Cancer Immunology, Immunotherapy (2019) 68:883–895
https://doi.org/10.1007/s00262-019-02319-7

ORIGINAL ARTICLE

HCT-116 colorectal cancer cells secrete chemokines which induce chemoattraction and intracellular calcium mobilization in NK92 cells

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Received: 9 September 2018 / Accepted: 28 February 2019 / Published online: 7 March 2019

Abstract

We recently reported that pretreatment of IL-2 activated human natural killer (NK) cells with the drugs dimethyl fumarate (DMF) and monomethyl fumarate (MMF) upregulated the expression of surface chemokine receptor CCR10. Ligands for CCR10, namely CCL27 and CCL28, induced the chemotaxis of these cells. Here, we performed a bioinformatics analysis to see which chemokines might be expressed by the human HCT-116 colorectal cancer cells. We observed that, in addition to CCL27 and CCL28, HCT-116 colorectal cancer cells profoundly express CXCL16 which binds CXCR6. Consequently, NK92 cells were treated with DMF and MMF for 24 h to investigate in vitro chemotaxis towards CXCL16, CCL27, and CCL28. Furthermore, supernatants collected from HCT-116 cells after 24 or 48 h incubation induced the chemotaxis of NK92 cells. Similar to their effects on human IL-2-activated NK cells, MMF and DMF enhanced the expression of CCR10 and CXCR6 in NK92 cells. Neutralizing anti-CXCL16 or anti-CCL28 inhibited the chemotactic effects of 24 and 48 supernatants, whereas anti-CCL27 only inhibited the 48 h supernatant activity, suggesting that 24 h supernatant contains CXCL16 and CCL28, whereas HCT-116 secretes all three chemokines after 48 h in vitro cultures. CXCL16, CCL27, and CCL28, as well as the supernatants collected from HCT-116, induced the mobilization of $(Ca)^{2+}$ in NK92 cells. Cross-desensitization experiments confirmed the results of the chemotaxis experiments. Finally, incubation of NK92 cells with HCT-116 induced the lysis of the tumor cells. In summary, these results might have important implications in directing the anti-tumor effectors NK cells towards tumor growth sites.

Keywords Colorectal cancer · NK92 cells · Chemokines · Dimethyl fumarate · Monomethyl fumarate · Calcium mobilization

Abbreviations

CCL/CCR	CC chemokine ligand/chemokine receptor
CTACK	Cutaneous T-cell-attracting chemokine/CCL27
CXCL/CXCR	CXC chemokine ligand/chemokine receptor
DMF	Dimethyl fumarate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EGTA	Ethylene glycol-bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FU	Fluorescence units
HRP	Horseradish peroxidase
MI	Migration index
MMF	Monomethyl fumarate
mRNA	Messenger RNA
MS	Multiple sclerosis
NK	Natural killer
PDL-1	Program death ligand-1
qRT-PCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
Treg	Regulatory T cells

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-019-02319-7) contains supplementary material, which is available to authorized users.

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Introduction

Natural killer (NK) cells are innate immune cells that serve as anti-tumor effectors [1]. These cells have been classified into two main subsets; based on CD56 and CD16 expression. The cells that express CD56 but not CD16 known as $CD56^{bright/+}$ represent about 10–20% of total NK cells in the blood, whereas those that express CD16 and low CD56 known as $CD56^{dim/-}$ cells represent about 80–90% of total circulating NK cells [2, 3]. In addition, NK cells have been classified into subsets based on their differential expression of chemokine receptors [4].

NK cells have been used for the treatment of cancer patients, and there are currently more than 900 NCI approved protocols for using these cells in the therapy of various cancers (https://clinicaltrials.gov/ct2/results?term=nk+cells+and+cancer&pg=9). The problems that face successful treatment with NK cells include changes in the tumor microenvironment as well as the inability of NK cells to migrate towards sites of tumor growth. It was also observed that, in colorectal cancer patients, NK-cell-activating receptors are downregulated while inhibitory receptors are upregulated, along with altered antibody-dependent cell-mediated cytotoxicity in those patients [5].

To obtain more effective methods utilizing NK cells in cancer immunotherapy, we examined the effect of drugs utilized for treating multiple sclerosis (MS) patients on the anti-tumor activity of NK cells. Dimethyl fumarate (DMF, commercial name Tecfidera, Biogen), has been approved for treatment of MS patients due to its efficacy and low toxicity [6]. Intriguingly, the beneficial application of this drug was extended beyond MS such as utilization in virotherapy [7], and as a potential therapy for treating many diseases [8]. In a similar approach, it was reported that DMF possesses an anti-tumor activity as illustrated in an experimental model of melanoma [9]. Although it was suggested that the effect of DMF might be related to its cleavage by esterases into the metabolite monomethyl fumarate "MMF" [10], it was reported that DMF could have distinct activities than MMF [11]. We showed that MMF ameliorates experimental autoimmune encephalomyelitis (EAE) clinical scores in mice, corroborated with the potentiality of this metabolite to boost NK-cell lysis of dendritic cells [12]. More important, we reported that MMF induces NK-cell lysis of two tumor cell lines: the erythroleukemia K562 cells and the B-cell lymphoma RAJI cells [13]. Recently, we demonstrated that DMF and MMF cause an upregulation in the expression of surface CCR10 on IL-2-activated NK cells [14]. This effect was corroborated with the chemotaxis potential of MMF- and DMF-treated NK cells towards different concentration gradients of CCR10 ligands, i.e., CCL27 and CCL28 [14]. These results support the other work where higher immunostaining of CCL27 in the supratumoral epidermis was correlated with survival against melanoma and a longer progression-free interval [15]. On the other hand, CCL28 is increased in the plasma of patients with colon cancer [16]. It is plausible that this secretion may aid the recruitment of anti-tumor effector cells to sites of colorectal cancer growth, and hence, these effector cells could be potential candidates for targeting into tumor sites. In this study, we investigated in detail the plausibility that colorectal cancer cells may secrete chemokines and, consequently, recruit the anti-tumor effector NK cells.

To gain insights into the mechanisms of recruiting NK cells, we used NK92 cells. These cells are easy to expand in vitro and have been used for therapeutic purposes, due to their highly anti-tumor activity and growth manipulations [17]. Furthermore, NK92 cells were used to examine the signaling pathways in NK cells [18], and for their ability to induce death in colorectal cancer cells [19]. Recently, it was reported that haNK cells, derivative of NK92, can be used for treating human carcinomas with anti-program death ligand-1 (PDL-1) antibody, due to their participation in antibody-dependent cell-mediated cytotoxicity [20]. However, more studies are needed to delineate the importance of NK92 in treating cancer patients. In fact, nothing is known about chemokine receptor expression in NK92 cells despite the wealth of knowledge regarding non-activated or IL-2-activated NK cells. Consequently, we collected supernatants from HCT-116 colorectal cancer cells and examined whether these supernatants contain chemokines that may attract NK92 expressing the corresponding chemokine receptors. In addition, we combined the migration assay with the release of intracellular calcium and the ability of NK92 to lyse the colorectal cancer cells.

Materials and methods

Cell culture of human cell lines

The human natural killer cell line NK92 was cultured in RPMI-1640 medium supplemented with 4.5 g/L glucose, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 71.5 µM 2-mercaptoethanol, and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), in addition to 200 U IL-2/mL. Erythroleukemia K562 and colorectal cancer cell line HCT-116 were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 71.5 µM 2-mercaptoethanol, and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). To collect condition media (supernatants), 1×10^6 HCT-116 cells/mL were incubated for 24 or

48 h, after which the cell suspensions were centrifuged for 8 min, and the collected supernatants were stored at − 80 °C until further use.

Reagents

PE-conjugated mouse anti-human CCR10 and its PE-conjugated IgG2a isotype control, as well as PerCP-Cy5.5 mouse anti-human CD193 (CCR3) and its PerCP-Cy5.5 IgG2b isotype control, were purchased from Becton Dickinson (BD Biosciences, San Jose, CA, USA). APC-conjugated mouse anti-human CXCR6 and APC-conjugated mouse IgG2b isotype control were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Unconjugated mouse IgG1, unconjugated rat IgG2a, unconjugated mouse anti-human CCL27, unconjugated mouse anti-human CCL28, and unconjugated rat anti-human CXCL16 were purchased from R&D Systems Inc. (Minneapolis, MN, USA). CCL27 and CCL28 were acquired from R&D Systems, while CXCL16 was obtained from PeproTech (Rocky Hill, NJ, USA). Triton-X, ethylene glycol-bis (2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and ionomycin, were obtained from Sigma-Aldrich, St. Louis, MO, USA. Monomethyl fumarate (MMF) and dimethyl fumarate (DMF) were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO), according to manufacturer's instructions. Mouse anti-human CCR10 was purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA), rabbit anti-human CXCR6 was from Abcam (Cambridge, UK) and mouse anti-human β-actin from Sigma-Aldrich. Anti-rabbit IgG HRP-linked antibody and anti-mouse IgG HRP-linked antibody were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA).

In vitro chemotaxis assay

Neuro probe blind well chemotaxis chambers with a lower well volume of 200 µL were used. In the lower wells of the chamber, 200 µL of medium were placed in the presence or absence of chemokines, HCT-116 supernatants, or K562 supernatants. In the antibody neutralization experiments, antibodies specific for CCL27, CCL28, or CXCL16 were incubated with the supernatants for 1 h. Upper and lower compartments were separated by 8 µm Nuclepore polycarbonate filters (Whatman International Ltd., Maidstone, UK), and 2×10^5 NK92 cells were placed in the upper compartments, whereas the supernatants either untreated or pretreated with the neutralizing antibodies were placed in the lower compartment of the chambers. After the 2 h incubation period, filters were removed and dehydrated with methanol, after which they were stained with 15% modified Giemsa stain for 25 min and fixed on glass slides. NK92 cells were counted in × 10 power field, and an average count was calculated for each sample. Migration index (MI) was calculated using the equation where the number of cells migrating towards chemoattractant (chemokines or supernatant) is divided by the number of cells migrating towards medium only. Mean ± SEM of 3–4 experiments was calculated for each experiment, where 4–6 filters were used and averaged in each experiment.

Flow cytometric analysis

NK92 cells were cultured at a concentration of 1×10^6 cells/mL and were treated with 100 µM MMF or DMF or control vehicle and left for 24 h at 37 °C in a 5% CO_2 incubator. Subsequently, 3×10^5 NK92 cells, whether untreated or DMF- or MMF-treated, were taken and washed with FACS buffer (PBS, 2% FBS, 0.1% sodium azide). Next, the antibodies were added to the cells and left for 45 min incubation in the dark at room temperature. The cells were washed twice before being examined in the flow cytometer (FACS Aria III, Becton–Dickinson Biosciences, San Jose, CA, USA). Viable cells were selected, and gating was adjusted based on the isotype control. Flow cytometry data analysis was executed using the FlowJo software (Ashland, Oregon, USA).

Western blot analysis

NK92 cells were cultured at a concentration of 1×10^6 cells/mL and were treated with 100 µM MMF or DMF or control vehicle and left for 24 h at 37 °C in a 5% CO_2 incubator. The cells were collected and washed with PBS, after which the proteins were extracted using the Laemmli lysis buffer. Protein extracts were quantified using Bradford Protein Assay Kit, according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). A 50 µg of protein was separated on SDS-PAGE and transferred to nitrocellulose membrane. Expression of CCR10, CXCR6, and β-actin was assessed using mouse anti-human CCR10, rabbit anti-human CXCR6, and mouse anti-human β-actin, respectively. Anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibodies were used along with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) for chemiluminescent detection of protein bands.

RNA extraction and quantification of CCR10 and CXCR6

NK92 cells were cultured at a concentration of 1×10^6 cells/mL and were treated with 100 µM MMF or DMF or control vehicle and left for 24 h at 37 °C in a 5% CO_2 incubator. They were collected and washed with PBS, after which total RNA was extracted using the Total RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON, Canada). RNA was further purified by removing genomic DNA using

TURBO DNA-free Kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). A 1 µg of purified RNA was reverse transcribed to cDNA using TruScript First Strand cDNA Synthesis Kit. The expression of CCR10 and CXCR6 was quantified relative to the house-keeping gene 18S using real-time quantitative polymerase chain reaction qRT-PCR (StepOne, Applied Biosystems, Foster City, CA, USA). Primers used for 18S, CCR10, and CXCR6 are shown in Supplementary Table 1.

Calcium mobilization

NK92 cells were washed twice and incubated in Ca^{2+} buffer-containing 5 mM KCl, 145 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Na/MOPS, 1 mM $CaCl_2$, 10 mM HEPES, 0.25% BSA with a pH equal to 7.4, and 5 µM fura-2-AM (all from Sigma-Aldrich, St. Louis, MO, USA) for 45 min at 37 °C. Consequently, the cells were washed and resuspended at a concentration of 3×10^5 cells/mL to be incubated with 100 ng/mL of the chemokines CCL27, CCL28, CXCL16, or with the 24 h or 48 h HCT-116 collected supernatants. Fluorescence is measured using the fluorescence spectrometer system (LS55, Perkin-Elmer, Waltham, Massachusetts, USA), where excitation was measured at 340 and 380 nm, and the emission was determined at 510 nm. In these assays, the intensity was assessed using a photomultiplier tube system, and the fluorescence ratio of bound /free fura-2 was then calculated.

Quantification of chemokines in HCT-116 supernatants using ELISA

Collected supernatants were used for the quantification, where the levels of chemokines (CXCL16, CCL27/CTACK, and CCL28) were determined using ELISA (Enzyme-Linked Immunosorbent Assay) Duoset kits from R&D Systems Inc. (Minneapolis, MN, USA). Measurement of the color intensity was performed using the BioTek ELx808 plate reader (BioTek Instruments, Winooski, VT, USA) at an absorbance wavelength of 450 nm.

NK92 cytotoxicity assay

HCT-116 cells were used as target cells, where 1×10^6 cells/mL were incubated with 1 µM Calcein-AM (Sigma-Aldrich, St. Louis, MO, USA) for 45 min. Target cells (1×10^4) were co-cultured with NK-92 cells in RPMI at 5:1 E:T ratio for 4 h. Total lysis was achieved by adding 5% Triton-X with the target cells, whereas total viability was attained by incubating the cells with RPMI medium alone. After 4 h, the 96-well plates were centrifuged to discard the supernatants, and PBS was added instead. Fluorescence units (FUs) were measured in the Synergy HTX plate reader (BioTek Instruments, Winooski, VT, USA). Percent cytotoxicity was calculated as previously described [21].

Exploring the gene expression of chemokines in publically available databases

We used an online resource named "Harmonizome" that is accessible through http://amp.pharm.mssm.edu/Harmonizome, as it provides a large comprehensive database about genes and proteins that are publicly available [22]. We searched for the databases that comprise most of the available cell lines in the Cancer Cell Line Encyclopedia containing cancer cell lines' mRNA expression profile, i.e., 18,022 genes in 1035 cell lines [23]. We downloaded all gene–attribute matrices that are standardized to have a uniform meaning across different cell lines and experiments, and then, we filtered the gene sets to all the chemokines in three cell lines used; HCT-116, K562, and DAUDI, and the heat map was then generated accordingly.

Statistical analysis

Normally distributed samples were compared using Student's *t* test. Comparisons between groups were made using one-way multiple range ANOVA test for multiple comparisons. A P value < 0.05 was considered to be statistically significant.

Results

HCT-116 cells express CXCL16, and to a much lower extent CCL27 and CCL28

In our attempt to find cancer cells that may induce the chemoattraction of NK cells, we examined by bioinformatics the chemokines expressed by HCT-116 colorectal cancer cells and compared this expression with two other tumor cell lines known to be targeted by NK cells, i.e., the erythroleukemia K562 cells and the B-cell lymphoma DAUDI cells. The results demonstrate that HCT-116 highly express CXCL16, and to a lower extent CCL27 and CCL28 (Supplementary Fig. 1). Although CCL28 was weakly expressed in HCT-116 using bioinformatics analysis, our previous results suggest that this chemokine was a robust chemoattractant for human NK cells [14]. Consequently, it was included in this study, as bioinformatics information does not always predict biological functions.

CXCL16, CCL27, and CCL28 induce the in vitro chemotaxis of NK92

Only a few reports showed the chemotaxis of NK92 cells despite the fact that these cells are used for therapy of certain

cancers, and have the potential of treating most cancer patients [17, 20]. Berahovich et al. extensively examined the chemotaxis of these cells towards most of the known chemokines [24]. Here, we examined the in vitro chemotaxis of these cells towards CXCL16, CCL27, and CCL28 chemokines. The results demonstrate that various concentrations of CXCL16 ranging between 0.1 and 100 ng/mL induced the chemotaxis of NK92 cells ($P < 0.03$). Incubation of NK92 with MMF or DMF did not significantly increase their migration towards CXCL16 (Fig. 1a).

NK92 cells also migrated toward only the highest concentration, i.e., 100 ng/mL of CCL27. Incubation with MMF highly induced their chemotaxis towards the 10 ng/mL CCL27 ($P < 0.0001$, as compared to the control). However, incubation with DMF did not significantly increase their chemotaxis (Fig. 1b). The effect of CCL28 on the chemotaxis of NK92 was also examined. The data showed that these cells do not migrate towards CCL28. In contrast, incubation with MMF induced their chemotaxis towards 1, 10, and 100 ng/mL CCL28, whereas incubation with DMF induced their chemotaxis towards only the 100 ng/mL CCL28 (Fig. 1c). Berahovich et al. [24] reported that NK92 do not migrate towards the concentration gradients of CCL27. Our results do not conflict with these findings, as the chemotaxis towards the CCR10 ligands is either very low as in the case of CCL27 or non-existent in case of CCL28. However, only after pretreatment with MMF or DMF, the cells significantly migrated towards these chemokines.

MMF and DMF upregulate the expression of chemokine receptors on NK92 cells

In a previous publication, we demonstrated that treatment of human IL-2-activated NK cells overnight with MMF or DMF upregulated the expression of CCR10 [14]. Here, we examined whether treatment of NK92 with MMF or DMF may result in a similar upregulation of CCR10, the receptor for chemokine ligands CCL27 and CCL28, or CXCR6, the receptor for chemokine ligand CXCL16. Results in Fig. 2a demonstrate that incubating NK92 cells with MMF or DMF upregulated the expression of the surface CCR10 on these cells. There was also a slight increase in the percentages of $CXCR6^+$ cells after incubation with MMF or DMF (Fig. 2a). Because CCL28 also binds CCR3 in addition to CCR10

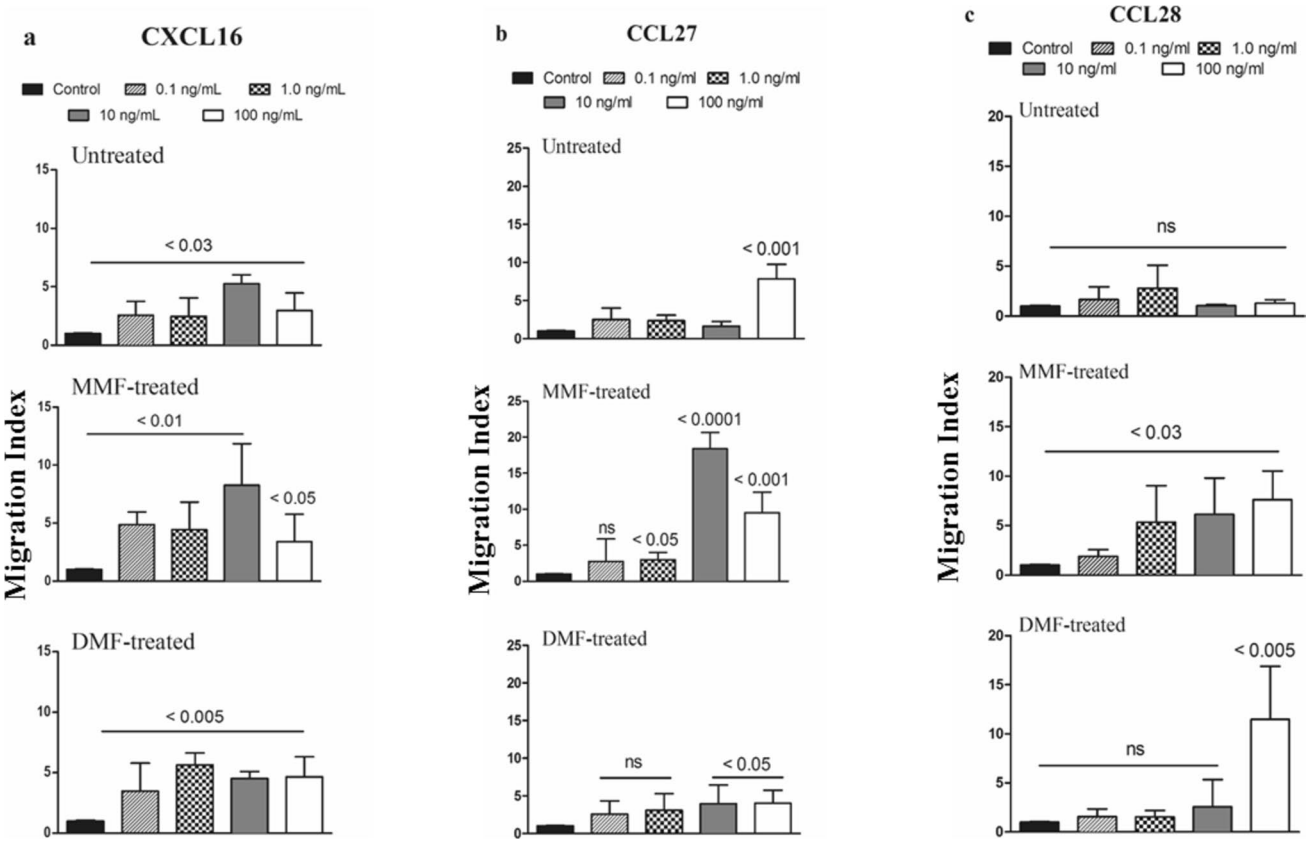

Fig. 1 Recombinant CXCL16, CCL27, and CCL28 induce the chemotaxis of NK92 cells. NK92 cells were either untreated or pretreated overnight with 100 µM MMF or 100 µM DMF. The cells were washed, and chemotaxis assay was performed using different concentrations of **a** CXCL16, **b** CCL27, and **c** CCL28. *P* values are placed above the columns comparing the migration of cells towards the chemokine and those migrating in the absence of chemokine (control, black columns)

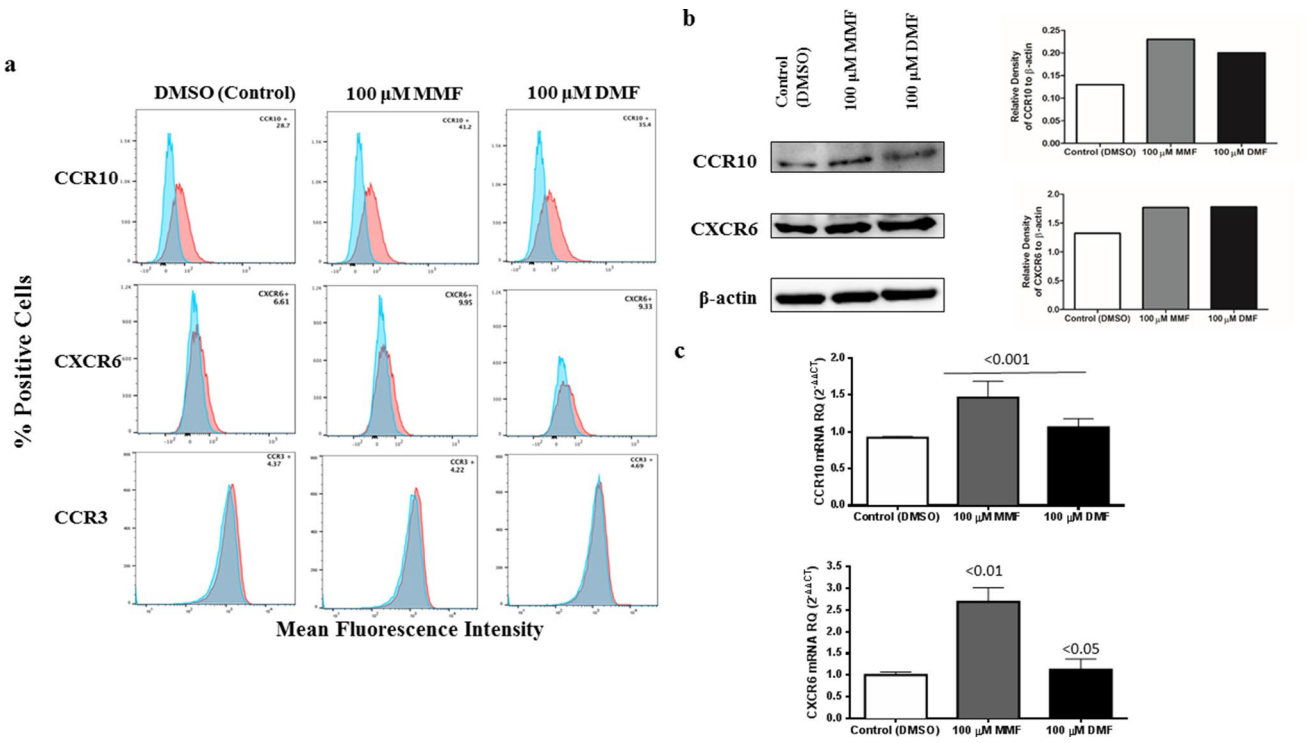

Fig. 2 Expression of CCR10 and CXCR6 levels using flow cytometry, western blot, and qRT-PCR. NK92 cells were either pretreated with DMSO, or with 100 µM of MMF or DMF. **a** NK92 cells were washed, and the expression of CCR10 or CXCR6 or CCR3 was examined using flow cytometry. Percentages of positive cells are shown on the *y*-axis and staining with isotype control antibodies is shown in blue. One of two representative experiments is shown. **b** Immunoblot analysis showing the protein levels of CCR10 and CXCR6 as compared to the level of the house-keeping β-actin. **c** qRT-PCR analysis showing the receptors' mRNA expression in NK92 cells after treatment with MMF or DMF as normalized to the house-keeping 18S

[16], we examined the effects of the drugs on CCR3 expression. The results demonstrate that only about 4% of NK92 express CCR3 and that pretreatment with MMF or DMF did not increase the percentages of $CCR3^+$ NK92 cells.

We also utilized western blot analysis and qRT-PCR to confirm the effects of MMF and DMF on the expression of CCR10 and CXCR6. The immunoblot results show that both MMF and DMF increased the expression of CCR10 (Fig. 2b), although MMF was a slightly better inducer when the relative expression of CCR10 and CXCR6 were compared to the level of β-actin (Fig. 2b). In qRT-PCR, it was clear that MMF highly increased the mRNA level of both CCR10 and CXCR6, as compared to the effect of DMF (Fig. 2c). Of note, mRNA of CXCR6 was higher after MMF treatment when compared to the protein level of this receptor, which could be the result of protein instability or post-translational modifications.

Supernatants collected from HCT-116 colorectal cancer cells induce the chemotaxis of NK92 cells

Although the above findings established the basis of NK92 chemotaxis, it was imperative to demonstrate whether colorectal cancer cells may be able to recruit NK cells towards these cells. To investigate this issue, we collected supernatants from HCT-116 cells after 24 or 48 h incubation, which were examined for their ability to induce the in vitro chemotaxis of NK cells. As shown, the 24 h supernatants used either undiluted or diluted at 1:1, 1:10, and 1:100 with RPMI significantly induced the chemotaxis of NK92 cells. Treatment with MMF enhanced the chemotaxis of NK92 cells towards the undiluted 24 h supernatants (Fig. 3a). However, incubation with DMF did not increase their migration above the background level. One-way ANOVA analysis Berlett's ad-hoc shows that incubation with MMF but not DMF significantly enhanced the chemotaxis of these cells ($P < 0.001$, Fig. 3a).

Similarly, supernatants collected from HCT-116 colorectal cancer cells after 48 h incubation induced the chemotaxis of NK92 cells when used either undiluted or when diluted 1:1, 1:10, and 1:100. However, incubating the cells with MMF or DMF did not significantly improve their chemotaxis beyond the background level induced by supernatants of cells untreated with the drugs (Fig. 3b). As a control, we used supernatants collected from the K562 cells, which are considered the prototype of NK cell-sensitive targets.

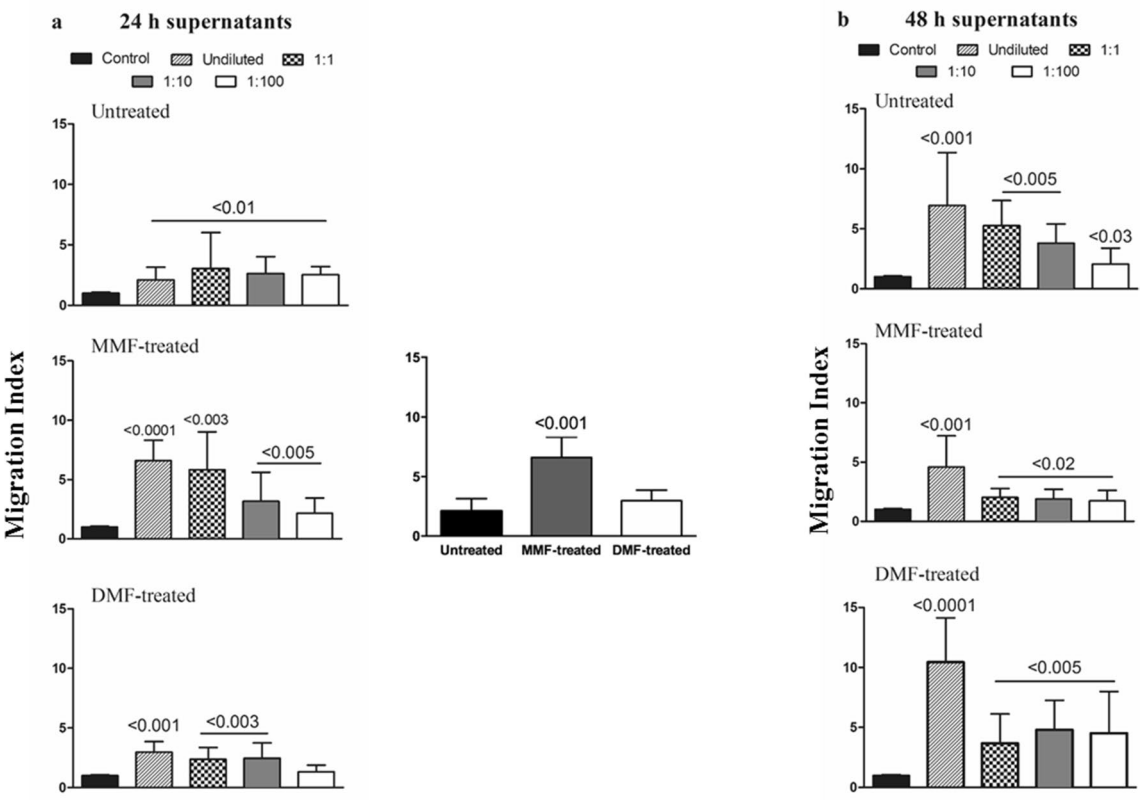

Fig. 3 Supernatants collected from HCT-116 induce the chemotaxis of NK92 cells. NK92 cells were either untreated or pretreated overnight with MMF or DMF. The cells were washed, and chemotaxis assay was performed using undiluted, 1:1, 1:10, or 1:100 diluted supernatants collected from HCT-116 colorectal cancer cells after **a** 24 h and **b** 48 h culture. *P* values are placed above the columns comparing the migration of cells towards the supernatant and those migrating in the absence of chemoattractants (control, black columns)

The results of these experiments indicate there is almost no chemotaxis of NK92 cells towards supernatants collected from K562 cells (Supplementary Fig. 2). These results support the bioinformatics analysis, showing that K562 express none of the three chemokines examined in this study.

To ascertain that the chemotactic effect exerted by the supernatants is related to the presence of chemokines in the 24 and 48 h supernatants, we incubated these supernatants with neutralizing antibodies. NK92 cells were placed in the upper wells of Boyden chambers, whereas undiluted 24 h HCT-116 supernatants were either left untreated or pretreated with isotype control antibody or with different concentrations (0.1 µg/mL or 1 µg/mL) of the neutralizing antibodies for each of the three chemokines 1 h before adding to the lower wells of the chambers. As shown in Fig. 4a, supernatants collected from HCT-116 cells after 24 h significantly induced the chemotaxis of NK92 cells. Isotype antibody did not affect this chemotaxis, and the cells robustly migrated towards the 24 h supernatant in the presence of this antibody. In contrast, pretreatment with 0.1 or 1 µg/mL of anti-CXCL16 significantly inhibited the chemotaxis of these cells as compared to pretreatment with isotype control antibody ($P < 0.03$ and $P < 0.01$, respectively, Fig. 4a upper panel). However, pretreatment with anti-CCL27 did not affect the chemotaxis (Fig. 4a, middle panel), suggesting that 24 h supernatants did not contain CCL27. On the other hand, 0.1 or 1 µg/mL of anti-CCL28 significantly inhibited the chemotaxis of NK92 towards HCT-116 cells 24 h supernatants ($P < 0.01$, and $P < 0.005$, respectively, Fig. 4a, lower panel).

Similarly, the 48 h supernatants either alone or in the presence of the isotype control antibody induced the chemotaxis of NK92 cells (Fig. 4b). Two concentrations of anti-CXCL16, anti-CCL27 or anti-CCL28, i.e., 0.1 or 1 µg/mL, significantly inhibited the chemotaxis of HCT-116 towards the 48 h supernatants, with the exception of the 0.1 µg/mL anti-CCL28 (Fig. 4b, lower panel), although this concentration was effective against the 24 h supernatants (Fig. 4b, lower panel). This discrepancy could be attributed to the differential migration index among the two supernatants (compare lower panels in Fig. 4a and b). These results suggest that 48 h supernatants collected from HCT-116 cells contain CXCL16, CCL27, and CCL28.

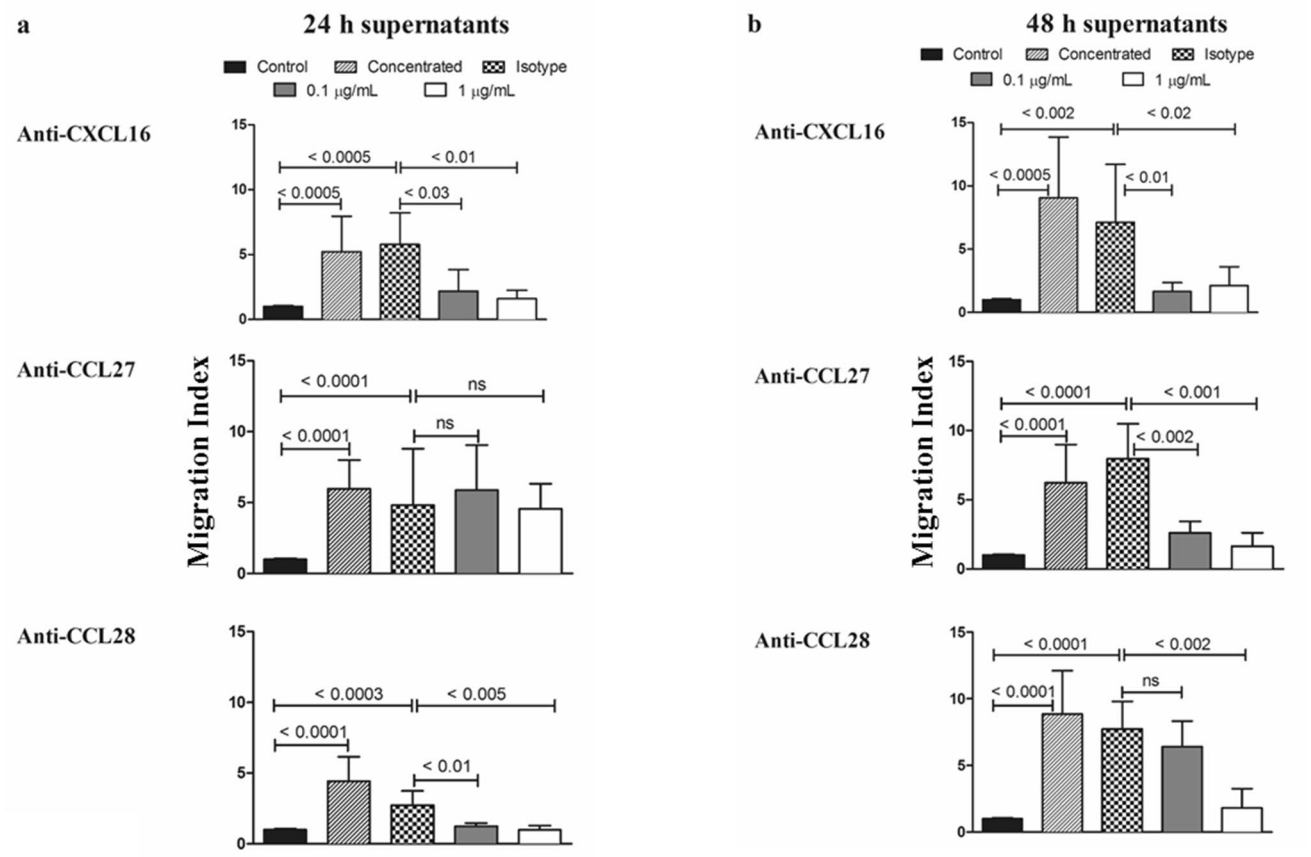

Fig. 4 Neutralizing anti-CXCL16, anti-CCL27, and anti-CCL28 inhibit the migration of NK92 cells. Using Boyden chambers, NK92 cell migration was assessed where undiluted supernatants were incubated with neutralizing antibodies for CXCL16, CCL27, and CCL28 (0.1 μg/mL or 1 μg/mL). **a** Chemotaxis assay was performed utilizing 24 h supernatants collected from HCT-116. **b** This is similar to panel **a** except the supernatants obtained from HCT-116 cells after 48 h incubation were used. *P* values are placed above the columns comparing the migration of cells in the presence of supernatants, either alone or pretreated with isotype control antibody to the migration in the absence of supernatants (control, black columns)

To confirm the presence of chemokines in the supernatants collected from HCT-116 cells, we measured the levels of the three chemokines by ELISA assay. Our results demonstrate that HCT-116 supernatants collected after 24 h contain CXCL16 and CCL28 but not CCL27, whereas the 48 h supernatants contain all three chemokines, i.e., CXCL16, CCL27, and CCL28 (Fig. 5a, respectively). These results support the chemotaxis results in utilizing neutralizing antibodies.

DMF and MMF potentiate NK92 lysis of HCT-116 colorectal cancer cells

Furthermore, we examined whether MMF and DMF might have any effect on in vitro NK92 cell lysis of HCT-116. Pretreatment of NK92 with MMF significantly enhances NK92 cell killing of HCT-116 cells ($P < 0.01$, Fig. 5b). Mean ± SEM of four experiments was calculated. Although there was a trend of increased cytotoxicity after the DMF treatment, this did not reach statistical significance.

Recombinant chemokines and supernatants collected from HCT-116 colorectal cancer cells stimulate the mobilization of intracellular Ca^{2+} in NK92 cells

It is known that there is a strong association between chemotaxis and the mobilization of intracellular calcium [25]. To confirm such principles in NK92 cells, we assessed the mobilization of intracellular Ca^{2+}. First, we used Triton-X as a control to induce the release of intracellular calcium and EGTA to chelate this response, as previously shown [26]. Next, we examined two different concentrations of CXCL16, CCL27, and CCL28, namely 10 and 100 ng/mL, and observed that the 100 ng/mL concentration of the three chemokines induced the mobilization of $(Ca^{2+})_i$; however, only the 10 ng/mL concentration

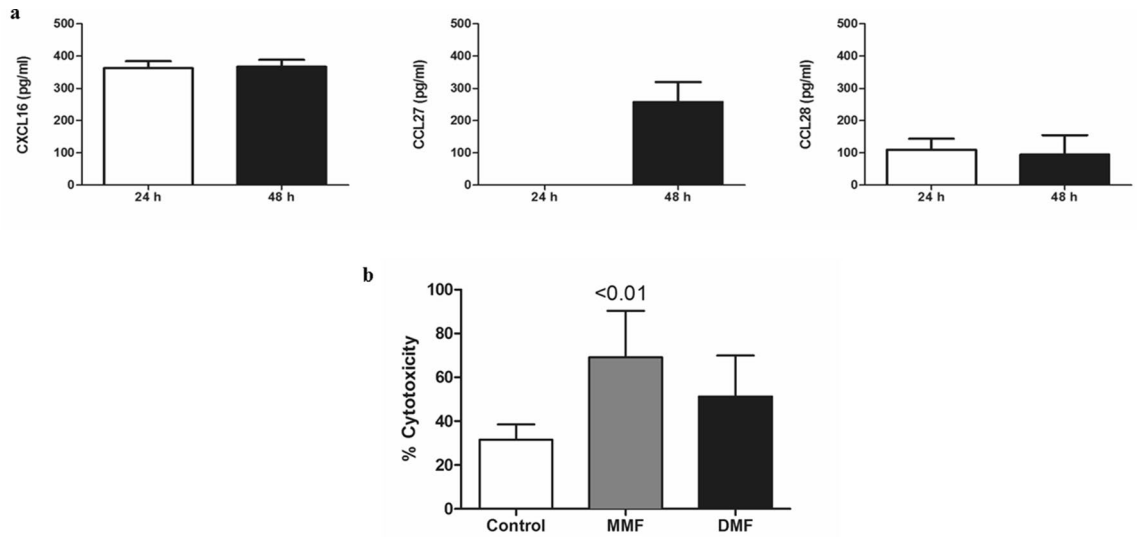

Fig. 5 Measurement of chemokine levels in HCT-116 supernatants, and influence of DMF or MMF on NK-cell cytotoxicity. **a** The levels of CXCL16, CCL27, and CCL28 were measured in the supernatants of HCT-116 collected after 24 h or 48 h using ELISA assay. **b** NK92 cells were pretreated with either DMSO, 100 μM MMF or 100 μM DMF overnight, after which the cells were incubated with HCT-116 at 10:1 E:T ratio for 4 h. *P* values are shown to compare the percent cytotoxicity of each treatment with the control groups

of CCL28 produced a positive response (Supplementary Fig. 3). Intriguingly, the undiluted, 1:1 dilution, or 1:10 dilution of supernatants collected after 24 or 48 h from HCT-116 cells also provoked the mobilization of $(Ca^{2+})_i$ in NK92 cells (Supplementary Fig. 3).

To verify whether the supernatants collected from HCT-116 may utilize similar receptors to that of recombinant chemokines, we performed desensitization experiments. To gain insight into this issue, fura-2-AM-labeled NK92 cells were incubated with undiluted supernatants collected from HCT-116 cells after 24 h or 48 h incubation. The results indicate that addition of 24 h supernatants entirely desensitized the effects of the 24 h supernatants, the CXCL16 and CCL28, but there were a partial desensitization of the 48 h supernatants and no effect on CCL27 (Fig. 6a). However, addition of the 48 h supernatants desensitized the calcium mobilization effects of the 48 h supernatants, 24 h supernatants, CXCL16, CCL27, and CCL28 (Fig. 6a). We also performed the reciprocal experiments where recombinant chemokines were added prior to the addition of the supernatants. CXCL16 desensitized CXCL16 activity but not the activity of the supernatants (Fig. 6b). Similarly, CCL27 and CCL28 desensitized CCL27 and CCL28 activity, respectively, but not the supernatants with the exception where CCL28 partly desensitized the effect of 24 h supernatants (Fig. 6b). These results confirm that 24 h supernatants contain CXCL16 and CCL28, whereas 48 h supernatants contain all the three chemokines examined.

Discussion

There are currently more than 900 approved protocols utilizing human NK cells or NK92 cells for treatment of cancer patients. Among the many obstacles, utilizing NK cells as a therapeutic modality is the insufficient migration of these cells towards the sites of tumor growth. Chemokines are highly chemoattractant molecules for NK cells [4]. However, it is not known whether tumor cells might secrete chemokines that attract NK cells. NK cells isolated from colorectal cancer patients are suppressed and are inefficient killers [5]. Hence, it is imperative to devise strategies where NK cells are harnessed in vitro before using them for therapy. The purpose of this paper is to find a procedure of harnessing NK cells and to seek the possibility that colorectal cancer cells might secrete chemokines that could facilitate the recruitment of NK cells. In this report, we focused our efforts on three chemokines, namely CXCL16, CCL27, and CCL28 due to the bioinformatics analysis, showing that the colorectal cancer cell line HCT-116 expresses these three chemokines. Our choice of HCT-116 was based on the previous studies, showing that HCT-116 cells could be targeted by NK cells [27, 28]. It was also reported that the use of HCT-116 in an in vivo model aids in understanding the molecular mechanisms involved in the metastatic phase of colorectal cancer [29].

One of the chemokines examined CCL27 (CTACK) plays a vital role in the tumor microenvironment. It was

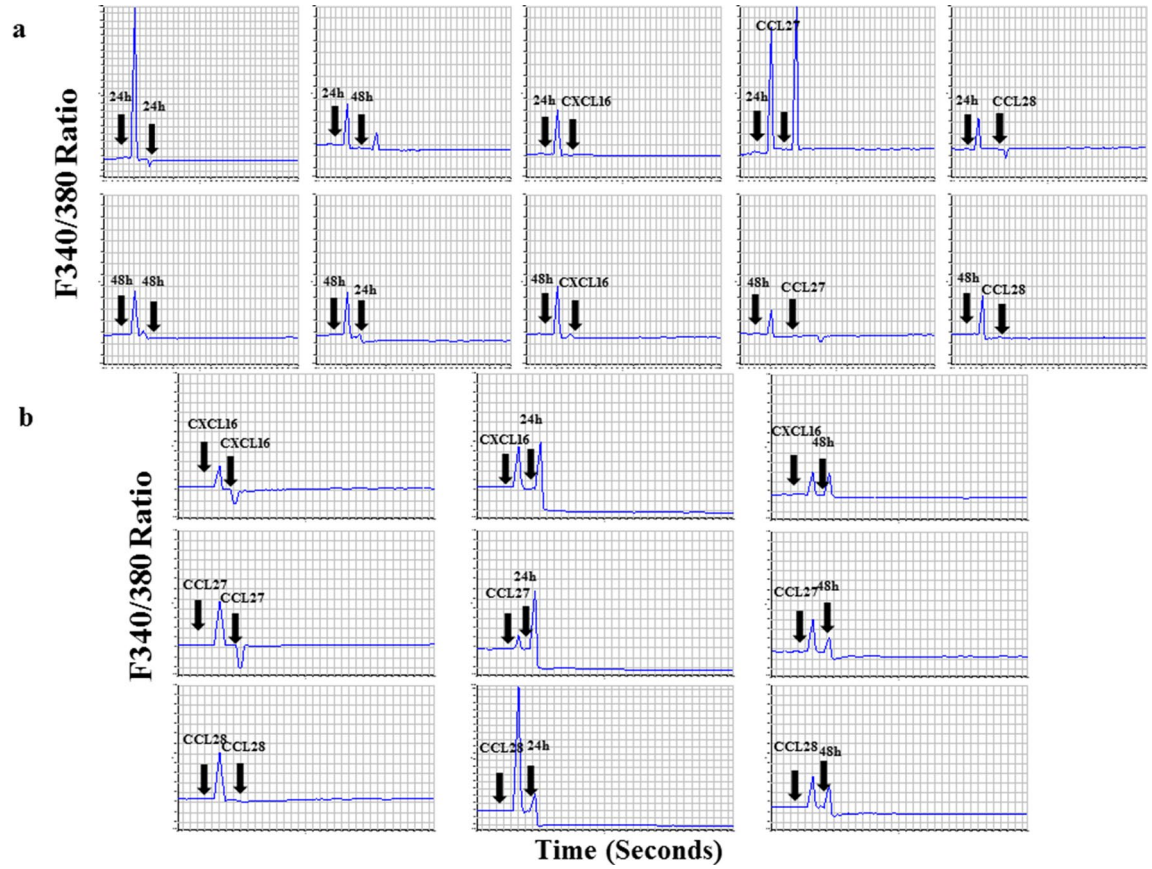

Fig. 6 Desensitization of chemokine receptors with recombinant chemokines and HCT-116 supernatants. **a** Fura-2-AM-labeled NK92 cells were incubated with 24 h or 48 h undiluted supernatants collected from HCT-116 cells prior to the addition of 24 h supernatants, 48 h supernatants, CXCL16, CCL27, or CCL28. **b** Reciprocal experiments were performed where recombinant chemokines (CXCL16, CCL27, and CCL28) were added prior to the addition of the respective chemokine or the supernatants. Calcium mobilization was determined by measuring the ratio of F340/380. Vertical arrows indicate the time of addition. This figure is representative of more than four performed experiments

reported that T-cell recruitment and activation at tumor sites occurred upon administration of CCR10 intra-tumorally [30]. In addition, a reduction in tumor growth by immune responses was noted upon transfection of CCL27 into ovarian carcinoma cells [31]. Likewise, neutralization of CCL27 caused a decline in leukocyte migration to cutaneous tumor sites, thus promoting tumor growth [32].

On the other hand, CCL28 which also binds CCR10 is upregulated in inflamed tissues [33] and is suggested to bridge the innate and adaptive immune responses [34]. Similar to CCL27, there could be different levels of CCL28 in different areas of the tumor microenvironment. For example, Dimberg et al. [16] demonstrated that CCL28 levels are higher in the plasma than the rectum of the same patients with colon cancer. In addition, CCL28 released by hypoxic tumor cells was found to employ Treg cells expressing the CCR10 receptor, subsequently leading to tumor growth and angiogenesis where this activity was abolished by anti-CCL28 [35].

Our results indicating that NK92 cells upregulate the expression of CCR10 and migrate towards CCL27 and CCL28 after stimulating with DMF or MMF, may provide an explanation to these contradictory findings, and may give an impetus of harnessing these cells for therapeutic purposes. Furthermore, and in accordance with their effects on IL-2-activated NK-cell lysis of tumor cells [14], treatment of MMF or DMF enhanced NK92 lysis of HCT-116 cells. These findings support others, showing that NK cells express CCR10 and are accumulated inside tumors after intra-tumoral injection of CCL27-encoding fiber-mutant vector [36].

It is also intriguing that colorectal cancer cell supernatants contain CCL27 and CCL28 that stimulate the chemotaxis of NK92 cells and induce their mobilization of intracellular calcium. Calcium fluxes are an important initial step before cells start to polarize towards the concentration gradients of any chemoattractant [25]. For this purpose, we investigated whether supernatants collected from HCT-116 cells

might induce intracellular calcium fluxes in NK92 cells. The results clearly demonstrate that supernatants collected from these colorectal cancer cells induced the mobilization of intracellular calcium, an activity that sometimes exceeded those activities of the recombinant chemokines. This activity is plausible, because the supernatants contain more than one chemokine and the collective actions are exerted on several chemokine receptors such as CXCR6 and CCR10. This is confirmed by the observations, showing that addition of 24 or 48 h supernatant prior to the addition of CCL27 or CCL28 abolished the response of CCL27 and CCL28, except for the activity of CCL27 which induced calcium fluxes when added after the 24 h supernatant. These results support those reported in the chemotaxis assay, showing that 24 h supernatant collected from the colorectal cancer cells contains only CCL28 (and CXCL16), but not CCL27. In contrast, the cells secrete all three chemokines after 48 h culture.

On the other hand, CXCL16 is a key factor in the pathogenesis of acute kidney injury [37] as well as cardiovascular injury [38]. CXCL16 level is also increased in the lungs of cancer patients [39]. Lang et al. [40] observed that CXCL16 levels are high in patients with urothelial carcinoma and that these cells may shed this chemokine and hence, evade immune detection. Both CXCL16 and CXCR6 were increased and are positively correlated with late stages of non-small cell lung cancer patients [41]. On the other hand, blocking the complement 5a with its receptor decreased lung bone metastases by CXCL16-mediated activity [42]. However, only sporadic reports described the expression of CXCR6 in NK cells and the effect of its ligand CXCL16 on these cells. Yoon et al. [43] showed CXCR6 expression on activated NK cells and correlated it with their migration towards breast cancer cell lines which highly secrete CXCL16 after irradiation. Such expression may facilitate eradicating breast cancer cells. In addition, it was reported that resident liver NK cells express CXCR6 which aids them in localizing inside this organ [44]. Finally, Hojo et al. reported that colorectal cancer cells secrete CXCL16 which recruit $CD4^+$ and $CD8^+$ T cells [45], but the effect on NK cells is not known.

Here, we report that CXCR6 is expressed in about 6% of NK92 cells and that MMF or DMF slightly increased the percentages of $CXCR6^+$ NK cells, as well as increasing the mRNA level of this receptor, particularly after the MMF treatment. Intriguingly, NK92 cells highly migrated towards the concentration gradients of CXCL16, suggesting that the low percentages of $CXCR6^+$ NK92 cells are highly motile. Furthermore, supernatants collected after 24 and 48 h from colorectal cancer cells induced the chemotaxis of these cells. The effect of the supernatants was as effective, if not more robust than recombinant chemokines. Neutralizing anti-CXCL16 inhibited the chemotaxis of these cells suggesting that HCT-116 cells secrete CXCL16 at both time points. These results were confirmed using the calcium mobilization assay, where the addition of 24 or 48 h supernatants completely desensitized the activity of recombinant CXCL16, suggesting that they use a similar receptor. On the contrary, adding CXCL16 prior to the addition of the supernatants only partially inhibited the supernatant activity. This is most probably due to the effects of the supernatants on CCR10 in addition to CXCR6. These observations were ascertained by the ELISA assay, where the CXCL16 levels were measured in the supernatants of HCT-116 collected after 24 or 48 h incubation.

In summary, tumor microenvironment and particularly colorectal cancer cell microenvironment plays an important role in the anti-tumor effector cells. It appears that HCT-116 colorectal cancer cells secrete chemokines that attract effector cells towards the sites of tumor growth permitting them to perform their anti-tumor activity. The question arises as to why tumor cells attract these highly anti-tumor effector cells. Although the answer to this question is complicated, it is plausible that tumor cells might deplete NK cells from the circulation and sequester them in the tumor microenvironment as an attempt to suppress the NK-cell activity, similar to the effects of lysophospholipids that recruit NK cells and suppress their cytolytic activity [reviewed in 46]. In this regard, NK cells should be armed to become highly lytic before they accumulate at tumor sites. Molecules such as MMF or DMF, among others, should be used to harness NK cells before utilizing them for therapy. Although pretreatment with MMF or DMF did not significantly increase the chemotaxis of NK92 towards the 48 h supernatants, it was clear that pretreatment with MMF enhances their chemotaxis towards the 24 h supernatants. The precise knowledge of cancer microenvironment is not clear yet. Whether cancer cells secrete factors such as chemokines after they metastasize or at any other point during their growth is not understood. Furthermore, it is not known whether these factors might travel a distance and, hence, become diluted in the microenvironment milieu or function in close proximity.

Further enigma is the lack of knowledge of how long it would take tumor cells to secrete these factors after establishing metastatic lesions. Our results are essential in understanding, at least in vitro, the cross-talk among tumor and anti-tumor cells. Until we fully understand all the factors influencing this cross-talk, we suggest, at this juncture, that NK92 cells should be harnessed by in vitro pretreatment with MMF or DMF to enhance their migration towards the sites of tumor growth. These molecules not only regulate NK cells migration, but also augment their cytolysis of tumor cells.

Author contributions NME performed most of the experiments and wrote the paper; ZAJ performed the calcium assays; MYH performed the bioinformatics and qRT-PCR; AAM designed the experiments, performed the statistical analysis, and wrote the manuscript.

Funding This work was supported by the University of Sharjah Grants with numbers 1701090222-P and 1701090223-P.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.
The work described in this paper was performed using commercially available cell lines. This article does not contain any studies involving patients or experimental animals. Therefore, no study approval was required and no informed consent from the donors.

Cell line authentication The human natural killer cell line NK92 (CRL-2407), colorectal cancer cell line HCT-116 (CCL-247), and the erythroleukemia K562 (CCL-243) were obtained from the American type culture collection (ATCC, Manassas, VA, USA). No cell line authentication was necessary.

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