB-MDSC were enriched from cord blood mononuclear cells (CBMC) and added to peripheral blood mononuclear cells (PBMC) isolated from a healthy adult control. After 5 days of culture surface staining (a–c), stimulation for 5 hr with GolgiPlug and intracellular staining for interferon- γ (IFN- γ) and interleukin-4 (IL-4) (d–f) or intracellular staining for FoxP3 (g, h) was performed and cells were analysed by flow cytometry. (a) Gating strategies for CD4⁺ CD183⁺ CD194⁻ CD196⁻ Th1 cells and CD4⁺ CD183⁻ CD194⁺ CD196⁻ Th2 cells. (b, c) Scatter plots with bars show the percentage of CD183⁺ CD194⁻ CD196⁻ Th1 cells and CD183⁻ CD194⁺ CD196⁻ Th2 cells of CD4⁺ cells without addition of CB-MDSCs (white bars) and with addition of CB-MDSCs (grey bars). Bars represent pooled data from 10 independent experiments and each point represents an individual sample. $**P < 0.01$, $***P = 0.0003$; paired *t*-test. (d) Density plots for FL-1 versus IFN- γ (upper plots) and FL-1 versus IL-4 (lower plots) without addition of CB-MDSCs (w/o MDSC) and with addition of CB-MDSCs. Cells were pre-gated for CD4. (e, f) Scatter plots with bars show the percentage of IFN- γ ⁺ cells and IL-4⁺ cells of CD4⁺ cells without addition of CB-MDSCs (white bars) and with addition of CB-MDSCs (grey bars). Bars represent pooled data from 14 independent experiments each point represents an individual sample. $***P = 0.0002$, $****P < 0.0001$; Wilcoxon matched-pairs signed rank test and paired *t*-test. (g) Density plots for FoxP3 versus CD25 shows gating strategies for CD4⁺ CD25^{high} FoxP3⁺ regulatory T (Treg) cells without addition of CB-MDSC (left plot) and with addition of CB-MDSC (right plot). Cells were pre-gated for CD4. (h) Scatter plot with bars show percentage of CD4⁺ CD25^{high} FoxP3⁺ Treg cells without addition of CB-MDSC (white bar) and with addition of CB-MDSC (grey bar). $n = 10$, $****P < 0.0001$: paired *t*-test.

no effect on Th1 suppression by CB-MDSC (Fig. 2e). As we found high expression levels of the programmed death ligand 1 (PD-L1) on CB-MDSC (see Supplementary material, Fig. S4), we sought to examine whether Th1 suppression could be mediated through the PD1/PD-L1 axis. Blockade of PD-1, however, did not restore Th1 expression under addition of CB-MDSC (Fig. 2f). Lastly, we hypothesized that the CB-MDSC-induced decrease in Th1 cells could be caused by an induction of apoptosis in Th1 cells. Annexin V staining revealed that co-culture of PBMC with CB-MDSC led to a higher apoptosis rate in CD4⁺ T cells compared with culture of PBMC alone ($5.8\% \pm 2.7\%$ versus $9.7\% \pm 1.8\%$, $n = 6$, $P = 0.02$) (Fig. 2g). Counterstaining of CXCR3 and CCR4 showed a tendency towards higher apoptosis rates in CXCR3⁺ CD4⁺ T cells but not in CCR4⁺ CD4⁺ T cells (Fig. 2h,i). However, blockade of CD95L had no effect on Th1 suppression by CB-MDSC (Fig. 2j). On the whole, CB-MDSC suppressed Th1 cells in a cell-contact-dependent manner and without the help of other cell types, but the underlying mechanism remains unclear.

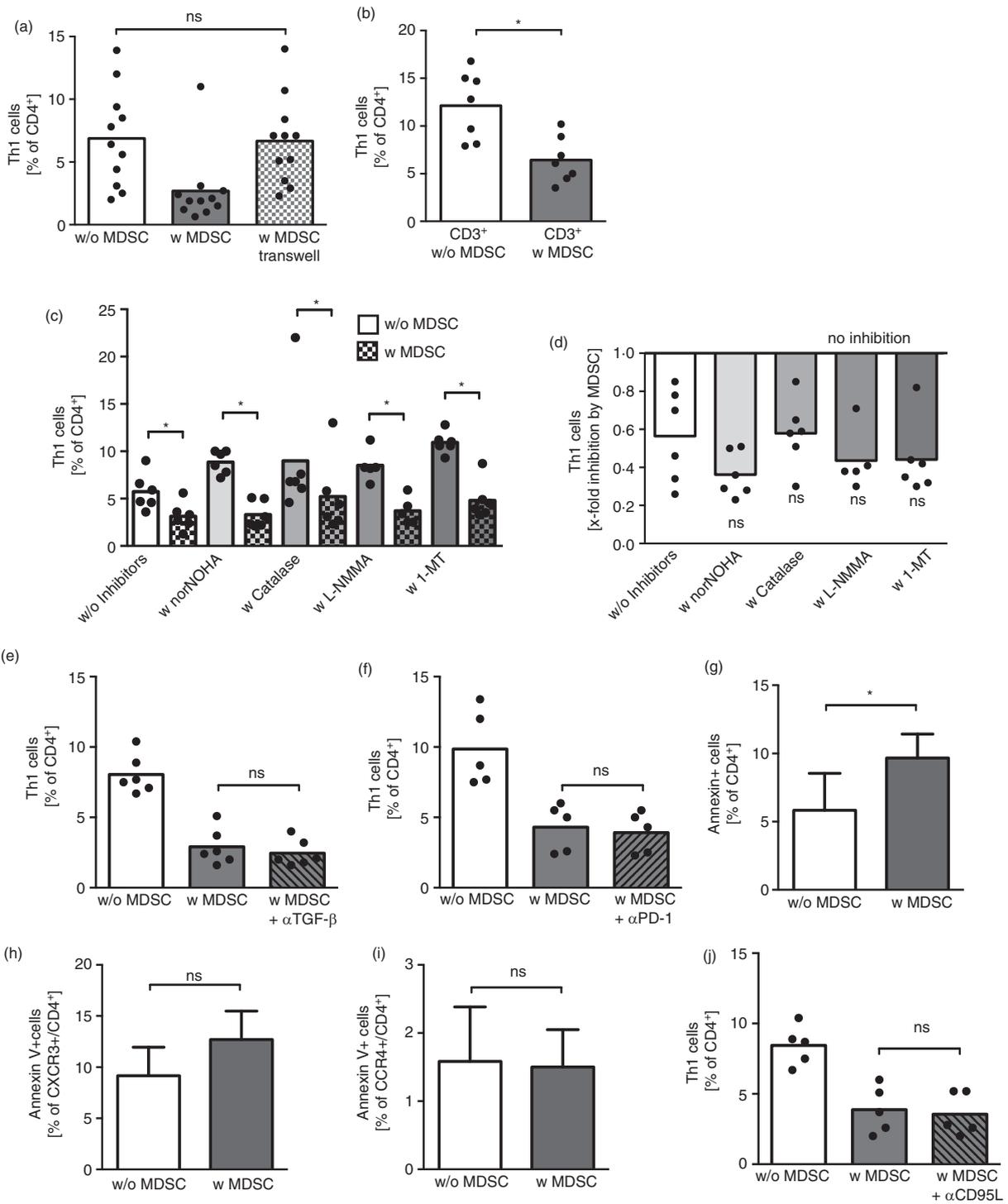
Induction of Th2 cells by CB-MDSC is mediated through ArgI and ROS and is partially dependent on an interaction with monocytes

Concerning induction of Th2 cells by CB-MDSC, transwell experiments revealed that there was no cell-contact-dependency ($7.9\% \pm 2.9\%$ versus $6.2\% \pm 2.0\%$ without CB-MDSC, $n = 11$, $P = 0.008$), suggesting that soluble factors produced by CB-MDSC were responsible for the effect. Addition of the ArgI inhibitor nor-NOHA reduced the CB-MDSC-mediated Th2 induction from 1.4-fold to a 1.1-fold induction ($n = 6$, $P = 0.04$). Addition of catalase also reduced Th2 induction, but this effect was not statistically significant (1.4-fold to 1.1-fold, $n = 6$, $P = 0.06$), whereas L-NMMA and 1-MT had no effect (Fig. 3b,c). Nor-NOHA also reduced the production of IL-4 by CD4⁺ T cells (see Supplementary material,

Fig. S5). Combined addition of nor-NOHA and catalase amplified the effects of nor-NOHA (1.5-fold induction without inhibitors, 1.2-fold induction with nor-NOHA alone, no induction with nor-NOHA and catalase, $n = 5$, $P = 0.03$) (Fig. 3d). As Th2 induction was reduced in co-culture experiments with PBMC selected for CD3 containing CD4 and CD8 T cells (1.1-fold versus 1.4-fold, $n = 8$, not significant, data not shown), and abrogated in co-cultures containing only CD4 T cells instead of whole PBMC (and see Supplementary material, Fig. S3b), we asked whether monocytes or B cells could be involved in CB-MDSC-mediated Th2 induction. Addition of CD14-enriched monocytes to CB-MDSC/T-cell co-cultures significantly increased Th2 induction by CB-MDSC ($12.8\% \pm 6.2\%$ without MDSC, $15.0\% \pm 6.8\%$ with MDSC alone, $17.8\% \pm 7.9\%$ with MDSC and monocytes, $n = 6$, $P = 0.02$), whereas addition of CD19-enriched B cells had no effect (Fig. 3e). Concordantly, depletion of CD14⁺ monocytes from PBMC significantly reduced the induction of Th2 cells by CB-MDSC from $15.9 \pm 2.8\%$ to $13.0\% \pm 3.9\%$ ($n = 8$, $P = 0.003$) (Fig. 3f). Taken together, CB-MDSC induced Th2 cells through the expression of ArgI, the production of ROS and an interaction with monocytes.

Induction of Treg cells by CB-MDSC is partially mediated through iNOS expression

To figure out the mechanism for Treg cell induction by CB-MDSC, we again tested cell-contact-dependency and contribution of other cell types. As shown in Fig. 4(a,b), neither separation of CB-MDSC and PBMC by transwells, nor co-culture with CD3-enriched T cells instead of PBMC altered the Treg cell-inducing effect of CB-MDSC (induction from $2.0\% \pm 0.5\%$ to $4.7 \pm 0.3\%$, $n = 5$, $P < 0.0001$ in transwells and from $2.0\% \pm 1.1\%$ to $4.9\% \pm 1.2\%$, $n = 5$, $P = 0.01$ with CD3-enriched T cells), suggesting that a soluble factor would be responsible for the induction and no other cell type was



involved. Blocking of ArgI, iNOS, ROS and IDO with appropriate inhibitors showed that Treg induction by CB-MDSC was partially abrogated by addition of L-NMMA (iNOS inhibitor) to the culture, whereas non-NOHA (ArgI inhibitor) and 1-MT (IDO inhibitor) had no effects on Treg cell induction by CB-MDSC (Fig. 4c, d). Addition of catalase (ROS inhibitor) led to a strong

induction of Treg cells even without the addition of CB-MDSC. This induction was not further increased by CB-MDSC (Fig. 4c,d). Blocking of TGF- β and PD-1, which has been shown to modulate T-cell responses in soluble isoforms,²⁵ also did not influence Treg cell induction (Fig. 4e,f). In summary, the Treg cell induction by CB-MDSC was partially mediated through iNOS

Figure 2. Cord blood myeloid-derived suppressor cells (CB-MDSC) inhibit T helper type 1 (Th1) cells in a cell-contact-dependent manner. CB-MDSC were enriched from cord blood mononuclear cells (CBMC) and added to peripheral blood mononuclear cells (PBMC) or CD3⁺ T cells isolated from a healthy adult control either with direct cell/cell contact or in transwells. After 5 days of culture, surface staining was performed and cells were analysed by flow cytometry. (a) Scatter plot with bars shows the percentage of CXCR3⁺ CCR4⁻ CCR6⁻ Th1 cells without addition of CB-MDSC (white bar), with addition of CB-MDSC in direct cell/cell-contact (grey bar) or separated by transwells (checked bar). Bars represent pooled data from 11 independent experiments and each point represents an individual sample. ns = not significant; Wilcoxon matched-pairs signed rank test. (b) Scatter plot with bars shows the percentage of CXCR3⁺ CCR4⁻ CCR6⁻ Th1 cells after culture of CD3⁺ T cells without (white bar) or with CB-MDSC (grey bar). $n = 7$, $P = 0.02$, Wilcoxon matched-pairs signed rank test. (c, d) Scatter plots with bars shows inhibition of Th1 cells by CB-MDSC alone (white bar) or under addition of inhibitors for ArgI (nor-NOHA), ROS (catalase), iNOS (L-NMMA) and IDO (1-MT) in ascending grey scales. (e) Percentages of Th1 cells without addition of CB-MDSC (blank bars) and with addition of CB-MDSC (checked bars). $n = 5$ or $n = 6$; $*P < 0.05$, ns = not significant; Wilcoxon matched-pairs signed rank test. (f) Inhibition of Th1 cells was determined as ratio between percentages of Th1 cells with addition of CB-MDSC and percentages of Th1 cells without addition of CB-MDSC. $n = 5$ or $n = 6$; ns = not significant in comparison to without (w/o) inhibitors; Wilcoxon matched-pairs signed rank test. (g, h) Scatter plots with bars show the percentage of CXCR3⁺ CCR4⁻ CCR6⁻ Th1 cells without addition of CB-MDSC (white bars), with addition of CB-MDSC (grey bars) and with addition of CB-MDSC and blocking antibodies (dashed bars) against transforming growth factor- β (TGF- β) (e) and programmed death 1 (PD-1) (f). $n = 5$ or $n = 6$, ns = not significant; Wilcoxon matched-pairs signed rank test. (g-i) Bar graphs show the percentage of Annexin V⁺ cells of all CD4⁺ T cells (g) and of CXCR3⁺ CD4⁺ (h) and CCR4⁺ CD4⁺ T cells (i) without the addition of CB-MDSC (white bars) and with the addition of CB-MDSC (grey bars). $n = 6$, $*P = 0.02$, ns = not significant; Wilcoxon matched-pairs signed rank test. (j) Scatter plot with bars shows the percentage of CXCR3⁺ CCR4⁻ CCR6⁻ Th1 cells without addition of CB-MDSC (white bars), with addition of CB-MDSC (grey bars) and with addition of CB-MDSC and blocking antibody (dashed bars) against CD95L, $n = 5$.

independent of other cell types, but the complete mechanism remains to be elucidated.

Discussion

Besides their role during pathological processes like cancer, infections or autoimmunity, a physiological role of MDSC during pregnancy^{23,26-28} and the neonatal period^{21,29} has recently been described. It was hypothesized that accumulation of MDSC in both mother and fetus during pregnancy play a role in maternal-fetal tolerance. Little is known, however, about the effects of elevated MDSC numbers on neonatal immune responses. Here, we investigated the impact of CB-MDSC on the phenotype of Th cells to determine whether CB-MDSC contribute to the altered immune response and high susceptibility to infections seen in neonates. In our experiments, we focused on the functional effects of GR-MDSC, because in our previous study²¹ we only found an accumulation of GR-MDSC and not MO-MDSC in cord blood. Our findings revealed that (i) CB-MDSC suppressed Th1 cells but induced Th2 cells and Treg cells from adult PBMC, (ii) Th1 cell suppression by CB-MDSC occurred in a cell-contact-dependent manner, independent of other cell types, (iii) CB-MDSC induced Th2 cells through expression of ArgI and production of ROS in a partially monocyte-dependent way, and (iv) CB-MDSC induced Treg cells through expression of iNOS and independent of other cell types.

GR-MDSC used in these experiments sometimes expressed low levels of CD14, which was originally not described to be expressed by this cell type. Compared with monocytes and MO-MDSC, however, the expression levels of CD14 on CB-MDSC were very low and rather

comparable to those of mature granulocytes (data not shown). In our studies on MDSC during pregnancy, we repeatedly observed that GR-MDSC are not always completely CD14 negative, but sometimes primarily exhibit or secondarily gain during culture a dim CD14 expression comparable to that found in the present study (discussed in refs 22,30). We therefore assume that no static surface phenotype of GR-MDSC and probably no strict division between GR-MDSC and MO-MDSC in humans exists, but varying expression of surface markers rather reflects variable differentiation stages and plasticity of MDSC depending on environmental factors.

In our experiments, the addition of CB-MDSC to PBMC of healthy donors led to a reduced proportion of Th1 cells but an increased proportion of Th2 cells and Treg cells. These findings are in line with our previous human data²³ that maternal GR-MDSC isolated from placenta had similar effects on Th1/Th2 polarization as now shown for CB-MDSC and other groups' data on mice.³¹⁻³⁷ Given the known bias towards Th2 responses¹⁴ and the elevated levels of Treg cells in preterm infants,^{38,39} our data, together with increased numbers of MDSC during neonatal life shown previously,²¹ clearly suggest a role of MDSC in neonatal immunity.

Concerning the mechanism for Th1 suppression by CB-MDSC, we found that Th1 suppression occurred in a cell-contact-dependent manner without contribution of other cell types, but we could not identify an underlying mechanism. In previous studies, inhibition of T-cell IFN- γ production by MDSC was shown to be reversible through blockade of ArgI and/or iNOS,^{40,41} whereas we observed no effects of ArgI, iNOS, IDO or ROS blockade on CB-MDSC-mediated Th1 suppression. However, the above studies analysed IFN- γ production of total CD3⁺ T

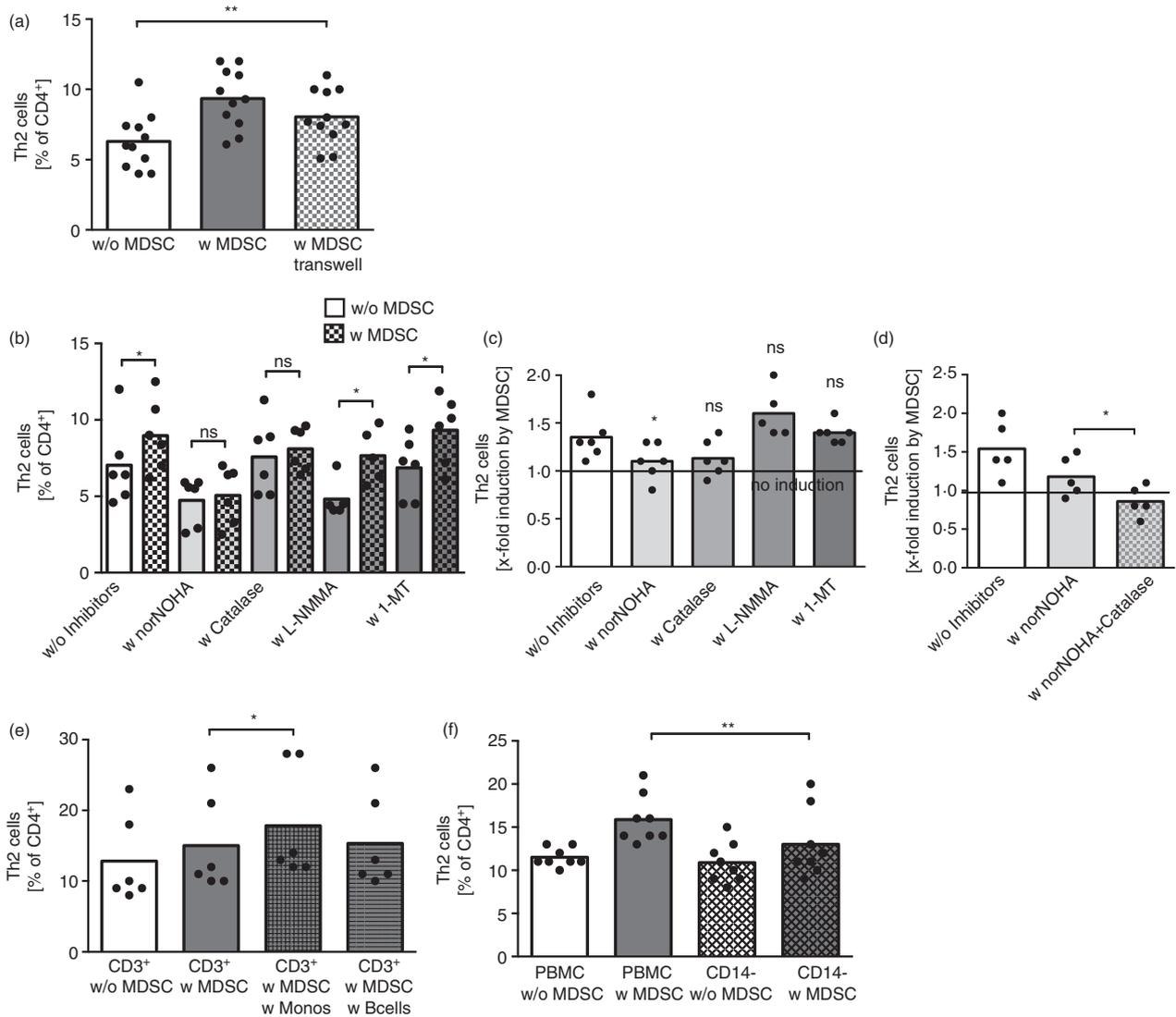


Figure 3. Cord blood myeloid-derived suppressor cells (CB-MDSC) induce T helper type 2 (Th2) cells via ArgI and reactive oxygen species (ROS) and partially dependent on monocytes. CB-MDSC were enriched from cord blood mononuclear cells (CBMC) and added to peripheral blood mononuclear cells (PBMC), CD3⁺ T cells or CD14-depleted PBMC isolated from healthy adult controls either with direct cell/cell-contact or in transwells. After 5 days of culture, surface staining was performed and cells were analysed by flow cytometry. (a) Scatter plot with bars shows the percentage of CD4⁺ CXCR3⁻ CCR4⁺ CCR6⁻ Th2 cells without addition of CB-MDSC (white bar), with addition of CB-MDSC in direct cell/cell-contact (grey bar) or separated by transwells (checked bar). Bars represent pooled data from 11 independent experiments and each point represents an individual sample. ***P* = 0.008; paired *t*-test. (b, c) Scatter plots with bars shows induction of Th2 cells by CB-MDSC alone (white bar) or under addition of inhibitors for ArgI (nor-NOHA), ROS (catalase), iNOS (L-NMMA) and IDO (1-MT) in ascending grey scales. (b) Percentages of Th2 cells without addition of CB-MDSC (blank bars) and with addition of CB-MDSC (checked bars). *n* = 5 or *n* = 6; ****P* < 0.01, **P* < 0.05, ns = not significant; Wilcoxon matched-pairs signed rank test. (c) Induction of Th2 cells was determined as ratio between percentages of Th2 cells with addition of CB-MDSC and percentages of Th2 cells without addition of CB-MDSC. *n* = 5 or *n* = 6, **P* < 0.05, ns = not significant in comparison to without (w/o) inhibitors; Wilcoxon matched-pairs signed rank test. (d) Scatter plot with bars show induction of Th2 cells by CB-MDSC alone (white bar) or under addition of nor-NOHA (light grey bar) or nor-NOHA and catalase in combination (checked bar). *n* = 5, **P* < 0.05, Wilcoxon matched-pairs signed rank test. (e) Scatter plot with bars show the percentage of CD4⁺ CXCR3⁻ CCR4⁺ CCR6⁻ Th2 cells after culture of CD3⁺ T cells without (white bar) or with CB-MDSC (grey bar) and with CB-MDSC and CD14⁺ monocytes (checked bar) or with CB-MDSC and CD19⁺ B cells (striped bar). *n* = 6, **P* = 0.02, Wilcoxon matched-pairs signed rank test. (f) Scatter plot with bars shows the percentage of CD4⁺ CXCR3⁻ CCR4⁺ CCR6⁻ Th2 cells after culture of PBMC (filled bars) or CD14-depleted PBMC (checked bars) without addition of CB-MDSC (white bars) or with addition of CB-MDSC (grey bars). *n* = 8, ***P* = 0.003; Wilcoxon matched-pairs signed rank test.

cells or CD8⁺ T cells, whereas we focused on CD4⁺ T cells, so analysing different target populations.

Although TGF- β has been reported to suppress Th1 responses⁴² and to be expressed in a membrane-bound form by MDSC,⁴³ blockade of TGF- β did not restore Th1 suppression by CB-MDSC in our setting. Recently, MDSC were found to express the co-inhibitory molecule PD-L1 under inflammatory conditions.^{44,45} Consistent with this, we measured high expression levels of PD-L1 on CB-MDSC. Although others have shown a diminished production of Th1 cytokines after activation of PD-1 by its ligand PD-L1 and reversal of this effect by addition of blocking antibodies against PD-1 to the culture,^{46,47} we could not demonstrate a role for the PD-1/PD-L1-axis in suppressing Th1 cells by CB-MDSC. In contrast to previous studies measuring Th1 suppression upon T-cell receptor stimulation with anti-CD3/CD28 antibody or specific antigens, we did not use any additional stimulus. Therefore, PD-L1 expression by CB-MDSC may have biological effects under T-cell receptor stimulation, but not in the setting used in our study.

Taken together we found CB-MDSC induced Th1 suppression to be independent from major effector enzymes ArgI, iNOS, IDO and not mediated by ROS, TGF- β or the PD-1/PD-L1 axis. Further potential mechanisms for Th1 inhibition are production of the cytokine IL-6⁴⁸ and expression of Galectin-1,⁴⁹ however, both of these molecules are secreted as soluble factors. As Th1 inhibition in our experiments was mediated in a cell-contact-dependent manner we did not further analyse these pathways.

As co-culture of CB-MDSC with PBMC led to a higher apoptosis rate in CD4⁺ T cells and especially in CXCR3⁺ CD4⁺ T cells, induction of apoptosis selectively in Th1 cells seems to be a potential mechanism of Th1 inhibition by CB-MDSC. Consistent with our results, two other studies showed apoptosis induction in T cells by MDSC.^{50,51} Apolloni *et al.*⁵⁰ found that apoptosis induction by MDSC occurred in a cell-contact-dependent manner and analysed the main pathways for apoptosis induction (TRAIL, CD95/CD95L and tumour necrosis factor- α), but could not identify an underlying mechanism. Similarly, in our experiments, blockade of CD95/CD95L-interaction also could not restore Th1 cells in PBMC/CB-MDSC co-cultures. A more detailed analysis of apoptosis induction pathways in the interaction between CB-MDSC and Th cells should be performed in further studies.

Next, we found that CB-MDSC induced Th2 cells through the expression of ArgI and production of ROS. One study described an induction of Th2 cells by MDSC after adoptive transfer in mice,³⁵ whereas others observed an association between elevated MDSC numbers and Th2 cytokine expression.^{31,32,52} However, these studies did not provide mechanisms for Th2 induction by MDSC. We found ArgI expressed by CB-MDSC to be responsible for

CB-MDSC-mediated Th2 induction. This is in line with previous results showing an increased ArgI expression and decreased arginine levels in cord blood on the one hand, and a Th2-polarized T-cell response in neonates on the other hand.^{53,54} Furthermore, ArgI-expressing macrophages (also known as M2 macrophages) are known to drive T cells to produce Th2 cytokines (reviewed in ref. 55), whereas the Th2 cytokines IL-4 and IL-13 were shown also to induce ArgI expression in myeloid cells.^{56,57}

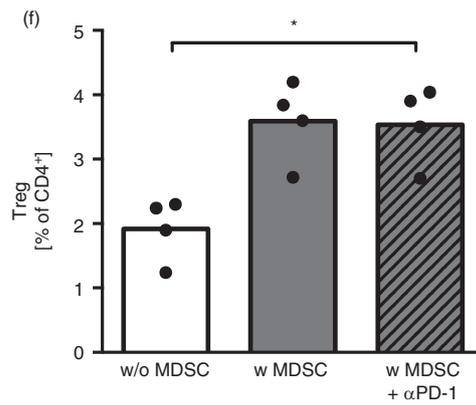
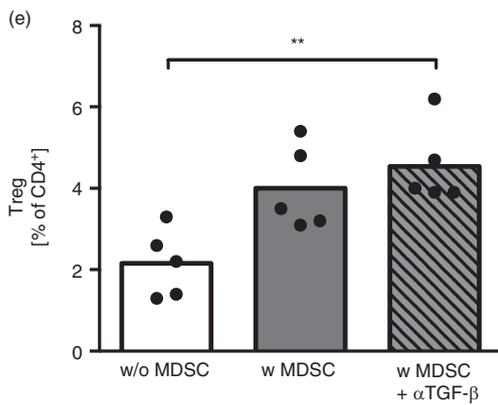
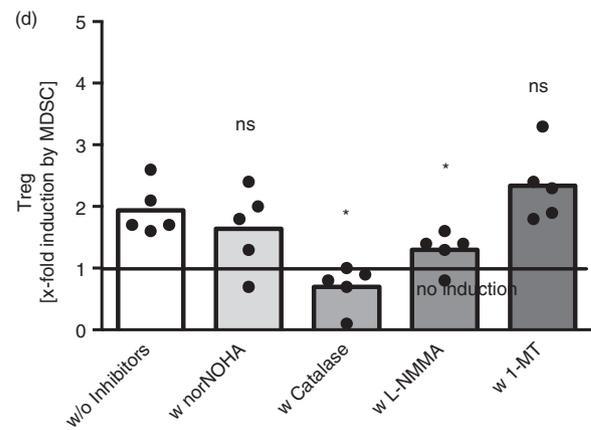
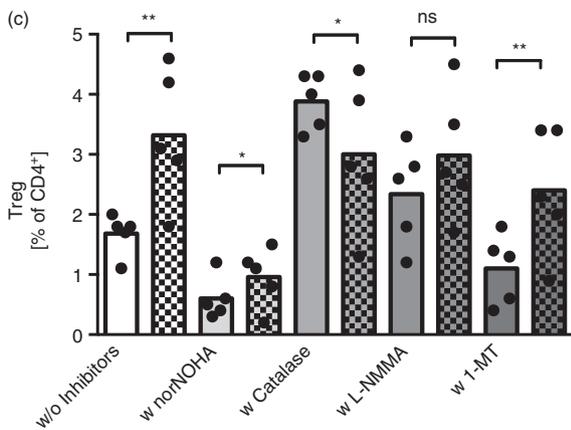
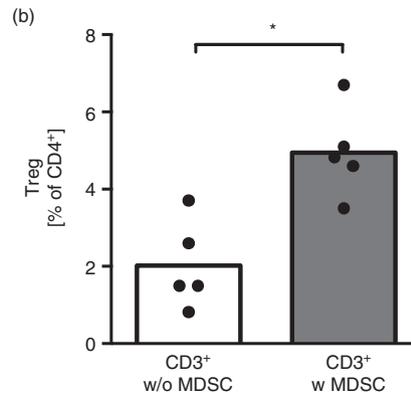
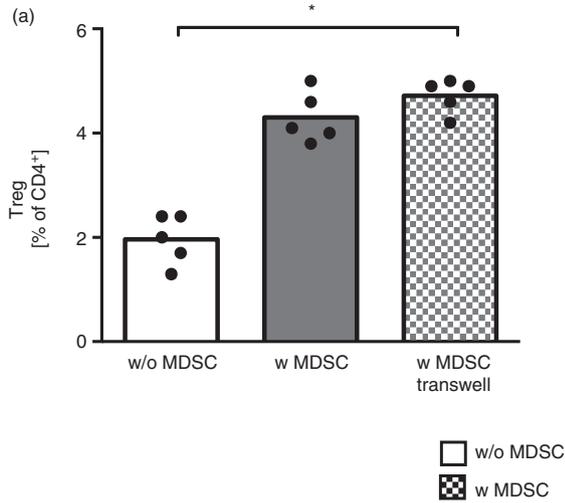
Concerning the impact of ROS, combined blockade of ArgI and ROS had synergistic effects on CB-MDSC-mediated Th2 induction in our experiments. This is in line with results from Tang *et al.*,⁵⁸ who showed that ROS produced by dendritic cells orchestrate Th cells towards Th2 responses. Consequently, also ROS production by CB-MDSC is likely to exert these effects. Taken together, elevated numbers of MDSC during fetal and early postnatal life may contribute to the Th2-biased immune response in neonates.

Besides this, we observed that Th2 induction by CB-MDSC was partially dependent on monocytes. Little is known about interactions between MDSC and monocytes/macrophages. Sinha *et al.*⁵² found tumour-derived MDSC to polarize macrophages towards an M2 phenotype by lowering production of IL-12, leading to a reduced secretion of the pro-inflammatory cytokines IL-6 and tumour necrosis factor- α ⁵⁹ and influencing their expression of co-inhibitory molecules.⁶⁰ We now add that cross-talk between MDSC and monocytes seems to establish a milieu that induces Th2 cells. Impact of CB-MDSC on monocytes will be the subject of future studies.

As induction of Th2 cells was slightly lower in our transwell experiments, and addition of the ArgI inhibitor nor-NOHA did not completely abrogate Th2 induction by CB-MDSC, it is likely that two methods of Th2 induction by CB-MDSC exist: a direct method mediated through ArgI, independent of cell-contact, and an indirect route requiring an interaction with monocytes, possibly dependent on direct cell-contact and independent of ArgI.

Lastly, we found an induction of Treg cells by CB-MDSC, which was partially mediated through expression of iNOS. Although Treg cells in the mother were frequently described as being relevant for an uncomplicated pregnancy,^{61,62} little is known about Treg cells in neonates and their role during neonatal infections. Recently, it was shown that preterm infants with sepsis had higher Treg cell numbers than non-infected infants.³⁹

Results on the interaction between MDSC and Treg cells are conflicting: MDSC were reported to impair differentiation of Treg cells,⁶³ but most studies describe an induction of Treg cells by MDSC.^{37,64–66} Considering our results, elevated MDSC levels in cord blood could be responsible for a more tolerogenic state of the neonatal immune system, both directly and by inducing Treg cells.



In our experiments, induction of Treg cells by CB-MDSC was cell-contact-independent and partially mediated through iNOS. To our knowledge, no direct relation between iNOS expression and Treg cells has yet been described. One study of patients with prostate cancer showed that elevated numbers of high iNOS-expressing MO-MDSC correlated with elevated numbers of Treg cells,⁶⁷ while another study described decreased iNOS expression in parallel with increased Treg cells.⁶⁸

Interestingly, distinct Treg cell subsets have been shown to express iNOS, so mediating their own suppressive properties.⁶⁹ In our experiments, iNOS blockade without the addition of CB-MDSC had no suppressive effect on Treg cell number. Hence the effect of iNOS-blockade on Treg cell induction in PBMC/CB-MDSC co-cultures is likely to be mediated by CB-MDSC.

In previous studies, ROS was shown to be essential for Treg cell induction.^{70,71} In our experiments, blockade of

Figure 4. Cord blood myeloid-derived suppressor cells (CB-MDSC) induce regulatory T (Treg) cells through iNOS. CB-MDSC were enriched from cord blood mononuclear cells (CBMC) and added to peripheral blood mononuclear cells (PBMC) or CD3⁺ T cells isolated from a healthy adult control either with direct cell/cell-contact or in transwells. After 5 days of culture, surface staining for CD4 and CD25 and intracellular staining for FoxP3 were performed and cells were analysed by flow cytometry (a) Scatter plot with bars shows the percentage of CD4⁺ CD25^{high} FoxP3⁺ Treg cells without addition of CB-MDSC (white bar), with addition of CB-MDSC in direct cell/cell-contact (grey bar) or separated by transwells (checked bar). Bars represent pooled data from five independent experiments and each point represents an individual sample. *****P* < 0.0001; paired *t*-test. (b) Scatter plot with bars shows the percentage of CD4⁺ CD25^{high} FoxP3⁺ Treg cells after culture of CD3⁺ T cells without (white bar) or with CB-MDSC (grey bar). *n* = 5, **P* = 0.01, paired *t*-test. (c, d) Scatter plots with bars shows induction of Treg cells by CB-MDSC alone (white bar) or under addition of inhibitors for ArgI (nor-NOHA), ROS (catalase), iNOS (L-NMMA) and IDO (1-MT) in ascending grey scales. (c) Percentages of Treg cells without addition of CB-MDSC (blank bars) and with addition of CB-MDSC (checked bars). *n* = 5; ***P* < 0.01, **P* < 0.05, ns = not significant; paired *t*-test. (c) Induction of Treg cells was determined as ratio between percentages of Th2 cells with the addition of CB-MDSC and percentages of Th2 cells without addition of CB-MDSC. *n* = 5 or *n* = 6, **P* < 0.05, ns = not significant in comparison to without (w/o) inhibitors; Wilcoxon matched-pairs signed rank test. (d, e) Scatter plots with bars show the percentage of CD4⁺ CD25^{high} FoxP3⁺ Treg cells without addition of CB-MDSC (white bar), with addition of CB-MDSC (grey bar) and with addition of CB-MDSC and blocking antibodies (dashed bars) against transforming growth factor- β (TGF- β) (d) or programmed death 1 (PD-1) (e). *n* = 4 or *n* = 5, **P* = 0.02; paired *t*-test.

ROS by catalase led to a strong Treg cell induction in PBMC cultures independent of CB-MDSC. Treg cell numbers were not further increased by CB-MDSC so it remains unclear whether ROS produced by CB-MDSC indeed mediated Treg cell induction or if the seemingly blocking effect of catalase in CB-MDSC/PBMC co-cultures might be attributable to the high baseline level of Treg cells, from which no further induction was possible.

To identify further mechanisms for Treg cell induction by CB-MDSC we blocked TGF- β and PD-1 in co-culture experiments. In contrast to others, who found a cell-contact-dependent Treg cell induction by MDSC through membrane-bound TGF- β ,³⁶ we found that Treg cell induction by CB-MDSC was cell-contact-independent and blocking TGF- β had no effect. Besides, blockade of PD-1, which was found to be critical for Treg cell differentiation *in vivo*⁷² and to be produced in soluble isoforms,²⁵ could not abrogate Treg cell induction. In summary, we found that Treg cell induction by CB-MDSC occurred in a cell-contact-independent manner and was partially mediated through iNOS. In contrast to Th2 induction, Treg cell induction by CB-MDSC was independent of other cell types. Further studies are needed to identify other soluble factors involved in CB-MDSC-mediated Treg cell induction.

One limitation of our study is that we could only analyse CB-MDSC but not GR-MDSC from postnatal blood of healthy newborns confirming that the influence of GR-MDSC on T-cell responses is indeed relevant during early postnatal life. Unpublished data from our laboratory and data from Gervassi *et al.*²⁹ revealed that GR-MDSC counts are elevated after birth and decline after the first month of life. Due to the low sample volumes normally available during this period, it seems challenging to analyse GR-MDSC from postnatal blood functionally so that we can only speculate that these cells have the same T-cell modulatory properties as CB-MDSC.

Taken together, we have shown that CB-MDSC may not only suppress immune responses in general, but

rather modulate the Th cell phenotype towards a Th2-biased and regulatory phenotype. Interestingly, Th1 suppression, Th2 and Treg cell induction by CB-MDSC were all mediated by different mechanisms, together leading to an anti-inflammatory and tolerogenic T-cell response (Fig. 5). Whereas these features seem to be indispensable for the prevention of fetal rejection,^{61,73,74} after birth the biased immune response has to be considered a relict

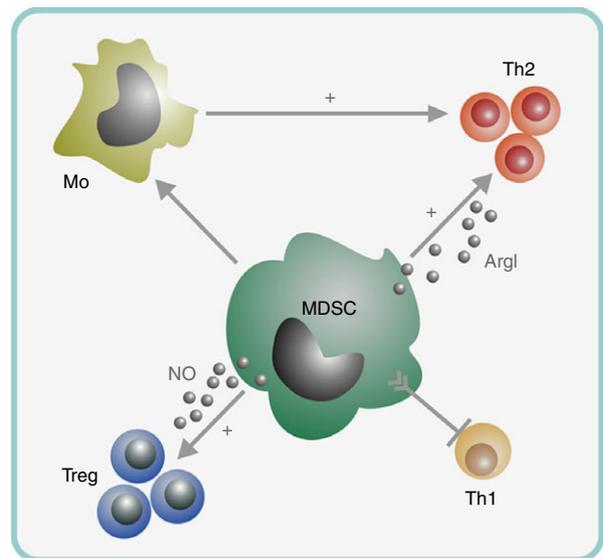


Figure 5. Cord blood myeloid-derived suppressor cells (CB-MDSC) modulate the T helper (Th) cell phenotype towards a Th2-biased and regulatory phenotype. CB-MDSC inhibited Th1 responses in a cell-contact-dependent manner, potentially by inducing apoptosis. In contrast, they induced Th2 cells through expression of arginase I and partially dependent of monocytes. Finally, CB-MDSC induced regulatory T (Treg) cells mediated through iNOS expression. Effects of CB-MDSC on Th cell subpopulations were all mediated by different mechanisms, together leading to an anti-inflammatory and tolerogenic T-cell response. Differentiated targeting of MDSC effector functions could improve neonatal host defence. [Colour figure can be viewed at wileyonlinelibrary.com]

from fetal life causing a transitory state with reduced pro-inflammatory capacity and sustained tolerance features, leading to a higher susceptibility to infections. Our results point towards a crucial role of CB-MDSC in modulating neonatal immune responses. Differentiated targeting of MDSC effector functions could be an option for selective modification of neonatal T-cell responses and improved neonatal host defence.

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Disclosures

There is no financial and commercial conflict of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gating strategy, phenotype and suppressive activity of cord blood myeloid-derived suppressor cells.

Figure S2. Cytokine production of CD4⁺ T cells after co-culture with polymorphonuclear cells.

Figure S3. Inhibition of T helper type 1 cells after co-culture of CD4⁺ T cells with cord blood myeloid-derived suppressor cells.

Figure S4. Expression of programmed death ligand 1 on cord blood myeloid-derived suppressor cells after stimulation with interferon- γ .

Figure S5. Interleukin-4 production of CD4⁺ T cells after co-culture with cord blood myeloid-derived suppressor cells under addition of ArgI inhibitor nor-NOHA.