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The immunomodulatory effects of cannabidiol on Hsp70-activated NK cells and tumor target cells

Fei Wang [a,b], Ali Bashiri Dezfouli [c], Gabriele Multhoff [b,*]

[a] Institute of Biological and Medical Imaging (IBMI), Helmholtz Center Munich and Department of Oncology, The second affiliated Hospital of Zunyi Medical University, Zunyi, China
[b] Radiation Immuno-Oncology Group, TranslaTUM – Central Institute for Translational Cancer Research and Department of Radiation Oncology, Klinikum rechts der Isar, TUM School of Medicine and Health, Munich, Germany
[c] Department of Otolaryngology, Head and Neck Surgery, Klinikum rechts der Isar, TUM School of Medicine and Health, Munich, Germany

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ABSTRACT

Background: Cannabidiol (CBD), the major non-psychoactive component of cannabis, exhibits anti-inflammatory properties, but less is known about the immunomodulatory potential of CBD on activated natural killer (NK) cells and/or their targets. Many tumor cells present heat shock protein 70 (Hsp70) on their cell surface in a tumor-specific manner and although a membrane Hsp70 (mHsp70) positive phenotype serves as a target for Hsp70-activated NK cells, a high mHsp70 expression is associated with tumor aggressiveness. This study investigated the immuno-modulatory potential of CBD on NK cells stimulated with TKD Hsp70 peptide and IL-2 (TKD+IL-2) and also on HCT116 p53wt and HCT116 p53-/- colorectal cancer cells exhibiting high and low basal levels of mHsp70 expression.

Results: Apart from an increase in the density of NTB-A and a reduced expression of LAMP-1, the expression of all other activatory NK cell receptors including NKp30, NKG2D and CD69 which are significantly up-regulated after stimulation with TKD+IL-2 remained unaffected after a co-treatment with CBD. However, the release of major pro-inflammatory cytokines by NK cells such as interferon-γ (IFN-γ) and the effector molecule granzyme B (GrzB) was significantly reduced upon CBD treatment. With respect to the tumor target cells, CBD significantly reduced the elevated expression of mHsp70 but had no effect on the low basal mHsp70 expression. Expression of other NK cell ligands such as MICA and MICB remained unaffected, and the NK cell ligands ULBP and B7-H6 were not expressed on these target cells. Consistent with the reduced mHsp70 expression, treatment of both effector and target cells with CBD reduced the killing of high mHsp70 expressing tumor cells by TKD+IL-2+CBD pre-treated NK cells but had no effect on the killing of low mHsp70 expressing tumor cells. Concomitantly, CBD treatment reduced the TKD+IL-2 induced increased release of IFN-γ, IL-4, TNF-α and GrzB, but CBD had no effect on the release of IFN-α when NK cells were co-incubated with tumor target cells.

Conclusion: Cannabidiol (CBD) may potentially diminish the anti-tumor effectiveness of TKD+IL-2 activated natural killer (NK) cells.

1. Introduction

As a representative Danger Associated Molecular Pattern (DAMP), extracellular heat shock protein 70 (Hsp70) exhibits immunostimulatory properties that support multiple cell based anti-tumor immune responses (Srivastava, 1998). Hsp70 chaperones activating immunogenic peptides to T cells (Massa et al., 2004; Sharapova et al., 2021; Sashchenko et al., 2017) and antigen presenting cells (APCs) (Vega et al., 2008; Li et al., 2008) and stimulates NK cells even in the absence of antigenic peptides (Elsner et al., 2010; Multhoff, 2009a). Furthermore, a selective cell surface localization of Hsp70 has been found on a large variety of different tumor types where it acts as a tumor-specific biomarker (Ferrarini et al., 1992; Shin et al., 2003). The membrane expression density of Hsp70 (membrane Hsp70, mHsp70) is stronger on metastases compared to primary tumor cells (Botzler et al., 1998), and tumors expressing mHsp70 release nano-sized extracellular lipid

* Correspondence to: Radiation Immuno-Oncology Group, TranslaTUM – Central Institute for Translational Cancer Research, Klinikum rechts der Isar, TUM School of Medicine and Health, Einsteinstr. 25, Munich 81675, Germany.
E-mail address: gabriele.multhoff@tum.de (G. Multhoff).

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vesicles (EVs) presenting Hsp70 on their surface and in their lumen (Vega et al., 2008). Our group has previously demonstrated that mHsp70 provides a tumor-specific target for NK cells that have been co-incubated with the Hsp70-derived peptide TKD which has stimulatory activities for NK cells but not T cells, in combination with low dose IL-2 (Multhoff et al., 2001, 1997; Gross et al., 2003a). TKD+IL-2-activated NK cells which exhibit an up-regulated expression density of CD94 and CD69 efficiently recognize and kill highly aggressive, mHsp70 positive tumor cells *in vitro* and in tumor mouse models (Multhoff, 2009b). Membrane Hsp70, which is not expressed on normal cells, is predominantly recognized by the heterodimeric C-type lectin receptor complex consisting of CD94 and prominent members of the NKG2 family (Gross et al., 2003b; Borrego et al., 2002). Clinical Phase I and II trials involving the adoptive transfer of *ex vivo* TKD+IL-2-stimulated, autologous NK cells have shown beneficial clinical responses in patients with advanced tumor diseases (Krause et al., 2004; Specht et al., 2015).

The medicinal properties of Cannabis sativa have been known since ancient times and cannabidiol (CBD), one of the major non-psychoactive components of Cannabis sativa, has been used in many different disease settings (Seltzer et al., 2020). In animal models, CBD has shown beneficial effects in a number of different autoimmune and inflammatory diseases including multiple sclerosis (MS) (Kozela et al., 2011), diabetes mellitus (Weiss et al., 2008), autoimmune hepatitis (Hegde et al., 2011). A suppression of lymphocyte proliferation and production of inflammatory cytokines is speculated to be responsible for the anti-inflammatory effects of CBD on the immune system (Borrelli et al., 2009; Weiss et al., 2006; Kaplan et al., 2008). However, very little is known about the immunomodulatory capacity of CBD in anti-tumor immune responses, especially those mediated by activated NK cells. Therefore, we investigated the effects of CBD on both the function of Hsp70-activated NK cells such as cytotoxic activity and NK cell ligand expression by tumor target cells exhibiting differential basal levels of mHsp70 expression, which has been shown to be related to the p53 status. We wanted to address the question whether it is reasonable to combine an NK cell-based therapy addressing highly aggressive mHsp70 positive tumor cells with an anti-inflammatory approach using CBD. Our *in vitro* findings suggest that CBD impairs the cytolytic capacity of TKD+IL-2-activated NK cells which is most likely attributed to a reduced release of pro-inflammatory cytokines such as IFN-γ and TNF-α by NK cells and a CBD-induced downregulation of Hsp70 on the cell surface of tumor cells.

2. Materials and methods

2.1. Cell lines

The human colon adenocarcinoma cell line HCT116 (p53wt) was purchased from ATCC (CCL-247) and the HCT116 p53 double knock out (HCT116 p53-/-) was kindly provided by the laboratory of Prof. Bert Vogelstein (Johns Hopkins University, Baltimore, USA). Both HCT116 cell lines are maintained in McCoy's 5 A medium (Sigma-Aldrich) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) at 37°C in 95 % (v/v) humidity and 5 % (v/v) CO_2. Both cell lines were passaged twice a week and were screened regularly as negative for mycoplasma contaminations. The mHsp70 status was determined by flow cytometry using the FITC-conjugated cmHsp70.1 monoclonal antibody (mAb, multimmune GmbH, Munich, Germany). CBD (Sigma-Aldrich) was dissolved in methanol/cell culture medium solution at a final concentration of 0.3 % (v/v) and this was also used as a vehicle control in all experiments.

2.2. Ethics

Ethical approval for taking blood samples of healthy human volunteers was obtained by the Institutional Ethical Review Board of the Klinikum rechts der Isar, TU München. Written informed consent was obtained from all donors before the start of the study. This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

2.3. Human PBMC isolation and in vitro stimulation of human NK cells with TKD+IL-2

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque. After separation, PBMCs were washed and resuspended in RPMI-1640 supplemented with 10 % (v/v) FCS and antibiotics (100 IU/ml Penicillin G and 100 µg/ml Streptomycin) at a cell density of 10 $\times 10^6$/ml. Following a standard protocol of an NK Cell Isolation Kit (Miltenyi Biotech, Dreieich, Germany), NK cells were separated from peripheral lymphocytes by a CD3 T cell and monocyte depletion using specific beads (Gehrmann et al., 2004). NK cells were stimulated with a sterile 14-mer TKD peptide (TKDNNLLGRFELS, 2 µg/ml, Bachem, Bubendorf, Switzerland) and low dose IL-2 (100 IU/ml) (Multhoff et al., 2001) in the presence or absence CBD (10 µM) for 3 days. Flow cytometry was performed on day 3 after the indicated treatment using the following fluorescence-labeled antibodies directed against: CD3, CD56, CD16, CD94, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46, KIR2DL2-DL3, CD69, NTB-A, LAMP-1, NKG2D, DNAM-1, OX40 and PD1. All antibodies were purchased from Miltenyi Biotec. Flow cytometry analysis was performed on a MACSQuant® flow cytometer (Miltenyi Biotec, Germany). Only viable cells were gated and analysed.

2.4. Analysis of NK cell ligands on the surface of HCT116 p53wt and HCT116 p53-/- cells by flow cytometry

HCT116 (p53wt) and HCT116 p53-/- cells were incubated with FITC-conjugated cmHsp70.1 mAb (multimmune GmbH, Munich, Germany), MICA/B (BD Biosciences), ULBP (BD Biosciences), B7-H6 (BD Biosciences) and the corresponding isotype-matched control antibodies (IgG1, BD Biosciences) after a treatment with CBD (10 µM) for 24 h. Dead cells were excluded from analysis by propidium iodide staining (PI, Sigma-Aldrich). Viable cells were analyzed by flow cytometry using a BD FACSCalibur™ flow cytometer (BD Biosciences). The percentage of positively stained cells was determined following subtraction of the number of cells which were stained with an isotype-matched negative control antibody.

2.5. Cytotoxicity assay

HCT116 p53wt and HCT116 p53-/- cells were used as target cells for analysis of the cytolytic activity of TKD+IL-2 activated NK cells which were isolated from peripheral blood of healthy human volunteers. Target cells were incubated in the presence or absence of CBD (10 µM) for 24 h and were further co-incubated with the following effector cells for another 4 h: (1) NK cells without stimulation; (2) NK cells following a 3-day stimulation with TKD+IL-2; (3) NK cells stimulated with TKD+IL-2 in combination with CBD (10 µM) for 3 days. The effector to target cell ratio was always 4:1. Since we have previously shown that only NK cells, but not T cells, get stimulated by a co-incubation with TKD+IL-2 for 3–5 days (Multhoff et al., 2001), only NK cells contribute to the stimulated effector cell population.

Following the manufacturer's instructions, the Annexin-V/PI assay (ab14085, Abcam) was used to determine the number of dead tumor target cells after co-incubation with the effector cells by flow cytometry. For the assay, CD45 (BD Biosciences) positive effector cells were excluded from the analysis by gating on the CD45 negative tumor cell population.

2.6. Multiplex cytokine assay

Cytokine levels in cell culture supernatants harvested 4 h after co-incubation of effector and target cells at a ratio of 1:1 were determined using the human MACSPlex Cytokine Kit (Miltenyi Biotec, Germany) following the manufacturer's instructions. Briefly, samples containing unknown levels of analytes were incubated with antibody coated MACSPlex Capture Beads having distinct and defined fluorescence properties which react with one of the respective analytes within the sample. The analytes are labeled with fluorescence-conjugated antibodies and the biotinylated Detection Reagent. Sandwich complexes consisting of MACSPlex Capture Beads, analytes, antibodies and Detection Reagent that are formed are then analyzed on a MACSQuant® flow cytometer (Miltenyi Biotec, Germany). Standards of known quantities of the given analytes were used to determine the concentrations of the analytes of each sample.

2.7. Human Granzyme B ELISA

Granzyme B (GrzB) levels in cell culture supernatants harvested 4 h after co-incubation of effector and target cells at a ratio of 1:1 were determined using a human GrzB ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, plates were coated with the GrzB coating antibody and incubated overnight at room temperature. Supernatants or standards were placed on 96-well pre-coated plates and incubated for 1 h at room temperature. After washing, plates were incubated with equal volumes of the detection antibody for 2 h at room temperature. After a 30 min incubation period with the substrate solution, plates were analyzed on a microplate reader (VICTOR X4 Multilabel Plate Reader, PerkinElmer, Waltham, MA, USA).

2.8. Statistical analysis

Statistical analyses were performed using the GraphPad Prism (version 8.0, Graphpad Software, USA). Normally distributed groups of two were analyzed with the Student's t-test, groups greater than two with a single variable were compared using one-way ANOVA test. A value of $p < 0.05$ was considered statistically significant. Data are presented as mean values of at least 3 independent experiments with standard deviation (SD).

3. Results

3.1. CBD does not affect the expression density of activatory NK cell receptors

The neuronal adhesion molecule CD56, the low affinity Fc-γ receptor CD16, C-type lectin receptors (NKG2D, CD94/NKG2C), natural cytotoxicity receptors (NKp30, NKp44, NKp46), activator killer-cell immunoglobulin-like receptors (KIRs), co-stimulatory receptors (CD69, NTB-A), LAMP-1, DNAX accessory molecule-1 (DNAM-1), OX40 and the immune checkpoint inhibitor PD1 are differentially expressed during NK cell activation. Depending on the expression density of their respective ligands on tumor cells, NK cells either maintain their cytolytic activity (Yokoyama and Plougastel, 2003; Martinet and Smyth, 2015) or become immunosuppressed.

Activatory receptors such as CD94/NKG2C and CD94/CD69 are involved in the recognition and killing of mHsp70 positive tumor cells (Gross et al., 2003b), whereas inhibitory receptor complexes such as CD94/NKG2A counterbalance the cytolytic activity of NK cells and thereby prevent NK cell mediated autoimmunity (Borrego et al., 2002). OX40, a member of the TNF-receptor family, is predominantly expressed on activated CD4+ and CD8+ T cells (Paterson et al., 1987; Mallett et al., 1990; Calderhead et al., 1993), but can also be expressed on activated NK cells.

In the present study, we aimed to study the effects of CBD on the receptor expression of unstimulated and TKD+IL-2-activated NK cells. The treatment of unstimulated NK cells with CBD had no effect on the expression of any of the receptors (data not shown). The gating strategy of CD3-/CD56+ NK cells and CD3+/CD56+ NK like T cells before and after stimulation with TKD+IL-2 and after co-incubation with CBD (10 μM) is illustrated in Fig. 1a. As shown in Fig. 1a (right), a stimulation of PBMCs with TKD+IL-2 did not affect the frequency of CD3-/CD56+, CD3-/CD56^{dim}, CD3-/CD56^{bright} NK cells (p=0.5885, p=0.1045 respectively) or that of CD3+/CD56- T cells. NK cells were stimulated with TKD+IL-2 and CBD (10 μM) had no significant effect on the TKD+IL-2-induced upregulation of the expression density (mean fluorescence intensity, MFI) of the activatory receptors CD94, NKG2A, NKG2D, NKp30 and CD69 on NK cells, but the proportion of their expression (Fig. 1b,c). The expressions of CD16, NKG2C, NKp44, NKp46, KIR2DL2-DL3, DNAM-1, OX40 and PD1 on NK cells remained unaffected upon treatment with TKD+IL-2 and CBD (Fig. 1b, c). With respect to the CD3+ T cell populations, the expression of none of the receptors was affected after an incubation with either TKD+IL-2 alone or in combination with CBD (Supplementary Figure 1a,b).

3.2. CBD attenuates the release of pro-inflammatory cytokines and Granzyme B after TKD+IL-2-activation

A multiplex cytokine analysis was used to determine whether CBD influences the TKD+IL-2-induced release of pro-inflammatory cytokines *in vitro*. A stimulation with TKD+IL-2 induces the release of major pro-inflammatory cytokines including IFN-γ and GrzB (Fig. 2d,f), whereas a co-treatment with CBD during the stimulation period attenuated the release of all tested cytokines, apart from IFN-α (Fig. 2a) and IL-17A (Fig. 2b). With respect to cytotoxic effector molecules, CBD significantly reduces the up-regulated release of IFN-γ and GrzB upon TKD+IL-2-stimulation (Fig. 2f).

3.3. CBD affects mHsp70 expression on HCT116 p53 wt cells, but not on HCT116 p53-/- cells

The expression density of NK cell ligands can impact the function of NK cell receptors and thereby modulate cytolytic responses. Based on the changes in the receptor expression on NK cells after stimulation with TKD+IL-2 (Fig. 1a-c), we focused on the expression of the NK cell ligands mHsp70, MICA and MICB which are known to interact with the NK cell receptors CD94 and NKG2D, respectively. Although both HCT116 p53wt and HCT116 p53-/- tumor cell lines expressed mHsp70, the percentage of mHsp70 positive cells and the mean fluorescence intensity (MFI) of mHsp70 expression was higher in HCT116 p53wt compared to HCT116 p53-/- cells (Fig. 3a,b). An incubation with CBD (10 μM) significantly reduced the proportion of high basal mHsp70 expressed HCT116 p53wt cells but had no effect on the proportion of low basal mHsp70 expressed HCT116 p53-/- cells (Fig. 3b). In contrast to Hsp70, the membrane expression of the non-classical MHC ligands MICA/B recognized by NKG2D on NK cells were not affected by CBD, with both ligands being expressed on a higher proportion of HCT116 p53-/- cells compared to HCT116 p53wt cells (Fig. 3c,d). ULBP, another ligand of the human NK cell receptor NKG2D (Table 1), as well as B7-H6, a prominent ligand of NKp30 on a large variety of different tumor cells (Brandt et al., 2009) were not expressed on HCT116 p53wt or HCT116 p53-/- cells (Table 1).

3.4. CBD attenuates the cytotoxic activity of TKD+IL-2-stimulated NK cells against tumor cells with a high mHsp70 expression

Previous studies indicated that NK cells incubated with TKD (2 μg/ml) and low dose IL-2 (100 IU/ml) display an enhanced cytolytic activity against mHsp70 positive tumor cells. CD94 could be identified as a potential receptor for mHsp70 on tumor cells since the cytolytic activity

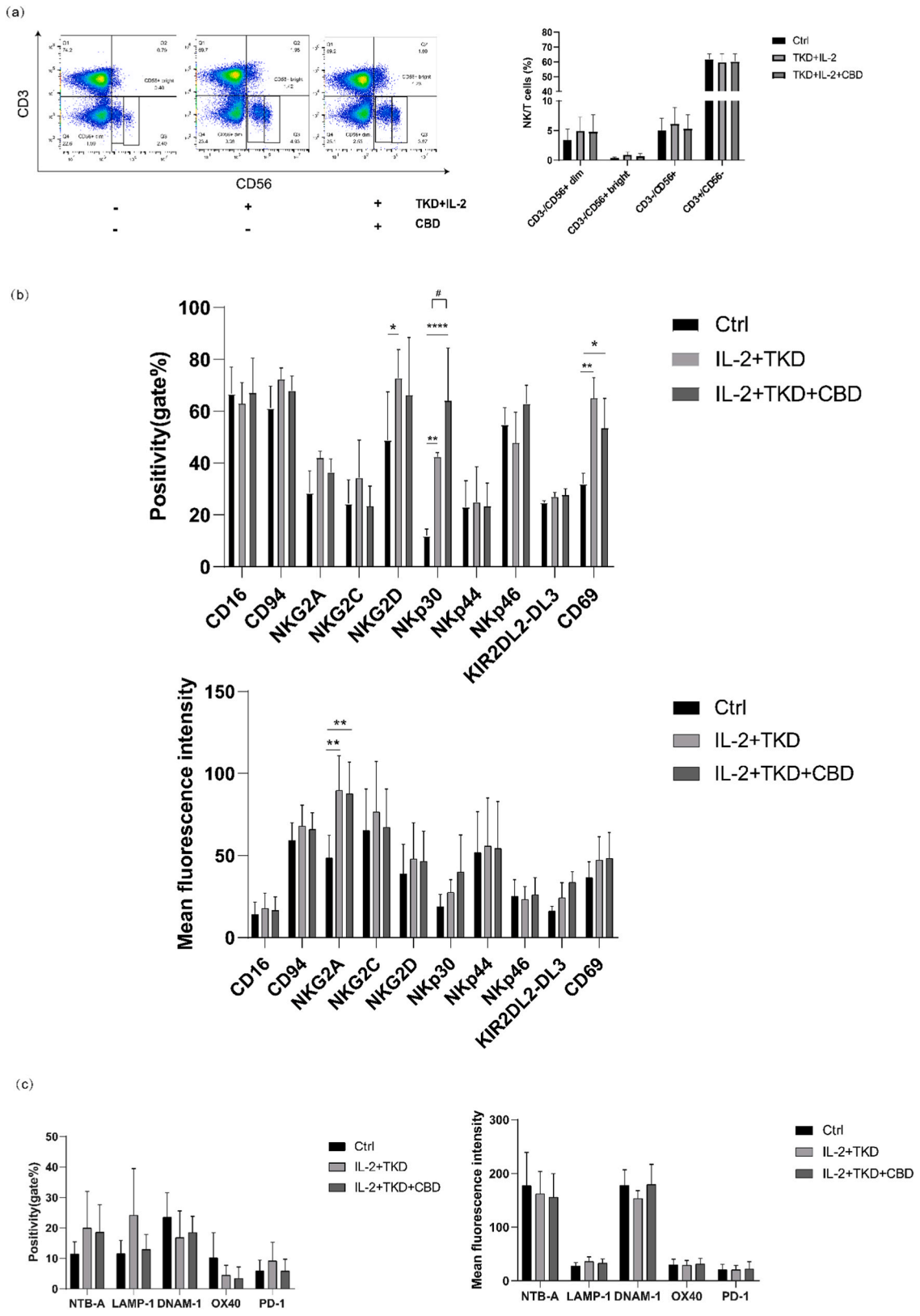

Fig. 1. Phenotypic characterization of cell surface receptors on NK cells upon CBD treatment. (a) Representative graphs of the gating strategy and distribution of different immune cell subsets following CBD treatment. Quantitative bar charts are shown on the right. (b,c) Expression of NK cell receptors in unstimulated cells (Ctrl), TKD+IL-2 stimulated NK cells (TKD+IL-2) in the absence and presence of CBD (TKD+IL-2+CBD); the proportion of positively stained cells (% gated) and the median fluorescence intensity (MFI) are shown in the adjacent bar chart. The data represent mean values ± SD of 3–4 healthy individuals. One-way ANOVA analysis was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$).

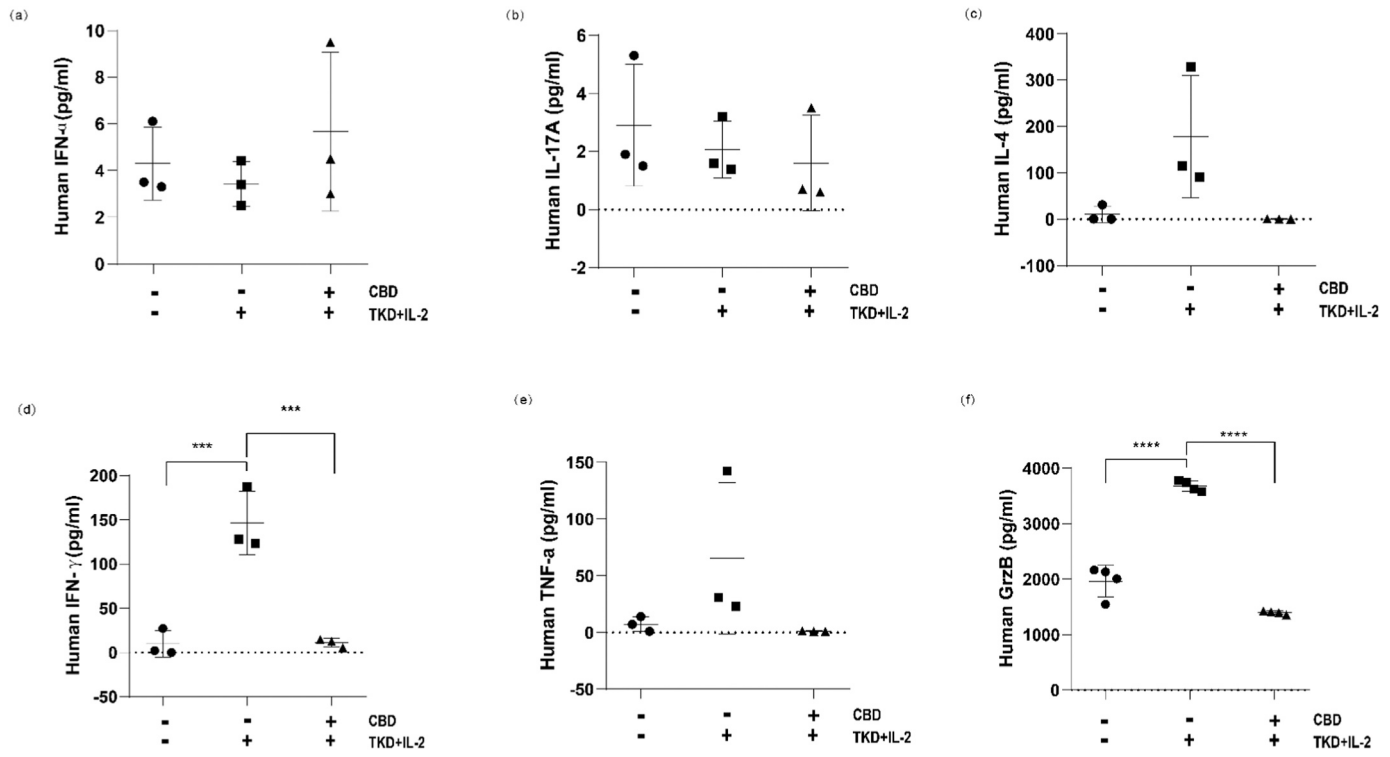

Fig. 2. Cytokine and granzyme B (GrzB) release by unstimulated and TKD+IL-2 stimulated NK cells in the absence and presence of CBD. Cytokine and GrzB release was examined in cell culture supernatants. (a) IFN-α; (b) IL-17A;(c) IL-4; (d) IFN-γ; (e) TNF-α;(f) GrzB. NK cells were stimulated with TKD+IL-2 in the absence or presence of CBD (10 μM) for 3 days. Supernatants of the indicated treatments were collected for GrzB and multiple cytokine analysis. One-way ANOVA analysis was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$). All data are expressed as the mean values of 3 independent experiments ± SD with the blood of three different healthy donors.

of NK cells could be completely inhibited using a CD94 specific blocking antibody (Gross et al., 2003a). The MHC class I chain-related (MIC) A and B are expressed on many human tumors and act as a ligand for the natural killer group 2D (NKG2D) receptor (Raulet et al., 2013; Hayakawa et al., 2002).

The cytolytic activity of TKD+IL-2-stimulated NK cells against HCT116 p53wt and HCT116 p53-/- cells which were exposed to CBD (10 μM) was determined at an effector to target (E:T) cell ratio of 4:1. A co-incubation of the effector cells with CBD (10 μM) concomitant with the stimulatory agents TKD+IL-2 for 3 days reduced the cytotoxic response of NK cells against both HCT116 p53wt and HCT116 p53-/- cell lines, especially for HCT116 p53wt cells (Fig. 4a). This finding is most likely due to the significantly decreased cell surface expression density of mHsp70 on HCT116 p53wt cells compared to HCT116 p53-/- cells upon CBD exposure, which indicates that the density of mHsp70 is crucial for mediating the cytotoxic response in NK cells. However, the significantly reduced expression of mHsp70 on HCT116 p53-/- cells might be compensated for, in part, by a higher expression density of the ligands MICA/B which can be recognized by the NK cell receptor NKG2D (Fig. 3b,c).

The effects of CBD on cytokine release in the presence of HCT116 p53wt and HCT116 p53-/- tumor cells were also analyzed. We found that an increased release of IL-4 (Fig. 4d), IFN-γ (Fig. 4e), TNF-α (Fig. 4f) were observed when NK cells were co-incubated with CBD pretreated HCT116 p53-/- tumor target cells but not with HCT116 p53wt cells (Fig. 4). The release of pro-inflammatory cytokines such as IFN-γ (Fig. 4e), TNF-α (Fig. 4f) and GrzB (Fig. 4g) upon TKD+IL-2-stimulation in the presence of CBD were significantly reduced after co-incubation with both target cell lines HCT116 p53wt and HCT116 p53-/-.

4. Discussion

Heat shock proteins with a molecular weight of 70 kDa (Hsp70) play a crucial role in many diseases, and Hsp70 overexpression in malignant tumors typically serves as a diagnostic biomarker for poor prognosis (Calderwood et al., 2006; Ciocca and Calderwood, 2005). Cytosolic Hsp70 is abundantly overexpressed in tumor cells and provides a survival advantage to malignant cells by inhibiting multiple apoptotic pathways, bypassing the cellular senescence system, interfering with tumor immunity, and assisting the maintenance of the tumor protein homeostasis (Daugaard et al., 2007; Beere, 2001; Martine et al., 2019). However, it is now generally accepted that Hsp70 is present not only in the cytosol but also on the cell membrane of tumor, but not normal cells (Yeh et al., 2009; Hartl and Hayer-Hartl, 2002; Arispe et al., 2004), and that it can be released into the extracellular milieu from viable tumor cells with intact plasma membrane (Pockley et al., 2003). Membrane Hsp70 positive tumor cells actively release Hsp70 surface positive exosomes which can stimulate anti-tumor immune responses in NK cells in the presence of pro-inflammatory cytokines (Gastpar et al., 2005). Based on our previous studies, TKD, an Hsp70-derived 14mer peptide in combination with IL-2, is sufficient to stimulate NK cells to subsequently mediate an enhanced cytotoxicity against mHsp70 positive tumor cells (Multhoff et al., 2001; Stangl et al., 2008, 2011). Upon stimulation with TKD+IL-2 the expression of activatory NK cell receptors such as CD69, NKp30 and NKG2D on CD3-/CD56+ NK cells are upregulated and a treatment with CBD did not significantly alter this surface phenotype on NK cells (Fig. 1). An important role of CD94 in the interaction of NK cells with mHsp70 has been demonstrated by CD94 antibody blocking assays which completely abrogated the cytolytic activity of NK cells against mHsp70 positive tumor cells (Gross et al., 2003a). The CD94/NKG2A heterodimeric receptor conveys an inhibitory signal when binding to its ligand HLA-E (Braud et al., 1998). In contrast, signaling through

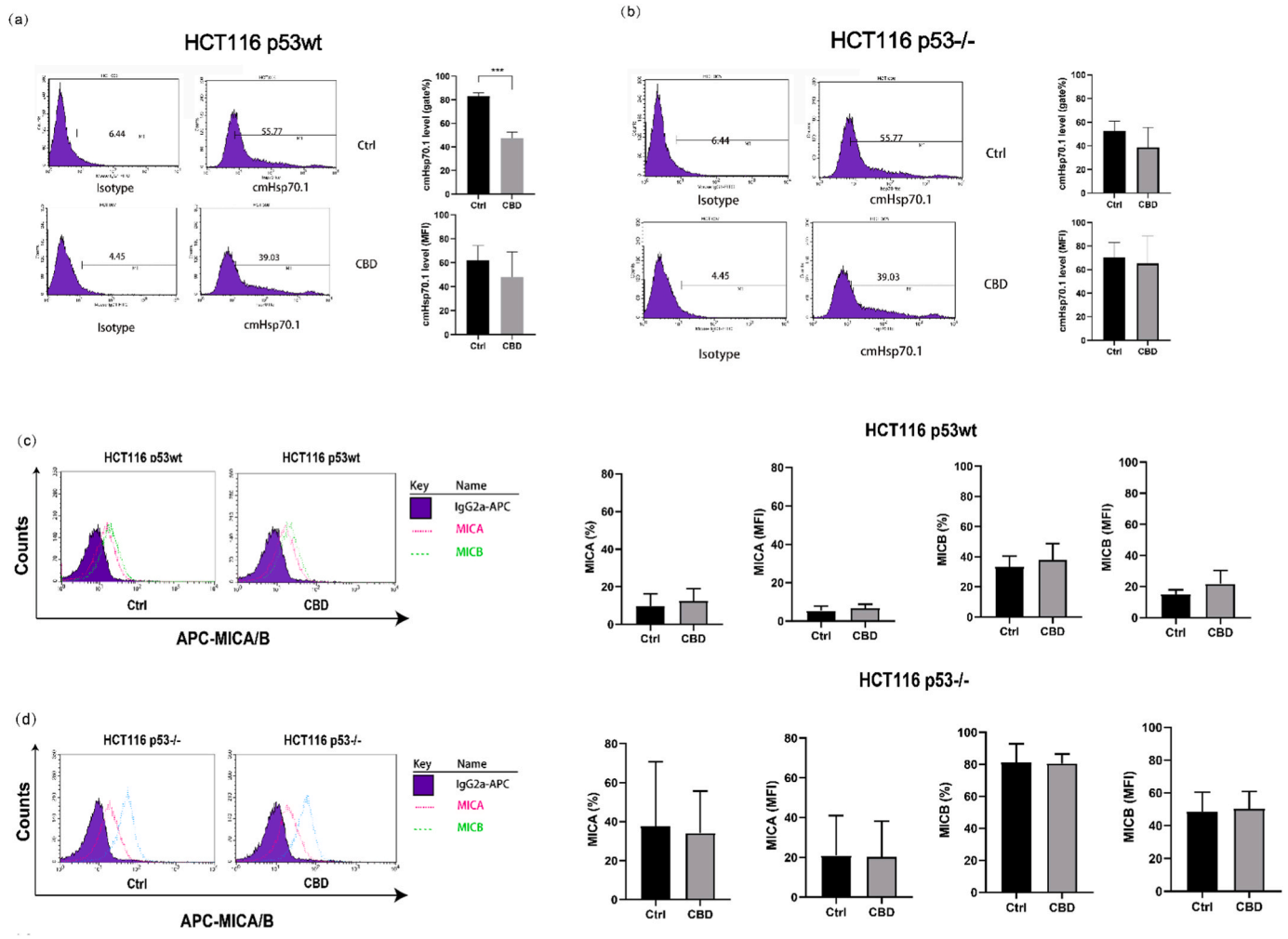

Fig. 3. Expression of NK cell ligands on the surface of HCT116 p53wt and HCT116 p53-/- tumor cells upon CBD treatment. (a-b) Expression of mHsp70 (% positively stained cells, left; mean fluorescence intensity, right) on HCT116 p53wt(a) and HCT116 p53-/-(b) tumor cells. (c) Expression of MICA (% positively stained cells, left; mean fluorescence intensity, right) on HCT116 p53wt and HCT116 p53-/- tumor cells. (d) Expression of MICB (% positively stained cells, left; mean fluorescence intensity, right) on HCT116 p53wt and HCT116 p53-/- tumor cells in the absence (Ctrl) and presence of CBD (10 μM). One-way ANOVA analysis or t-test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). All data are expressed as the mean ± SD of 3 independent experiments.

Table 1
Effect of CBD on the expression of NK ligands on HCT116 p53wt and HCT116 p53-/- cells.

	HCT116 p53wt				HCT116 p53-/-			
Ligands	Gate (%)		MFI		Gate (%)		MFI	
	Ctrl	CBD	Ctrl	CBD	Ctrl	CBD	Ctrl	CBD
Hsp70	82.83±3.12	47.66±5.06	62.06±12.17	47.88±20.97	52.75±8.02	38.67±16.98	70.50±12.64	65.20±23.06
MICA	9.70±6.44	12.47±6.42	5.44±2.38	6.75±2.05	37.69±32.98	34.19±21.56	20.95±20.04	20.38±17.71
MICB	33.26±7.31	38.05±10.74	15.04±2.87	21.83±8.38	81.28±11.52	80.53±5.92	48.59±11.85	50.43±10.51
ULBP	0.08±1.47	−1.90±1.49	−0.82±3.03	−3.94±4.48	−1.26±2.52	−2.21±0.37	−3.47±4.77	−4.62±1.82
B7-H6	−1.49±0.51	−1.56±1.05	−1.79±0.30	−2.56±0.88	−0.65±0.50	−0.01±0.64	−1.79±0.44	−1.51±0.54

Data represented mean values with standard deviations (SD) from 3 independent experiments. For all treatments 10 μM CBD and an incubation period of 24 h was used. Tumor cells were stained with antibodies directed against mHsp70 (cmHsp70.1), MICA, MICB, ULBP and B7-H6. The percentage of positively stained cells (%) and mean fluorescence intensity (MFI) within the tumor cell gate of each ligand are presented. The number of cells stained positively with an isotype-matched control antibody was subtracted from the number of cells stained with a specific antibody.

CD94/NKG2C stimulates NK cells (Pérez-Villar et al., 1995). Together with the upregulation in the activating receptors CD69 and NKG2D (Fig. 1b), we found that a slight decline in expression of the NKG2C receptor, whereas NKG2A receptor expression remained stable in comparison after a co-treatment with TKD+IL-2 and CBD (Fig. 1b). This finding might suggest that CBD does potentially impair the cytotoxicity of NK cells which is induced after stimulation with TKD+IL-2. An impact of CBD on APCs can be excluded since NK cells can be stimulated with TKD+IL-2, even in the absence of any other cell populations (Multhoff et al., 2001; Gross et al., 2003a). However, previous studies indicated that the NK cell cytotoxicity mediated by CD69 can be abrogated by a stimulation of NK cells expressing the heterodimeric receptor CD94/NKG2A (Borrego et al., 1999). Therefore, the interaction between CD69 and the inhibitory CD94 receptor complex on the activity of NK

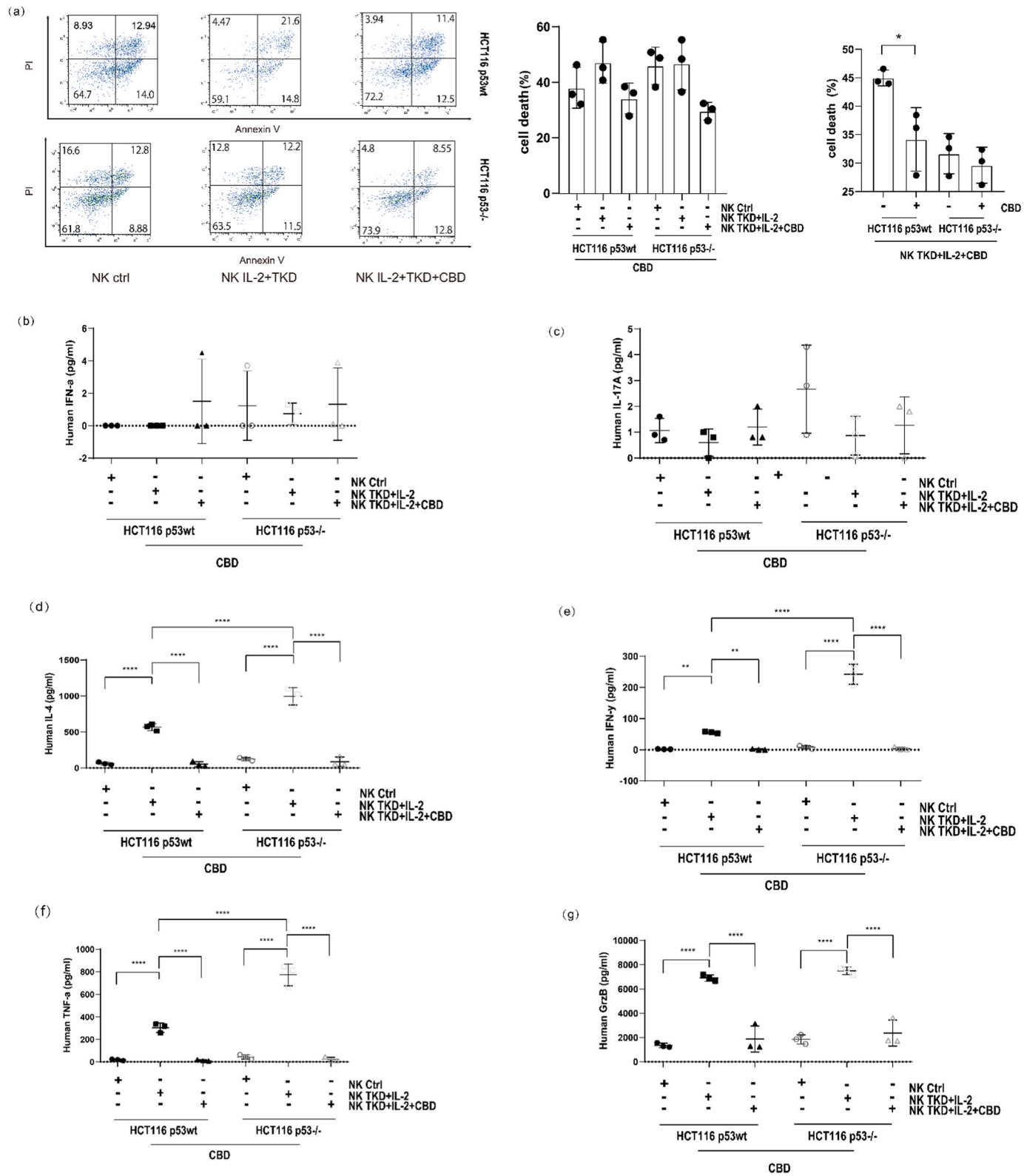

Fig. 4. Cytokine and granzyme B (GrzB) release by unstimulated and TKD+IL-2 stimulated NK cells after co-incubation with HCT116 p53wt and HCT116 p53-/- tumor cells in the absence and presence of CBD. (a) Comparative analysis of the cytotoxic activity of TKD+IL-2-stimulated NK cells in the presence or absence of CBD against membrane Hsp70^{high} HCT116 p53wt cells and membrane Hsp70^{low} HCT116 p53-/- tumor cells. Tumor cells were exposed to CBD (10 μM) for 24 h and then co-incubated with preconditioned NK cells at effector:target (E:T) ratios of 4:1 for another 4 h. Cell death was determined by Annexin-V+/PI+ staining. The killing assay was performed using NK cells from 3 healthy donors. (b-g) GrzB and cytokine profiles of unstimulated or TKD+IL-2 stimulated NK cells after co-incubation with HCT116 p53wt and HCT116 p53-/- tumor cells in the presence of CBD. (b) IFN-α; (c) IL-17A; (d) IL-4; (f) IFN-γ; (f) TNF-α; (g) GrzB. Supernatants of the indicated treatments derived from 3 healthy individuals were collected for GrzB and multiplex cytokine analysis. One-way ANOVA analysis or t-test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). All data are expressed as the mean data of 3 independent experiments ± SD.

cells against mHsp70 positive tumor cells needs to be further investigated. $CD56^{dim}$ NK cells are essential for natural and antibody-mediated cell killing (Farag et al., 2003), whereas $CD56^{bright}$ NK cells predominantly perform immunoregulatory functions by producing large amounts of pro-inflammatory cytokines (Cooper et al., 2001; Carson et al., 1997). As shown in Fig. 1, CBD maintained the percentage of $CD56^{dim}$ NK cells stimulated with TKD+IL-2 but did not result in an upregulation of the $CD56^{bright}$ NK cell population.

The proliferative and cytolytic activity of NK cells are induced through pathways that require interactions between NK cell receptors and their ligands (NKG2D-MICA and/or MICB) and cytokines secrete in a paracrine manner such as IFN-α, IL-2, IL-12 (Degli-Esposti and Smyth, 2005; Marçais, 2013). MICA/B positive tumor cells, even with a strongly reduced mHsp70 expression, can be lysis by TKD+IL-2-stimulated NK cells due to the presence of other NK cell ligands such as MICA and MICB, although the presence of mHsp70 provide a better target for NK cell activation and cytolysis (Elsner, 2007). Based on the overexpression of NKG2D upon co-treatment with TKD+IL-2 and CBD in NK cells, we further analyzed the membrane expression density of their corresponding ligands on HCT116 tumor cells with differential p53 status. In this study we had shown that the basal mHsp70 levels were higher in HCT116 p53wt cells compared to HCT116 p53-/- cells (Fig. 3a,b), whereas MICA/B were expressed at a much higher density on HCT116 p53-/- cells (Fig. 3c,d). The expression of Hsp70 on the cell membrane was, however, only significantly reduced following the administration of CBD in HCT116 p53wt cells (Fig. 3a). With respect to the cytolytic activity of NK cells, the higher levels of MICA/B on HCT116 p53-/- cells might compensate for the downregulated expression of the CD94 ligand mHsp70, as TKD+IL-2-stimulated NK cells showed a comparable killing activity of both tumor target types (Fig. 4a).

IFN plays an important role in the induction of apoptosis (Clemens, 2003). Some studies assume a link between these interferons and p53. For instance, an increased sensitivity of IFN-producing melanoma cell lines to apoptosis induced by cisplatin is associated with an IFN-dependent accumulation of p53 (Mecchia et al., 2000). IFN induces the transcriptional activity of p53 which is most likely mediated by the IFN-stimulated response elements (ISREs), a site which harbors p53 genes (Vilček, 2003). Elevated p53 levels facilitate an increased apoptosis by IFN (Takaoka et al., 2003; Fukui et al., 2003). In our study, we found that TKD+IL-2 stimulated NK cells co-cultured with HCT116 p53-/- produced greater IFN-γ (Fig. 4e) and TNF-α(Fig. 4f) than HCT116 p53wt cells, which might be the factor that the cell death ratio of HCT116 p53-/- cells was similar as HCT116 p53wt cells upon stimulated NK cells exposure (Fig. 4a). The potential mechanism in this case still needs to be clarified. IL-4, released by tumor cells, acts as an activator of tumor-associated macrophages and myeloid-derived suppressor cells, mediating pro-tumor activity (Suzuki, 2015). Similar as those two cytokines above, IL-4 was another one produced in large quantities in HCT116 p53-/- group, which corroborated the conclusions that loss of p53 in cancer cells can impact the myeloid and T cells recruitment, allowing immune evasion (Blagih et al., 2020). Some other studies showed that in PBMCs, CBD increased the IL-10 production when B cells were dependent on activated T cells and decreased TNF levels when T cells were activated independently (Lowin et al., 2023). Besides, CBD impaired both, the frequency of IL-4-producing CD4+ cells and IFN-γ/IL-17-producing cells, and diminished the stimuli-induced cytokine mRNA expression (Furgiuele et al., 2023). Other studies suggested that CBD often displayed an inhibitory effect on the activation and functional activity of NK cells by an interference of the GPR55/GPR18 signaling pathway (Chiurchiù et al., 2015). In early studies, NK cells were found to have the highest P-gp expression and the inhibition of P-gp by CBD reduced NK cell cytotoxicity in a dose-dependent manner (Klimecki et al., 1995; Takahashi et at., 1999; Zhu et al., 2006). Here, CBD significantly reduced the release of some pro-inflammatory cytokines, such as IFN-γ, which was induced by TKD+IL-2 stimulated NK cells (Fig. 2). This finding confirms the anti-inflammatory effect of CBD which was shown in previous studies (Jan et al., 2007). Moreover, the release of GrzB by TKD+IL-2-stimulation was likewise significantly attenuated by CBD (Fig. 2). A similar trend was found in the case of pre-conditioned NK cells co-incubated with target cells that have been treated with CBD (Fig. 4).

5. Conclusions

Our data suggests that a CBD treatment might exert immunosuppressive activities in a cell-based therapy with TKD+IL-2-stimulated NK cells by reducing the expression of the NK cell ligand (mHsp70) and the release of pro-inflammatory cytokines and GrzB by NK cells. This suggests that although CBD has been shown to exert an anti-tumor activity, in the context of a combined effector cell-based immunotherapy, CBD might limit the anti-tumor efficacy of activated NK cells. However, further research in immunocompetent tumor mouse models is needed to fully understand the potential impact of CBD on activated NK cells in a combined treatment schedule in vivo.

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Author Statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

CRediT authorship contribution statement

FEI WANG: Conceptualization, Methodology, Writing – original draft. **Ali Bashiri Dezfouli:** Methodology. **Gabriele Multhoff:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

Conflict of Interest

The authors declare no conflict of interest.

Data availability

All data are available in the main text or the supplementary materials.

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Informed Consent Statement

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

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethical Review Boards.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2024.07.008.

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