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Research Article

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Allogeneic NK cells induce the *in vitro* activation of monocyte-derived and conventional type-2 dendritic cells and trigger an inflammatory response under cancer-associated conditions

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Abstract

Natural killer (NK) cells are innate lymphocytes capable to recognize and kill virus-infected and cancer cells. In the past years, the use of allogeneic NK cells as anti-cancer therapy gained interest due to their ability to induce graft-versus-cancer responses without causing graft-versus-host disease and multiple protocols have been developed to produce high numbers of activated NK cells. While the ability of these cells to mediate tumor kill has been extensively studied, less is known about their capacity to influence the activity of other immune cells that may contribute to a concerted anti-tumor response in the tumor microenvironment (TME). In this study, we analyzed how an allogeneic off-the-shelf cord blood stem cell-derived NK-cell product influenced the activation of dendritic cells (DC). Crosstalk between NK cells and healthy donor monocyte-derived DC (MoDC) resulted in the release of IFNγ and TNF, MoDC activation, and the release of the T-cell-recruiting chemokines CXCL9 and CXCL10. Moreover, in the presence of prostaglandin-E2, NK cell/MoDC crosstalk antagonized the detrimental effect of IL-10 on MoDC maturation leading to higher expression of multiple (co-)stimulatory markers. The NK cells also induced activation of conventional DC2 (cDC2) and $CD8^+$ T cells, and the release of TNF, GM-CSF, and CXCL9/10 in peripheral blood mononuclear cells of patients with metastatic colorectal cancer. The activated phenotype of MoDC/cDC2 and the increased release of pro-inflammatory cytokines and T-cell-recruiting chemokines resulting from NK cell/DC crosstalk should contribute to a more inflamed TME and may thus enhance the efficacy of T-cell-based therapies.

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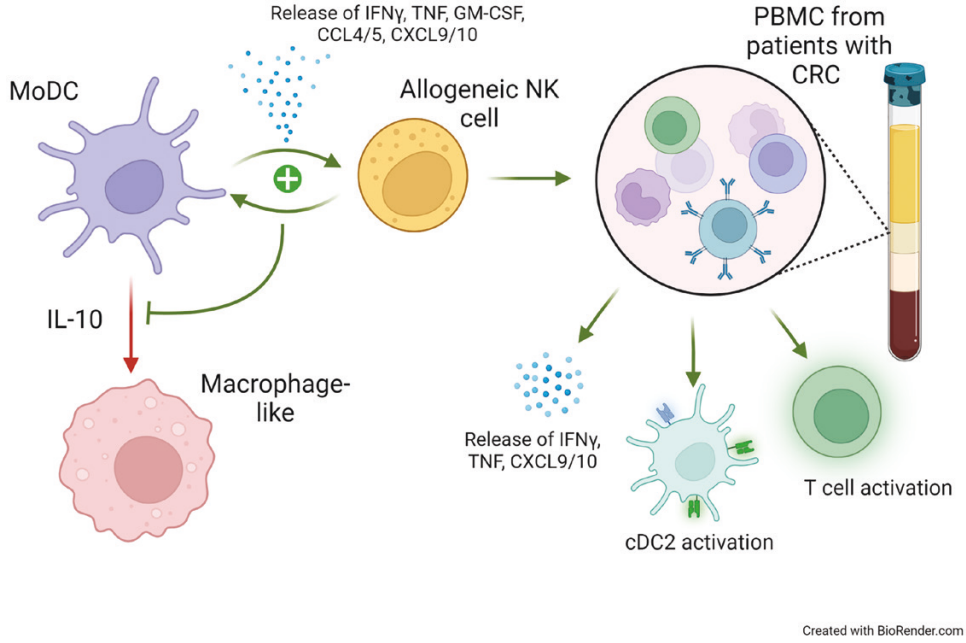
Graphical Abstract



Keywords: monocytes, natural killer cells, dendritic cells

Abbreviations: AML: acute myeloid leukemia; CBA: cytometric bead array; CCL: like C–C motif chemokine ligand; cDC1: conventional DC1; cDC2: conventional DC2; CRC: metastatic colorectal cancer; DC: dendritic cells; Flt-3L: Fms-related tyrosine kinase 3 ligand; GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin; mAbs: monoclonal antibodies; MC: maturation cocktail; MNCs: mononuclear cells; MoDC: monocyte-derived DC; NK: natural killer; PBMC: peripheral blood mononuclear cells; PD-L: programmed death-ligand; PGE2: prostaglandin-E2; SCF: stem cell factor; TAMs: tumor-associated macrophages; TME: tumor microenvironment; TNF: tumor necrosis factor; TPO: thrombopoietin; UCB: umbilical cord blood; XCL: X-C motif chemokine ligand.

Introduction

Natural Killer (NK) cells are lymphocytes with an innate ability to recognize and kill virus-infected and tumor cells without the need for prior immune sensitization [1]. Besides their cytotoxic capacity, NK cells can exert their anti-tumor function also by influencing the activity of other immune cells [1]. In particular, a functional link between NK cells and dendritic cells (DC) has been observed and the ability of these two cell subsets to crosstalk and induce reciprocal activation has been demonstrated [2, 3]. DC are professional antigen-presenting cells with the unique ability to prime and attract effector T cells [4]. In keeping with these observations, a growing number of publications in recent years have shown the dependence of effective immune checkpoint blockade on the presence of T-cell stimulatory DC in the tumor microenvironment (TME) [5–8]. Major precursors of DC in the TME are monocytes which are recruited in great numbers to developing tumors [8, 9]. Indeed, a recent publication pointed to the crucial role of tumor-associated monocyte-derived DC (MoDC) in the response to PD-1 blockade [8]. Unfortunately, rather than supporting their differentiation into T-cell stimulatory DC, suppressive conditions prevalent in the TME of solid tumors will most often convert these monocytes to M2-like tumor-associated macrophages (TAMs), which drive immune suppression, angiogenesis, tumor growth, and invasion [10]. Even fully differentiated CD1a$^+$ MoDC, when exposed during maturation to interleukin (IL)-10 (i.e. a dominant tumor-derived immune suppressive cytokine) can transdifferentiate into a CD14$^+$ M2-like state, characterized by low expression levels of co-stimulatory molecules, high levels of programmed death-ligand (PD-L)1, and the ability to induce proliferative T-cell anergy [11]. Healthy donor-derived human NK cells have been shown to induce MoDC differentiation from CD14$^+$ monocytes in a granulocyte-macrophage colony-stimulating factor (GM-CSF) and CD154-dependent manner [12]. Moreover, upon MoDC-mediated NKp30 triggering, NK cells were shown to induce MoDC maturation via the release of tumor necrosis factor (TNF) and interferon (IFN)γ [13]. In the TME, NK cells may attract DC through the release of chemokines like C-C Motif Chemokine Ligand (CCL)5 and X-C Motif Chemokine Ligand (XCL)1 and regulate their abundance by the production of FLT3L [14]. However, little is known about their ability to activate DC in the TME against the immune suppressive pressure mediated by soluble tumor-derived factors like prostaglandin-E2 (PGE2) and IL-10. In recent years, the use of allogeneic NK cells as a therapeutic anti-cancer strategy has caught the attention of the scientific community thanks to the demonstration of their ability to induce tumor lysis without inciting graft versus host reactions [15–17]. However, while multiple studies investigated the cytotoxic potency of these products, less is known of their immune-modulatory effects, particularly of their ability to crosstalk with DC. In this study, we analyzed the effects of an off-the-shelf allogeneic NK cell product, derived from expanded and differentiated umbilical cord blood (UCB) CD34$^+$ hematopoietic stem cells, on MoDC. These allogeneic NK cells have already been tested in a phase I clinical trial of patients with acute myeloid leukemia (AML) and no NK-cell infusion related toxicities or signs of graft vs host disease were observed [18]. Currently, a follow-up multicenter phase I/IIa trial in AML to further evaluate safety and efficacy is underway (NCT04632316).

Our data show that allogeneic NK cells have the ability to induce MoDC/conventional DC2 (cDC2) activation and effect a pro-inflammatory state, even in the presence of PGE2 and IL-10, which may support effector T cell activation and recruitment to the TME.

Material and methods

Hematopoietic stem cell isolation and NK cell culture

Glycostem Therapeutics developed a cryopreserved off-the-shelf allogeneic NK-cell product using a GMP-compliant culture platform. For research purposes, small scale cultures were generated. Mononuclear cells (MNCs) were isolated from fresh umbilical cord blood (UCB) units (Anthony Nolan, Nottingham, UK) with Ficoll Paque Plus (1.077 g/ml; GE Healthcare, Chicago, IL, USA) density gradient centrifugation, followed by hematopoietic $CD34^+$ stem selection using the $CD34^+$ microbead kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's protocol. Isolated $CD34^+$ cells were seeded in 6-well tissue culture treated plates (Corning Incorporated, NY, USA) at a concentration of 10 000 cells/ml. The expansion medium consisted of Glycostem Basal Growth Medium (GBGM®; Fertipro, provided by Glycostem Therapeutics) supplemented with 10% Human Serum (HS; Sanquin, Amsterdam, the Netherlands) and a mixture of thrombopoietin (TPO), IL-7, Fms-related tyrosine kinase 3 ligand (Flt-3L), stem cell factor (SCF), GM-CSF, IL-6 (all from Cellgenix, Sartorius, Freiburg, Germany), and Neupogen (G-CSF; Amgen BV, Breda, the Netherlands). After 9 days of expansion, TPO was replaced by IL-15 (Cellgenix, Sartorius, Freiburg, Germany). After 14 days, the expansion medium was replaced by differentiation medium consisting of GBGM medium supplemented with 2% human serum, and a cytokine mixture of IL-7, IL-15, SCF, GM-CSF, IL-6, Neupogen, and Proleukin (IL-2; Novartis, Basel, Switzerland). Cells were cultured for a total of 28 days and cryopreserved until further use. Upon thawing, the cells were recovered in differentiation medium for 5–7 days before use.

MoDC generation

MoDC were generated as previously described [19]. In short, blood samples were obtained from healthy volunteers under written informed consent (Sanquin). Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep™ (STEMCELL Technologies, Vancouver, BC, Canada) for density gradient centrifugation. Next, monocytes were isolated by magnetic bead-activated cell sorting using CD14 MicroBeads (Miltenyi Biotec) according to manufacturer's instructions. Purity was assessed via flow cytometry based on Side and Forward Scatter. The mean purity was 88.7% (SD: 5.9). To obtain MoDC (immature MoDC), isolated monocytes were cultured for 6 days in Roswell Park Memorial Institute medium (RPMI, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal calf serum (FCS, Sartorius, Freiburg, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.3 mg/ml glutamine (PSG, Gibco, Thermo Fisher), 0.05 molM/L 2-mercaptoethanol (2-ME, Merck, Kenilworth, NJ, USA), 100 ng/ml GM-CSF (Immunotools, Friesoythe, Germany) and 20 ng/ml IL-4 (Strathmann Biotech, Hamburg, Germany).

NK cell/MoDC co-cultures

To assess if NK cells could induce maturation of MoDC, MoDC were cultured with NK cells at multiple NK cell to MoDC ratios (i.e. 1:10, 1:1, and 10:1) for 72 h in RPMI supplemented with 10% FCS, PSG, and 2-ME. To test the effects of NK cells on MoDC maturation, MoDC and NK cells were co-cultured for 48 h in the presence of a maturation cocktail (MC) consisting of 1 µg/ml PGE2, 2400 U/ml TNF, 100 ng/ml IL-6, and 25ng/ml IL-1β. Transwell assays were performed using 24-transwell plates with 0.4 µm pore filter size (Corning, Merck, Kenilworth, NJ, USA) by culturing MoDC in the presence of the MC with or without NK cells, which were plated either in the transwell insert or, together with the MoDC, in the lower compartment. To analyze the effects of NK cells on MoDC maturation in the presence of IL-10, MoDC were cultured for 48 h with the MC in the presence and absence of NK cells and/or 10 ng/ml IL-10 (eBioscience, Thermo Fisher Scientific).

MoDC survival assay

To explore the potential cytotoxic activity of NK cells against MoDC, survival assays were performed by co-culturing NK cells and MoDC for 48 h in a NK:MoDC ratio of 10:1 in the presence of the MC. The flowcytometric readout was based on absolute numbers of MoDC ($CD56^-$) quantified with QUANTI BEADS (Invitrogen, Thermo Fisher Scientific).

Mixed leukocyte reaction

To evaluate the ability of MoDC to induce allogeneic T-cell proliferation, a mixed leukocyte reaction (MLR) was performed. First, MoDC were cultured with the MC in the presence and absence of NK cells and/or 10 ng/ml IL-10. After 2 days, MoDC were harvested and washed to remove the excess of cytokines. Of note, MoDC were not separated from NK cells before subsequent use in the MLR. The absolute number of MoDC was assessed with flow cytometry using QUANTI BEADS (Invitrogen, Thermo Fisher Scientific). The absolute number of lymphocytes in PBMC was similarly assessed. To perform the MLR, the pre-incubated MoDC were co-cultured for 7 days with PBMC at a lymphocyte to MoDC ratio of 10:1. To avoid an NK-related allogeneic reaction, the PBMC used for the MLR were derived from the same UCB donor as the NK cell product, which also ensured the naïve state of the used lymphocytes.

The MLR readout was based on the percentage of 5(6)-Carboxyfluorescein $(CFSE)^{dim}$ (Sigma-Aldrich, Saint Louis, MO, USA) $CD8^+$ or $CD4^+$ T cells as measured by flow cytometry. The CFSE staining was performed by pre-incubating the UCB PBMC with 3 µM CFSE at 37°C. After 7 minutes, the cells were washed to remove excess CFSE and used for the co-culture. As positive control, the PBMC were cultured in the presence of only phytohemagglutinin (PHA, Thermo Fisher Scientific) a well-known selective T-cell mitogen. An example of the followed gating strategy is shown in Supplementary Figure S1.

PBMC derived from patients with colorectal cancer and NK cell co-culture

Blood samples were obtained from patients with metastatic colorectal cancer (CRC) under written informed consent at the Amsterdam UMC (location VU University Medical Center, Amsterdam). Peripheral blood mononuclear cells

(PBMC) were isolated using Lymphoprep™ (STEMCELL Technologies) on a density gradient centrifugation. The PBMC were then counted and cryopreserved. At the time of use, the PBMC were rapidly thawed and diluted in RPMI supplemented with 10% FCS, PSG, and 2-ME. NK cells and PBMC were then plated in a 48-well plate at a ratio of 1:1. NK cells and PBMC only controls were taken along. To ensure the survival of NK cells and lymphocytes for 6 days, 100 U/ml of IL-2 (Novartis, Basel, Switzerland) was added to the culture on day 2. The cells were cultured for 2 and 6 days. After 2 days, the readout of the frequency and activation state of myeloid subsets was performed and after 6 days the readout of the frequency and activation state of lymphocyte subsets, both by polychromatic flow cytometry.

Flow cytometry

Immunophenotypic analyses were performed using flow cytometry (LSRFortessa™, BD, Franklin Lakes, NJ, USA). A list of all the fluorochrome-conjugated monoclonal antibodies (mAbs) used for cell staining can be found in Supplementary Table S1. Cell surface staining was performed by incubating the cell suspensions with mAbs at 4°C in phosphate-buffered saline (PBS, Fresenius Kabi, Bad Homburg vor der Höh, Germany) supplemented with 0.1% bovine serum albumin (BSA, Thermo Fisher Scientific) and 0.02 % NaN3 (Merck, Kenilworth, NJ, USA) (FACS buffer). After 20–30 minutes, the cell suspensions were washed with FACS buffer to remove the excess of mAbs. Data were analyzed using FlowJo version 10.8.1 (BD biosciences, Franklin Lakes, NJ, USA) or Kaluza version 2.1 (Beckman Coulter, Brea, CA, USA).

Cytometric bead array

A cytometric bead array (CBA) was performed on the collected supernatants according to manufacturer's protocol. A customized LEGENDplex™ (Biolegend, San Diego, CA, USA) kit was used containing the following analytes: CXCL9, CXCL10, CCL4, CCL5, TNF, GM-CSF, IFNγ, IL-15, and IL-18.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.3.1 (San Diego, CA, USA). Normality was assessed using the Shapiro-Wilk test and subsequent analyses were selected accordingly: i.e. differences between two variables were examined either by two-tailed paired *T*-test or Wilcoxon test. Two-way ANOVA with Tukey multiple comparison analysis or Friedman test with Dunn multiple comparison analysis were used to assess differences between multiple variables. Finally, a correlation analysis was performed using R version 4.0.3 (Boston, MA, USA). The correlation coefficients were calculated using the Spearman rank-order correlation analysis. Significance is presented as $P < 0.05$*, <0.01**, 0.001***, 0.0001****.

Results

NK cells maintain CD14 downregulation and induce maturation of CD1a$^+$CD14$^-$ and CD1a$^+$CD14$^+$ MoDC

To determine whether NK cells could induce maturation of immature DC, we analyzed the effects of NK cells on immature MoDC, a well-known *in vitro* DC model. MoDC were produced by culturing freshly isolated monocytes with IL-4 and GM-CSF as previously described [19]. To separate MoDC and NK cells in flowcytometric analyses, MoDC were gated as CD56$^-$. During differentiation, monocytes downregulated the monocyte marker CD14 and acquired the DC marker CD1a leading to an average of 6.3% (SD: 5.4) CD1a$^-$CD14$^+$ cells and 58.6% (SD: 8.6) CD1a$^+$CD14$^-$ cells after 6 days (Figure 1A, 1B). MoDC were then cultured for 3 days in either medium only or in the presence of NK cells at 3 different NK cell-to-MoDC ratios (1:10, 1:1, and 10:1). Interestingly, whereas in the presence of medium only, MoDC reacquired CD14 and lost CD1a expression, in the presence of higher relative numbers of NK cells (ratio 1:1 and 10:1), CD1a expression was maintained and CD14 levels remained low (Figure 1C, 1D), showing the ability of NK cells to stabilize the DC phenotype. Of note, while no differences in CD14 expression were found between the 1:1 and 10:1 ratio, in the latter CD1a levels were significantly lower.

Three different subpopulations of MoDC were defined based on the expression of CD14 and CD1a: CD1a$^+$ CD14$^-$, CD1a$^-$CD14$^+$, and CD1a$^+$CD14$^+$, the latter of which likely represent an intermediate state between CD1a$^+$CD14$^-$ DC and CD1a$^-$CD14$^+$ monocyte/macrophage-like cells. The expression of multiple DC maturation/T-cell (co-)stimulatory markers (i.e. CD86, CD83, CD80, CD70, HLA-ABC) and PD-L1 was analyzed for each population (Figure 1E). Higher levels of all the markers were observed on CD1a$^+$CD14$^-$ cells after co-culture with a high ratio of NK cells:MoDC (ratio 10:1). Similarly, CD1a$^+$CD14$^+$ cells expressed increased levels of all the analyzed markers after culture with higher NK cell-to-MoDC ratios (ratio 1:1 and 10:1). In contrast, limited NK cell-mediated effects on the activation of CD1a$^-$CD14$^+$ cells were observed with increased expression of only CD86, HLA-ABC, and PD-L1 upon co-culture with high ratios of NK cells. In conclusion, at relatively high NK cell-to-MoDC ratios, MoDC maintained low CD14 surface levels and NK cells induced maturation and activation of CD1a$^+$ MoDC. Although higher levels of CD1a expression were observed at the 1:1 ratio, the 10:1 ratio was chosen for follow-up experiments as it showed the best effects on phenotypic MoDC maturation.

Besides phenotypic changes upon NK cell/MoDC crosstalk, also the release of several pro-inflammatory cytokines and chemokines was induced in the co-cultures (Figure 2). The observed induction of IFNγ and TNF should lead to activation of both DC and type-1 T cell responses [20] while the production of IL-18 by both MoDC and NK cells should drive the proliferation and differentiation of effector T and NK cells [1, 21]. Moreover, the induced release of CXCL9/10 could attract effector T cells [1, 21, 22]. Of note, no increases in CCL4/5 were observed. Thus, NK cell/MoDC crosstalk induced both DC activation and a pro-inflammatory cytokine/chemokine profile that under *in vivo* tumor conditions could potentially result in a more T-cell response permissive TME.

NK cells enhance cytokine/PGE2-mediated MoDC maturation

Next, we analyzed if NK cells could further enhance MoDC maturation in the presence of maturation stimuli. For this purpose, we cultured MoDC with a cytokine/PGE2 MC, which included IL-1β, IL-6, PGE2, and TNF, in the presence and absence of NK cells and we analyzed CD1a/CD14 levels as well as the expression of multiple DC maturation markers (i.e. CD80, CD83, CD86, and HLA-ABC) and PD-L1. Of

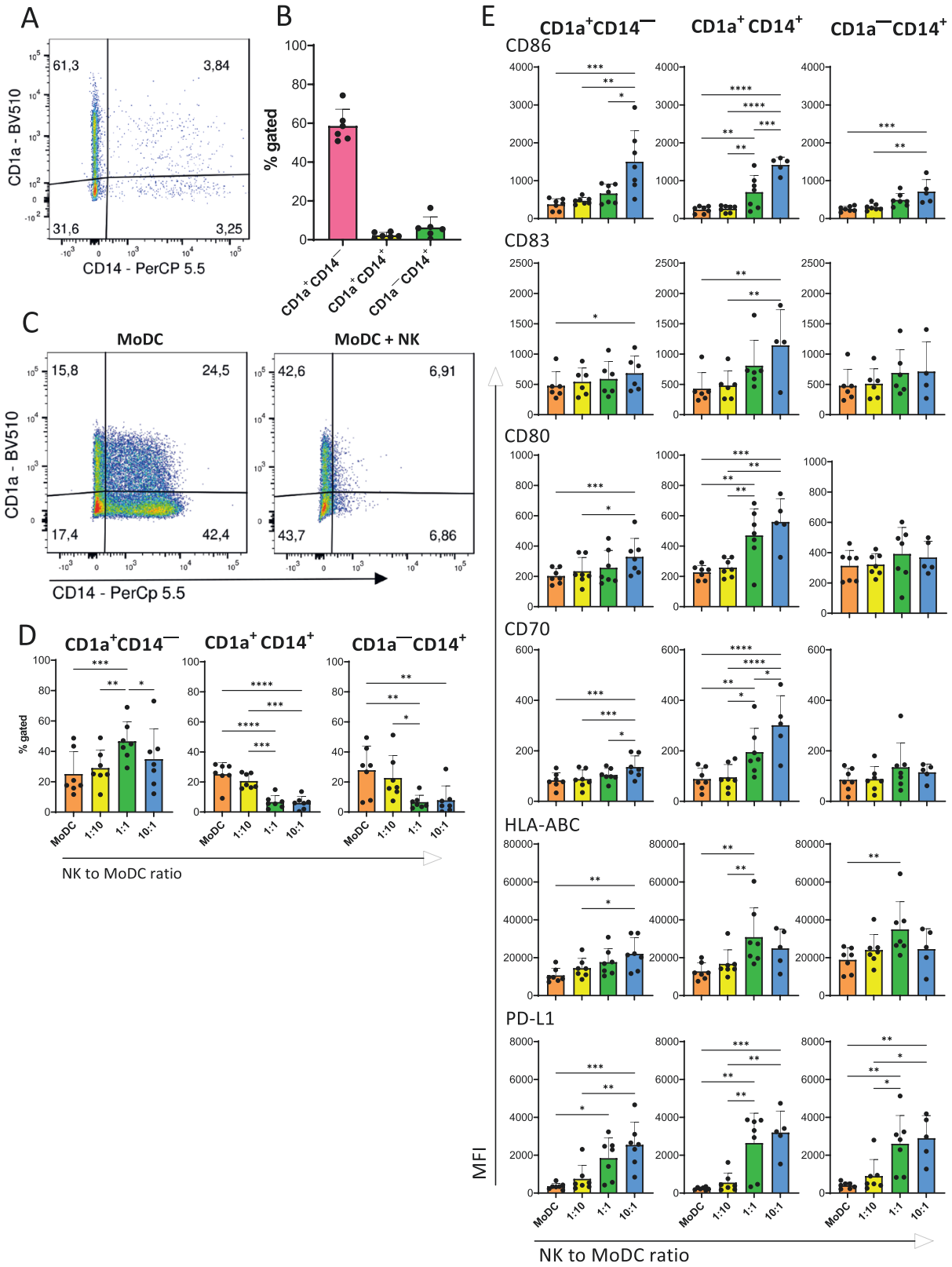


Figure 1. NK cells maintain CD1a expression and induce maturation of MoDC. Expression of CD1a and CD14 on MoDC after 5–7 days of culture in the presence of 20 ng/ml IL-4 and 100 ng/ml GM-CSF. (A) Representative example. (B) Bar graphs showing percentages of gated cells. $N = 6$. Expression of CD1a and CD14 on MoDC after 3 days of culture, either alone or in the presence of NK cells. (C) Representative example. NK cell-to-MoDC ratio 1:1. (D) Bar graphs showing percentages of gated cells. $N = 7$ (5 NK and 6 MoDC donors). Results of multiple NK cell-to-MoDC ratios are shown. (E) Expression of CD86, CD83, CD80, CD70, PD-L1, and HLA-ABC on MoDC after 3 days of culture either alone or in the presence of NK cells. Results of multiple NK cell-to-MoDC ratios are shown. $N = 7$ (except CD83: $N = 6$) (5 NK and 6 MoDC donors). Data are presented as mean ± SD (B, D, E). Significance is presented as $P < 0.05$*, <0.01**, 0.001***, 0.0001****. *P*-values were determined by one-way ANOVA with Tukey multiple comparison analysis (D, E). Abbreviations: NK, NK cells; MoDC, monocyte-derived dendritic cells; MFI, median fluorescence intensity.

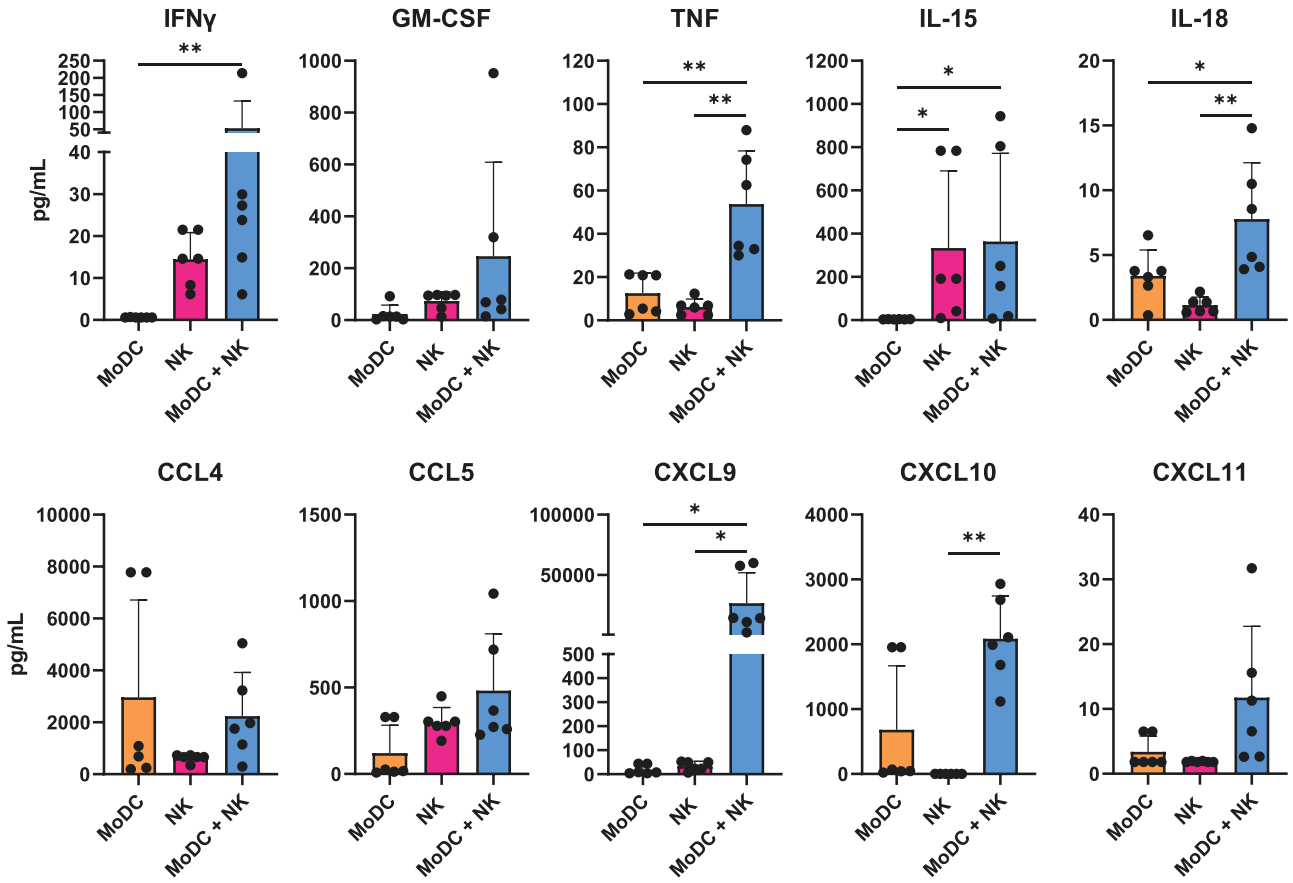


Figure 2. The culture of NK cells and MoDC results in higher release of proinflammatory cytokines and T-cell-recruiting chemokines. Cytometry bead array was performed on the supernatant of MoDC and/or NK cells after 3 days of culture. Optimal NK cell:MoDC ratios were selected per cytokine/chemokine (NK cell-to-MoDC ratio 10:1: IFNγ, GM-CSF, and IL-15; NK cell- to-MoDC ratio 1:1: TNF, IL-18, CCL4, CCL5, CXCL9, CXCL10, CXCL11). *N* = 6 (4 NK and 5 MoDC donors). Data are presented as mean ± SD. Significance is presented as $P < 0.05$*, <0.01**. *P*-values were determined by Friedman test with Dunn's multiple comparison analysis (IFNγ, GM-CSF, IL-15, CCL4, CCL5, CXCL10) or one-way ANOVA with Tukey multiple comparison analysis (TNF, IL-18, CXCL9, CXCL11). Abbreviations: NK, NK cells; MoDC, monocyte-derived dendritic cells.

note, this MC comprises a combination of factors that is often present in the TME of solid tumors, each of which has been implicated in either the disturbed differentiation of DC or the activation of myeloid regulatory cells [23–25]. After 2 days of culture, lower levels of $CD1a^{-}CD14^{+}$ and $CD1a^{+}CD14^{+}$ cells were observed when NK cells were added to the culture whereas the percentages of $CD1a^{+}CD14^{-}$ increased to near significance levels ($p = 0.054$) (Figure 3A). Moreover, the presence of NK cells induced higher expression levels of CD80, CD83, CD86, and HLA-ABC as well as of PD-L1 on all the MoDC subpopulations (Figure 3B, 3C). As NK cells were previously shown to increase the levels of DC maturation by the preferential killing of immature DC [2], we analyzed if these effects on MoDC maturation were accompanied by a reduction in the absolute numbers of MoDC. No differences in the absolute numbers of MoDC were observed in the presence of NK cells, indicating that, in this setting, the additional effect of the NK cells on MoDC maturation was not caused by the lysis of any residual immature MoDC subpopulations (Figure 3D). NK cells co-cultured with MC and MoDC led to enhanced levels of IFNγ and GM-CSF release (Figure 3E), which in the case of IFNγ was dependent on the presence of MC-matured MoDC and in the case of GM-CSF was related to a direct effect of MC on NK cells, which was demonstrated by the reduction of IFNγ release in a transwell set-up, whereas GM-CSF release was unaffected (Figure 3F). Moreover, higher levels of CXCL9 and CXCL10 were observed in the presence of both NK cells and MoDC likely induced by the increase in IFNγ. Indeed, a high correlation was observed between the CXCL9 and IFNγ ($r = 0.88$, $p < 0.0001$) as well as CXCL10 and IFNγ ($r = 0.72$, $p < 0.0001$) and the release of CXCL9 and CXCL10 was, similarly to IFNγ, contact dependent.

We also ascertained the effects of MC-matured MoDC and NK cell co-culture on the phenotype of NK cells (Figure S2). When cultured alone, NK cells showed an active profile with high expression of the activating receptors NKG2D, DNAM-1, NKp44, NKp46, NKp30, moderate expression of the activating receptor CD25, and low expression of the activating receptors NKG2C and CD16, with robust expression of the inhibitory receptor NKG2A and low expression of the inhibitory KIR2D receptor. These allogeneic umbilical cord-derived NK cells were previously described to harbor high levels of CD56 and low levels of CD16, despite having a high activation status and clearly exerting cytotoxic activity [18]. Co-culture with MC-matured MoDC left this phenotype mostly unaffected, apart from a slight, but significant downregulation of the NKp46 activating receptor. Interestingly, while the MC induced downregulation of CD25, matured MoDC were observed to counteract this effect.

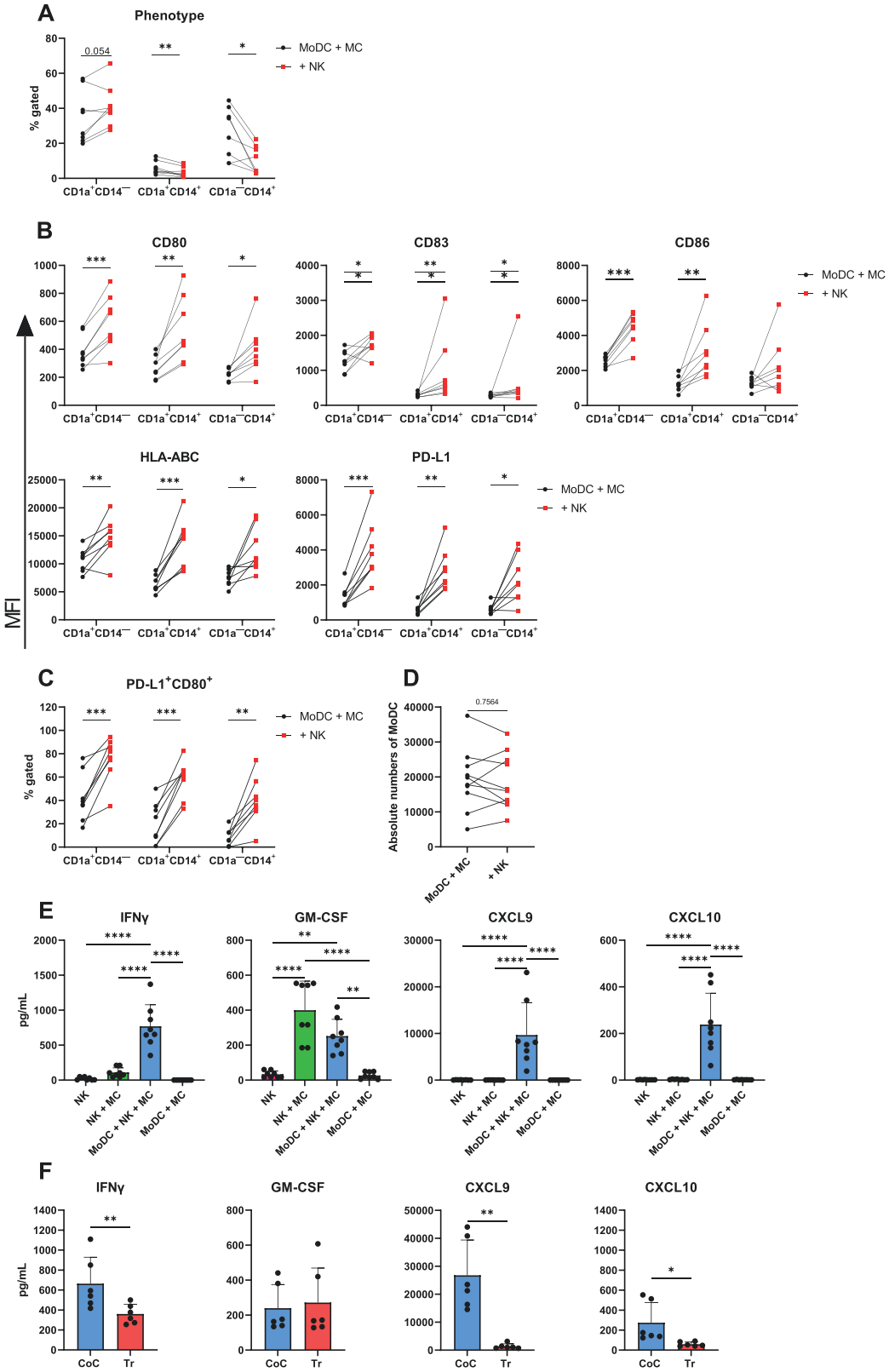


Figure 3. NK cells enhance cytokine-mediated MoDC maturation. Forty-eight hours culture of MoDC ± NK cells in the presence of a maturation cocktail (MC, consisting of 1 μg/ml PGE2, 2400 U/ml TNF, 100 ng/ml IL-6, and 25 ng/ml IL-1β). NK cell-to-MoDC ratio 10:1. (A) Percentages of $CD1a^{-}CD14^{+}$, $CD1a^{+}CD14^{-}$ and $CD14^{+}CD1a^{+}$. $N = 8$ (4 NK and 8 MoDC donors). (B) Expression of CD80, CD83, CD86, HLA-ABC, and PD-L1 on $CD1a^{+}CD14^{-}$, $CD14^{+}CD1a^{+}$, and $CD14^{+}CD1a^{-}$ MoDC. $N = 8$ (4 NK and 8 MoDC donors). (C) Percentages of $CD80^{+}PD\text{-}L1^{+}$ on $CD1a^{+}CD14^{-}$, $CD14^{+}CD1a^{+}$, and $CD14^{+}CD1a^{-}$ MoDC. $N = 8$ (4 NK and 8 MoDC donors). (D) Absolute numbers of MoDC. $N = 10$ (5 NK and 10 MoDC donors). (E) Cytometry Bead Array performed on the supernatant of 48-h culture of NK cells ± MoDC ± MC. $N = 8$ (4 NK and 8 MoDC donors). (F) Cytometric Bead Array performed on the supernatant of 48-h culture of MoDC ± NK cells in the presence of MC. NK cells were placed either in direct contact with the MoDC (co-culture) or separated by a transwell membrane (transwell). $N = 6$ (2 NK and 6 MoDC donors). The data are presented as mean ± SD (E, F). Significance is presented as $p < 0.05$*, <0.01**, <0.001***, <0.0001****. *P*-values were determined by two-tailed paired T test (A-D, F), Wilcoxon test (B: CD83) or one-way ANOVA with Tukey multiple comparison analysis (E). Abbreviations: NK, NK cells; MoDC, monocyte-derived dendritic cells; MFI, median fluorescence intensity; CoC, co-culture; Tr, transwell; MC, maturation cocktail.

Enhancement of cytokine-mediated MoDC maturation by NK cells is contact-dependent.

Previous reports have shown NK/DC crosstalk to be contact-dependent, with CD154/CD40L and NKp30 on the NK cell binding to counter-receptors on the DC surface, resulting in the release of IFNγ, TNF, and GM-CSF by the NK cells and subsequent DC activation [12, 13]. Indeed, transwell cultures with separated NK cells and MoDC fractions showed a clear contact dependence of the observed crosstalk effects on CD1a$^+$ DC activation (Figure 4) and, as previously mentioned, IFNγ release (Figure 3F). Although both CD154 and NKp30 were expressed on NK cells, the addition of antibodies blocking either CD154 or NKp30 did not affect the observed crosstalk, indicating the involvement of other, as yet unidentified, surface molecules (Figure S3).

NK cells partially counteract the detrimental effect of IL-10 on MoDC maturation

We previously demonstrated a detrimental effect of IL-10 on MoDC maturation. Compared to MoDC matured under standard conditions, the addition of IL-10 induced *de novo* trans-differentiation to CD14$^+$ cells with a more macrophage-like appearance that expressed high levels of PD-L1 and low levels of the T-cell co-stimulatory molecules CD80, CD83, and CD86 [11]. Moreover, these IL-10 conditioned MoDC were shown to have low T cell priming ability and to induce T-cell anergy [11]. As we demonstrated that NK cells enhanced cytokine-mediated MoDC maturation, we also assessed whether it could counteract the detrimental effects of IL-10 on MoDC maturation. To do so, MoDC were cultured with MC in the presence and absence of IL-10 and/or NK cells. IL-10 indeed induced downregulation of CD1a and the upregulation of CD14, as previously described, which could only modestly be counteracted by the presence of NK cells (Figure 5A, 5B). NK cells had a more profound effect on the maturation state of the IL-10 exposed MoDC, elevating expression levels of CD80, CD83, CD86, PD-L1, and HLA-ABC on all the analyzed MoDC subpopulations to levels comparable to MoDC matured under standard conditions, thus largely abolishing the suppressive effects of IL-10 (Figure 5C).

However, the MoDC activation by NK cells in the presence of IL-10 did not lead to preserved T cell priming capacity of the matured MoDC in an allogeneic mixed lymphocyte reaction (Figure S4A). This might well be related to the fact that NK cells largely failed to restore the IL-10-induced CD1a/CD14 unbalance despite their activating effect. As shown by the correlation plot in Figure S4B, proliferation induction of the allogeneic T cells (i.e. CFSE^{dim}) was indeed (and perhaps surprisingly so) related more to high CD1a and low CD14 expression levels than to expression levels of the activation markers CD80, CD83, and CD86, which actually were upregulated by NK cells. Another explanation may lie in the relatively stronger upregulation of the immune checkpoint PD-L1 as compared to the co-stimulatory molecules CD80 or CD86, tipping the balance in favor of T cell inhibition rather than activation (Figure 5C). Indeed, the expression of PD-L1 negatively correlated with the level of proliferation.

NK cells activate peripheral blood cDC2 and induce CD8$^+$ T cell activation and effector-memory differentiation

To study the ability of NK cells to activate conventional DC1 (cDC1) and cDC2, we co-cultured NK

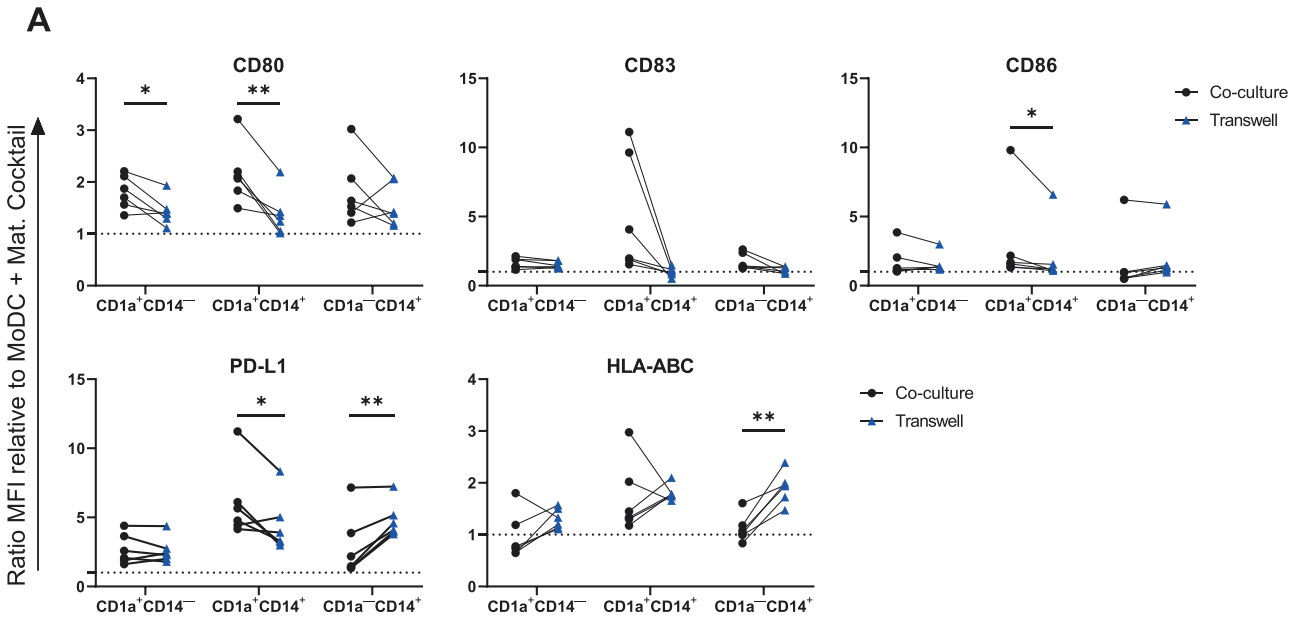


Figure 4. The effects of NK cells on MoDC are partially contact-dependent. Forty-eight hours culture of MoDC ± NK cells in the presence of a maturation cocktail (MC, consisting of 1 ug/ml PGE2, 2400 U/ml TNF, 100 ng/ml IL-6, and 25 ng/ml IL-1β). NK cells were placed either in direct contact with the MoDC (co-culture, CoC) or separated by a transwell membrane (transwell, Tr). NK cell-to-MoDC ratio 10:1. $N = 6$ (2 NK and 6 MoDC donors). Changes relative to MoDC + MC in the expression of CD80, CD83, CD86, HLA-ABC, and PD-L1 on CD1a$^+$CD14$^-$, CD14$^+$CD1a$^+$ and CD14$^+$CD1a$^-$ MoDC. Significance is presented as $P < 0.05$*, <0.01**. *P*-values were determined by two-tailed paired *T*-test except for CD86 where the Wilcoxon test was used. Abbreviations: NK, NK cells; MoDC, monocyte-derived dendritic cells; Tr, transwell; CoC, co-culture; MC, maturation cocktail.

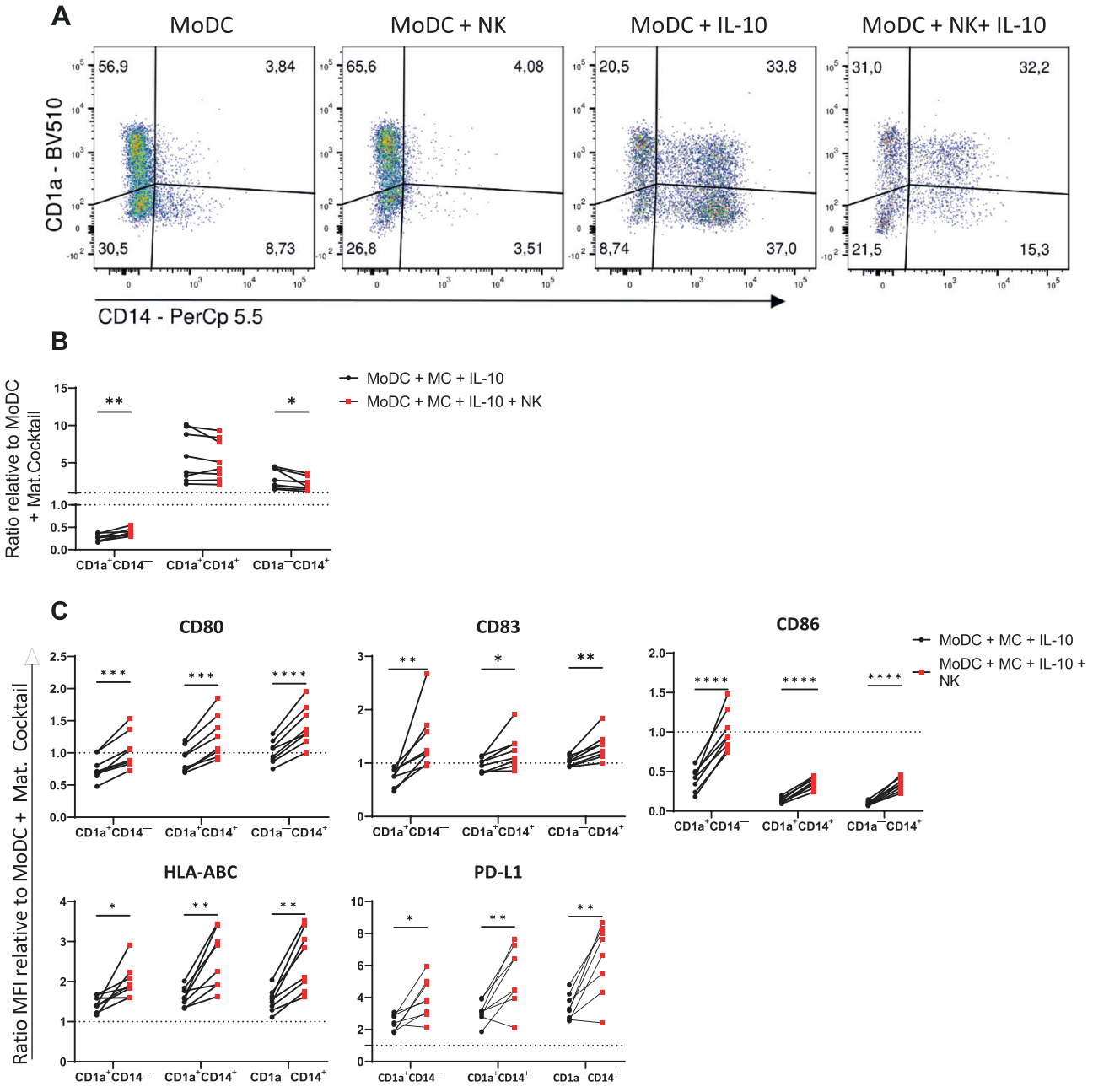


Figure 5. NK cells partially counteract the detrimental effect of IL-10 on MoDC maturation. Forty-eight hours culture of MoDC ± NK cells ± IL-10 in the presence of a maturation cocktail (MC, consisting of 1 μg/ml PGE2, 2400 U/ml TNF, 100 ng/ml IL-6, and 25 ng/ml IL-1β). NK cell-to-MoDC ratio 10:1. $N = 8$ (4 NK and 8 MoDC donors). (A) Representative example of the expression of CD14 and CD1a on MoDC. (B) Changes relative to MoDC + MC in the percentages of $CD1a^+CD14^-$, $CD14^+CD1a^+$, and $CD14^+CD1a^-$ MoDC. (C) Changes relative to MoDC + MC in the expression of CD80, CD83, CD86, HLA-ABC, and PD-L1 on $CD1a^+CD14^-$, $CD14^+CD1a^+$ and $CD14^+CD1a^-$ MoDC. Dotted lines represent MoDC + MC condition. Significance is presented as $P < 0.05$*, <0.01**, 0.001***, 0.0001****. *P*-values were determined by two-tailed paired *T*-test. Abbreviations: NK, NK cells; MoDC, monocyte-derived dendritic cells; MC, maturation cocktail.

cells with PBMC from patients with metastatic colorectal cancer (mCRC). cDC1 and cDC2 were gated as $CD11c^{hi}$HLA-$DR^+BDCA3^+CD14^-CD1c^-$ and $CD11c^{hi}$HLA-$DR^+CD1c^+CD14^-$ respectively. An example of the used gating strategy can be found in Figure S5. Unfortunately, the low numbers of cDC and the paucity of available PBMC numbers precluded the isolation of the cDC subsets. Upon 2-day co-culture of PBMC and NK cells, we observed clear activation of cDC2 [up-regulated expression of BDCA3, TIM3 and, although not statistically significant due variability in intensity levels, CD80 (p-value: 0.057) and PD-L1 (0.10)], whereas on cDC1, no significant changes in the expression of CD80, CD83, PD-L1 and TIM3 were observed (Figure 6A). NK cells also induced $CD8^+$ T cell activation, revealed by CD69 upregulation, and effected a shift away from a naïve state to an effector-memory state [26] (Figure 6B). The used T cell gating strategy is shown in Figure S6. The analysis of cytokines and chemokines revealed that NK

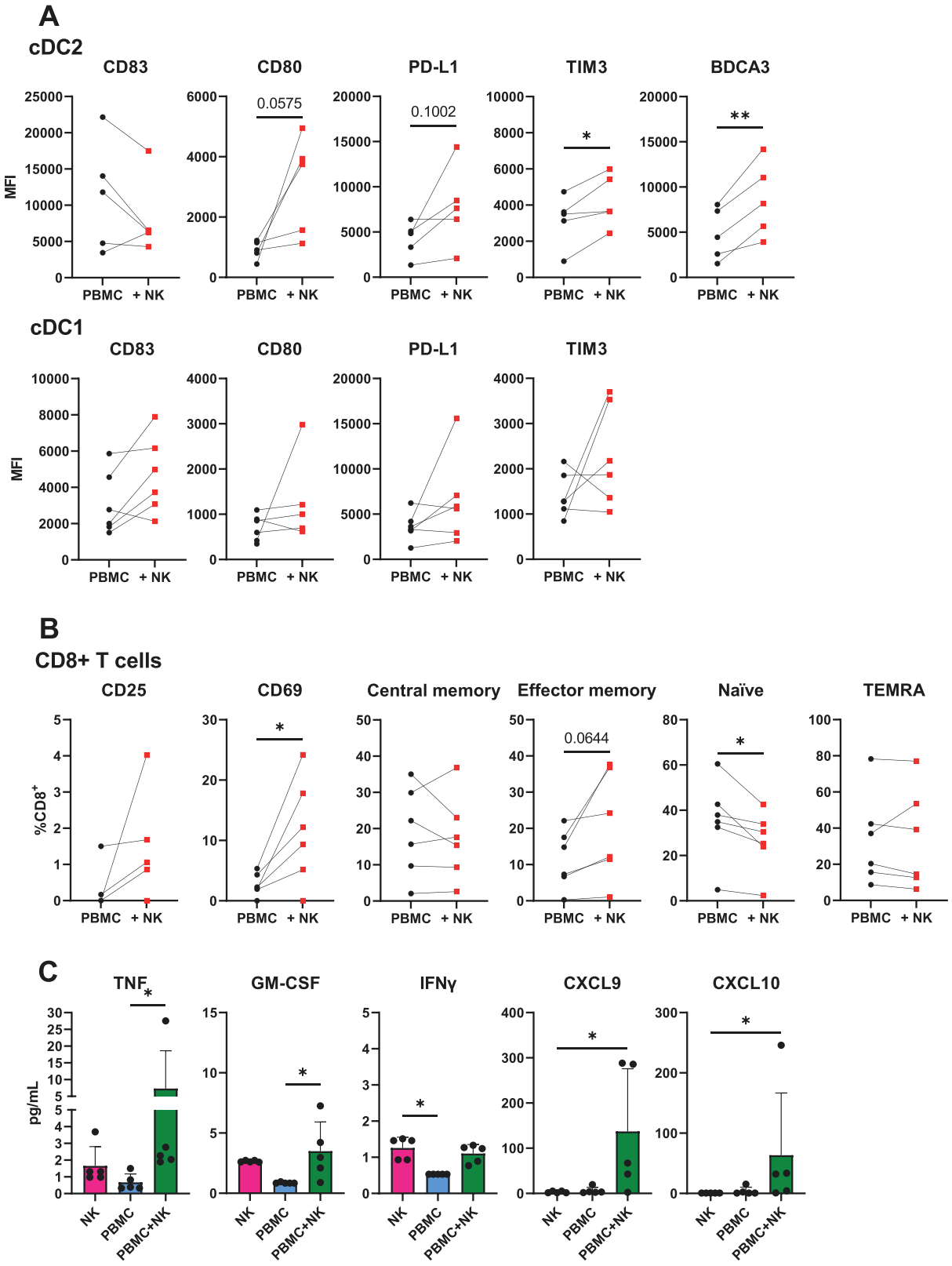


Figure 6. NK cells activate cDC2, induce CD8⁺ T-cell activation and effector-memory differentiation and induce the release of pro-inflammatory cytokines and chemokines in peripheral blood of patients with colorectal cancer. (A) Changes in MFI of various activation markers and immune checkpoints on cDC2 (pregated as CD11c^{hi}HLA-DR^{+}CD1c^{+}CD14^{-}) and cDC1 (pregated as CD11c^{hi}HLA-DR^{+}BDCA3^{+}CD1c^{-}CD14^{-}) in PBMC co-cultured with NK cells for 48 h. PBMC to NK cell ratio 1:1. cDC2 $N = 5$; cDC1 $N = 6$ (2 NK and 6 PBMC donors). (B) Changes in percentages of CD25 and CD69 on CD8⁺ T cells and differentiation state of CD8⁺ T cells after 6 days of co-culture with NK cells at a ratio of 1:1. At day 2, 100 U/ml of IL-2 was added to the culture. $N = 6$ (2 NK and 6 PBMC donors). (C) Cytometric Bead Array performed on the supernatant collected after a 48-h culture of PBMC and/or NK cells. $N = 5$ (2 NK and 5 PBMC donors). Significance is presented as $P < 0.05$*. *P*-values were determined by two-tailed paired *T*-test (A, B) or Friedman test with Dunn multiple comparison analysis (C). Abbreviations: NK, NK cells; PBMC, peripheral blood mononuclear cells.

cells induced an enhanced release of CXCL10 and CXCL9, both important chemokines for the homing of effector T cells and NK cells (Figure 6C). Moreover, in line with the findings in the MoDC model, higher levels of TNF, GM-CSF and, although not significant ($p = 0.17$), IFNγ were also observed in the presence of NK cells.

Discussion

Due to their ability to kill tumor cells while maintaining an acceptable safety profile, the interest for off-the-shelf allogeneic NK cell therapies is increasing [27]. However, NK cells have also been demonstrated to influence the activity of other immune cells [1]. It is important to study if allogeneic NK cell products have similar potential to understand their full mode of action. As NK cells were shown to crosstalk with DC [2], we analyzed in this study the effects of an allogeneic NK cell therapy on MoDC in the presence of IL-10, a well-known *in vitro* model of DC suppression (as under cancer conditions), and on DC in PBMC from patients with metastatic CRC.

Upon differentiation with IL-4 and GM-CSF, the vast majority of immature MoDC downregulated CD14 and acquired CD1a. This immature phenotype was not stable as MoDC, when subsequently cultured in medium only (i.e. starved of exogenous cytokines), tended to reacquire CD14 and downregulate CD1a, possibly regressing to monocytes or differentiating into a macrophage-like state, as might also be expected in the TME. However, in the presence of high numbers of NK cells the CD1a$^+$ DC phenotype with low levels of CD14 was maintained. At the same time, the MoDC were activated by NK cells in a dose-dependent manner with upregulated expression levels of CD80, CD83, CD86, CD70, and HLA-ABC. Slightly lower CD1a levels at the highest NK cell-to-MoDC ratio might have been due to this activation as CD1a has been known to be downregulated upon DC maturation induction. The *de novo* expression of CD14 was also observed and further enhanced during cytokine/PGE2(MC)-induced maturation in the presence of IL-10. We previously showed this CD14$^+$ population to have an M2-like macrophage phenotype with pro-tumor characteristics and a poor T cell stimulatory capacity [11]. While NK cells enhanced MC-mediated MoDC activation, both in the absence and presence of IL-10, at least in part in a contact-dependent fashion, it only marginally prevented the *de novo* expression of CD14 during exposure to IL-10, nor did it enhance the T-cell activating capacity of the maturing MoDC under this condition. The latter may be due to the minimal CD14-to-CD1a shift that NK cells induced during IL-10 driven maturation. Indeed, correlation analysis showed a clear and predominant relation between T-cell proliferation and the CD1a/CD14 subpopulation distribution. This, in turn, may be related to the previously reported finding that CD1a$^+$ MoDC, compared to CD14$^+$ MoDC, express higher levels of IL-12p70, a key pro-inflammatory cytokine for T-cell stimulation [28], leading to a higher IL-12p70/IL-10 ratio [11]. In addition, in the presence of IL-10, NK cells induced very high levels of PD-L1, which by far exceeded the enhanced levels of CD80 and CD86 and were also higher than the levels of PD-L1 found in MC-matured MoDC. This imbalance between PD-L1 and CD80 may well have prevented T-cell activation in the MLR [29] and would argue in favor of combining NK cells with PD-(L)1 blockade as a potential treatment strategy.

The MC used in this study, consisting of cytokines and PGE2, is often adopted in clinical trials to activate MoDC. Its composition is based on factors that are normally produced by activated monocytes [30]. It is noticeable that some of these factors (i.e. PGE2 and IL-6) are also present in high concentrations in the TME where they exert an immunosuppressive function hampering the activities of various immune cells, including NK cells and their crosstalk with DCs [31–33]. PGE2 in particular, was shown to induce the downregulation of NKG2D, NKp30, and NKp44 [34] and to hamper the NK-cDC1 crosstalk [32]. Similarly, IL-6 was demonstrated to decrease NKG2D and NKp30 expression on NK cells [33]. Remarkably, the phenotype of the NK cells used in our model remained mostly stable in the presence of the MC, and although CD25 expression was impacted, the addition of MoDC reverted this effect. Moreover, the crosstalk between NK cells and MoDC resulted in their activation and the release of pro-inflammatory factors despite the presence of these immune suppressive factors.

Multiple contact-dependent and -independent mechanisms have been shown to be involved in NK-DC crosstalk. NK cells have been shown to trigger the differentiation of monocytes to DC in a contact-dependent manner, mediated by CD154 and GM-CSF production [12]. Moreover, NK-dependent MoDC maturation was observed to be dependent on IFNγ and TNF production upon NKp30 triggering [13]. Healthy donor-derived NK cells and NK92 cells were also shown to induce cytolysis of both immature and mature MoDC in a NKp30/DNAM-1 dependent manner [35, 36]. As the NK cells used in this current study were an *in vitro* generated product different from healthy donor NK cells, we assessed if the mechanisms behind their DC-activating activity were similar to those observed for healthy donor-derived NK cells. Interestingly, while the transwell assays showed a partial contact-dependent effect of NK cells and the need for contact to induce enhanced IFNγ production, blocking of NKp30 and CD154 did not affect the ability of NK cells to induce activation of MoDC in the presence of the MC, suggesting that other receptors or a combination of receptors might be involved in this process. Of note, although further analyses are needed to establish the role of TNF, IFNγ, and GM-CSF, their release was consistently enhanced in most of our assays, suggesting that they might play an important role in the observed NK cell-induced DC activation.

Besides MoDC, we found that our NK cells also induced activation of cDC2 in co-cultures with PBMC from patients with CRC. Upon co-culture with NK cells, cDC2 in PBMC expressed higher levels of CD80, BDCA3, TIM3, and PD-L1, consistent with a higher state of maturation [11, 37, 38]. In this setting NK cells induced CD8$^+$ T cell activation and effector-memory differentiation, either directly, via the release of IFNγ [39], or perhaps indirectly, through cDC2 activation.

MoDC and CD1c$^+$ cDC2 are the most frequent DC subsets in the human TME [40] and while in mice cDC1 represent the predominant cross-presenting subset, in humans cDC2 and MoDC have both been shown to have similar CD8$^+$ T cell priming capacity [9, 14] underlining their importance in the anti-tumor response. Moreover, both cDC2 and MoDC were shown to play key roles in the response to anti-PD-(L)1 therapies [7, 8, 41], further suggesting that the combination of NK cells and PD-(L)1 blockade might be beneficial.

However, to further substantiate these findings, studies analyzing the effects of NK cells in more physiological (3D) models of the human TME, as patient-derived tumor samples, are needed.

Upon co-culture of NK cells and MoDC, increased levels of the pro-inflammatory cytokines TNF, IFNγ, and IL-18 [20, 21], as well as the lymphocyte attractant chemokines CXCL9 and CXCL10 [22]. In line with this, also higher levels of IFNγ, CXCL9 and CXCL10 were released upon co-culture of NK cells and MoDC in the presence of MC. Similarly, higher levels of TNF, GM-CSF, CXCL9, and CXCL10 were also observed upon co-culture of NK cells and PBMC from patients with CRC. These results suggest that through crosstalk with DCs, NK cells induce a more permissive, pro-inflammatory environment and have the potential, via the production of CXCL9 and CXCL10, to attract effector T cells to the TME, which is a key determinant of the success of immune checkpoint blockade [42, 43].

In conclusion, besides their direct cytolytic anti-tumor effect, allogeneic NK cell products have the potential to induce dendritic cell activation and thereby trigger a pro-inflammatory response, even under cancer-associated immune suppressive conditions. This is a key factor for the success of T-cell-based therapies and allogeneic NK cell-based cancer immunotherapies may therefore offer an attractive combination therapy to enhance the clinical efficacy of e.g. immune checkpoint blockade. As such, the effects of allogeneic NK cell therapies should be further studied in more physiologically relevant human tumor models and optimized for clinical translation.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical approval

Informed consent was obtained from all subjects involved in the study.

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Conflict of interests

J.S. is a chief scientific officer at Glycostem BV and H.J.v.d.V. is a chief scientific officer at Lava Therapeutics NV. T.D.d.G. is a scientific advisor to Mendus, GE Health and Lava Therapeutics. T.D.d.G. is a section editor of Clinical and Experimental Immunology and as such has been blinded from reviewing or making decisions on the manuscript. A.A.v.V. is an employee of Glycostem BV. No other conflict of interest is reported.

Data availability

Data available on request.

Author contributions

T.D.d.G., J.S, E.C.T., and H.J.v.d.V., conceived the study; T.D.d.G., J.S., H.M.W.V., and H.J.v.d.V. supervised the project; A.A., C.F., E.C.T., and A.A.v.V. performed experiments; J.T. provided patient samples; T.D.d.G., J.S, E.C.T., and H.J.v.d.V., analyzed the data and prepared the figures; T.D.d.G., J.S, E.C.T., and H.J.v.d.V., wrote the paper. All authors edited the manuscript and approved the final version. All authors have read and agreed to the published version of the manuscript.

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