**Attenuation of natural killer cell functions by capsaicin through a direct and TRPV1-independent mechanism**

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The assessment of the biological activity of capsaicin, the compound responsible for the spicy flavor of chili pepper, produced controversial results, showing either carcinogenicity or cancer prevention. The innate immune system plays a pivotal role in cancer pathology and prevention; yet, the effect of capsaicin on natural killer (NK) cells, which function in cancer surveillance, is unclear. This study found that capsaicin inhibited NK cell-mediated cytotoxicity and cytokine production (interferon-γ and tumor necrosis factor-α). Capsaicin impaired the cytotoxicity of NK cells, thereby inhibiting lysis of standard target cells and gastric cancer cells by modulating calcium mobilization in NK cells. Capsaicin also induced apoptosis in gastric cancer cells, but that effect required higher concentrations and longer exposure times than those required to trigger NK cell dysfunction. Furthermore, capsaicin inhibited the cytotoxicity of isolated NK cells and of an NK cell line, suggesting a direct effect on NK cells. Antagonists of transient receptor potential vanilloid subfamily member 1 (TRPV1), a cognate capsaicin receptor, or deficiency in TRPV1 expression failed to prevent the defects induced by capsaicin in NK cells expressing functional TRPV1. Thus, the mechanism of action of capsaicin on NK cells is largely independent of TRPV1. Taken together, capsaicin may have chemotherapeutic potential but may impair NK cell function, which plays a central role in tumor surveillance.

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**Introduction**

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the principal component of hot chili pepper, is among the most widely consumed spices in the world. An analgesic and anti-inflammatory agent used topically in neuropathic pain (1,2), it is a selective agonist of transient receptor potential vanilloid subfamily member 1 (TRPV1), which mediates many of its physiological effects (2,3). Recent studies also demonstrated that capsaicin exerts diverse biological effects through mechanisms distinct from TRPV1 receptor and sensory nerves (4–6).

Despite studies suggesting diverse therapeutic potentials, the benefit and safety of capsaicin consumption or medical use remains unclear, particularly in the context of carcinogenesis (7). Conflicting studies revealed that capsaicin may play a role in preventing or promoting cancer (7). It inhibits the growth of leukemia, hepatoma, glioblastoma and colon cancer cells through cell cycle arrest or apoptosis (8). In contrast, it is also a cocarcinogen and tumor promoter (7,9); capsaicin from chili extract promotes stomach and liver cancer in carcinogen-induced rodent tumor models (10,11), and epidemiologic studies suggest that consumers of large amounts of chili pepper are at high risk of stomach and gallbladder cancer (12–14). The focus of previous studies was to elucidate the interaction between capsaicin and cancer cells and to understand the cancer-modulating effect of capsaicin. In this context, studying the effect of capsaicin on the function of the immune cells that mediate the antitumor response is relevant, given the role of the immune system in tumor surveillance.

Natural killer (NK) cells take part in cancer surveillance (15,16). They are major effectors in the elimination of cancer cells through direct lysis and cytokine production, e.g. interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and contribute indirectly to antitumor defense by regulating antigen-presenting cells and adaptive T-cell responses. NK cells rely on a variety of germ line-encoded activating and inhibitory receptors to distinguish cancer cells from normal healthy cells (15,17). Thus, the capacity of NK cells to kill cancer cells is determined by a signal balance between activating and inhibitory receptors. Animal studies showed that depression of NK cell activity leads to high tumor incidence and metastasis (18,19). Moreover, patients suffering from various solid tumors, including gastric cancer, show defects in NK cell effector function (20,21), and the degree of NK cell dysfunction correlates with clinical prognosis (22,23).

This study aimed to assess the effect of capsaicin on NK cell function and its underlying mechanism. No such study has ever been conducted. Results showed that capsaicin attenuates the susceptibility of cancer cells to NK cells through a direct but largely TRPV1-independent suppression of NK cell function, thus cautioning against the consideration of capsaicin as a cancer preventive agent.

**Materials and methods**

**Reagents and antibodies**

Capsaicin, capsazepine (CPZ) and SB366791 (SB) were purchased from Tocris (Bristol, UK), Rp-SB-cAMP and AG1478 from Calbiochem (Darmstadt, Germany) and epidermal growth factor (EGF) from Sigma–Aldrich (St Louis, MO). The following monoclonal antibodies were used: anti-human CD3-PerCP (SK7), anti-human CD56-R-phycocerythrin (PE) (NCAM16.2), anti-human CD107a-FITC (1D4B), anti-mouse IFN-γ-FITC (XMG1.3) and anti-mouse CD16/CD32 (mouse Fc Block; 2.4G2) from BD Biosciences (San Jose, CA) and anti-human TNF-α-FITC (MAB11) from eBioscience (San Diego, CA). Rabbit anti-human TRPV1 polyclonal antibody (ACC-030) was from Alomone Labs (Jerusalem, Israel). Antibodies for p-EGFR, EGFR, p-ERKs and p-ERKs from Cell Signaling (Boston, MA). Anti-rabbit antibody conjugated to horseradish peroxidase was from Santa Cruz (Dallas, TX). Annexin-V-FITC and propidium iodide (PI) were from BD Biosciences.

**Cell isolation and culture**

Human blood samples from healthy donors were drawn for research purposes under a protocol approved by the Asan Medical Center Institutional Review Board with informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (MP Biomedicals, Solon, OH). Human NK cells were purified from PBMCs by negative selection using an NK cell isolation kit (StemCell Technologies) as described (24).

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1652
Capsaicin-induced NK cell dysfunction

NK cell dysfunction was assessed by cell surface expression of CD107a as described (25). For stimulation, resting IL-2-activated PBMCs or purified NK cells were stimulated with an equal number of K562 or 221 cells. Splenocytes from WT or TRPV1 KO mice were stimulated with Y AC-1 target cells for 4 h. Lymphocytes were gated on forward scatter/side scatter, and the CD107a expression of CD3-CD56+ human NK cells and CD3-NKp46+ mouse NK cells was analyzed by flow cytometry.

Intracellular cytokine assay

Cytokine production by NK cells was assessed by the intracellular expression of IFN-γ and TNF-α as described (26). PBMCs were stimulated with an equal number of K562 or 221 cells for 1 h at 37°C. Thereafter, brefeldin A (GolgiPlug; BD Biosciences) and monensin (GolgiStop; BD Biosciences) were added and the cells were incubated for an additional 5 h, making a total incubation time of 6 h.

NK cell cytotoxicity assay

The cytotoxicity of NK cells against sensitive target cells was assessed by europium-based cytotoxicity assay as described (24). Primary NK cells and NKL cells served as effector cells.

Western blot analysis for TRPV1

Cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM ethylenediaminetetraacetic acid, 1 mM NaVO₃, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche)) for 30 min on ice. Protein concentration of cell lysates was measured with the bichinchoninic acid protein assay kit (Pierce). Equal amounts of protein for each sample were resuspended in NuPAGE LDS sample buffer (Invitrogen) containing 50 mM dithiothreitol and boiled for 10 min at 70°C. The samples were resolved on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred onto polyvinylidene difluoride membrane (Millipore) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol]. Membranes were blocked with 5% skim milk in TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h at room temperature and incubated with primary anti-TRPV1 antibody (ACC-030, 1:500, Alomone Labs) overnight at 4°C and then with the horse-radish peroxidase-conjugated secondary antibody (sc-2301, 1:5000, Santa Cruz) for 1 h at room temperature. Blots were developed with SuperSignal West Pico (Pierce) and signals were detected with LAS-4000 (Fujifilm).

Measurement of intracellular Ca²⁺

Human NK and U87 glioma cells were loaded with fluo-4 AM (Invitrogen; 2 μg/ml) in Hanks’ balanced salt solution supplemented with 1% FBS and probenecid (Sigma; 4 mM) for 30 min at 37°C. After washing, cells were plated onto poly-l-lysine-coated coverslips and transferred to a recording chamber (Live Cell Instruments) positioned on an inverted microscope (Carl Zeiss LSM710). Changes in intracellular Ca²⁺ concentration were measured over time after addition of capsaicin and ionomycin (4 μM). Ca²⁺ responses in mouse dorsal root ganglion (DRG) neurons by capsaicin were included as positive controls. Intracellular Ca²⁺ flux was also measured by flow cytometry in cells labeled with 2 μg/ml fluo-4 AM as described (24).

Reverse transcription–polymerase chain reaction

To determine TRPV1 expression in human and mouse NK cells, total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized from 1 μg RNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to manufacturer’s protocols. The following PCR primers were used: 5′-GAT CCT TGC AGT AAC TCG GA-3′ (reverse) for human TRPV1 and 5′-TCA CGG TCA GCT CTG TTG TC-3′ (forward) and 5′-GGG TCT TTG AAC TCG CTG TC-3′ (reverse) for mouse TRPV1.

Results

Capsaicin attenuates the natural cytotoxicity and cytokine production of NK cells

To determine whether capsaicin affects NK cell function, we assessed the effect of capsaicin on peripheral blood NK cell degranulation (indicated by CD107a positivity) upon stimulation with K562 or 221 target cells. Target cell lysis by NK cells correlates with NK degranulation efficiency, and CD107a appears on the surface of NK cells during degranulation (25). PBMCs were isolated from healthy donors and incubated with target cells for 2 h after pretreatment with capsaicin for 1 h. NK cells were identified as CD3-CD56+ among size-gated lymphocytes. As shown in Figure 1A and C, 10–20 μM capsaicin reduced the degranulation of NK cells slightly. This inhibition was statistically significant after stimulation with K562 cells (Figure 1B) but not 221 cells (Figure 1D). Of note, 50–100 μM capsaicin impaired the degranulation of NK cells potently against both target cells (Figure 1B and D). Capsaicin did not affect NK cell viability for 3 h, assessed by annexin-V/PI staining (Supplementary Figure S1, available at Carcinogenesis Online) and trypan blue exclusion (data not shown), thus ruling out toxicity of capsaicin to NK cells during the time frame of degranulation assay. However, significant increase in NK cell apoptosis was detected upon exposure to 50–100 μM capsaicin for 24 h, showing exposure time-dependent toxicity of capsaicin to NK cells.

Next, we assessed the effect of capsaicin on NK cell proinflammatory cytokine production. The intracellular expression of IFN-γ and TNF-α was assessed in CD3-CD56+ NK cells upon stimulation with target cells. As observed with NK cell degranulation, NK cell production of IFN-γ and TNF-α was reduced modestly by capsaicin at low concentrations (10–20 μM) and potently at high concentrations (50–100 μM) (Supplementary Figure S2, available at Carcinogenesis Online). These results suggest that capsaicin attenuates the natural cytotoxicity and cytokine production of NK cells; such NK cell dysfunction was evident at high concentrations of capsaicin but could also be observed at low concentrations.

Capsaicin inhibits the cytolytic activity of NK cells in a direct manner

We investigated whether capsaicin caused NK cell dysfunction directly or indirectly via capsaicin-sensitive non-NK cells in PBMCs. We employed both the NK cell line NKL and highly purified primary NK cells. As observed in PBMCs, exposure of NKL cells to capsaicin caused a significant impairment in NK cell cytotoxicity; the effect was potent at high concentrations (50–100 μM) and modest at low concentrations (10–20 μM) (Figure 2A). To determine whether the observation made in NKL cells was applicable to primary NK cells, similar experiments were performed using purified NK cells negatively selected from PBMCs. As expected, capsaicin impaired degranulation (Figure 2B and C). The impairment was more pronounced at high concentrations (50–100 μM). Although the degree of NK cell dysfunction caused by capsaicin varied by donor, reproducible and consistent concentration-dependent inhibition of NK cell function was observed. These results suggest that capsaicin attenuates NK cell function through a direct mechanism.
Capsaicin attenuates the susceptibility of gastric cancer cells to NK cells

Capsaicin has potential value in cancer treatment owing to its ability to trigger apoptosis in cancer cells (2,8). To assess the effectiveness of capsaicin as a cancer preventive agent in our experimental setting, we measured the effect of capsaicin on cancer cell viability and compared, for each cancer cell population, sensitivity to capsaicin and susceptibility to NK cells. Given that the gastrointestinal tract is a site of frequent exposure to capsaicin and given the increased risk of gastric cancer among high chili pepper consumers, we chose gastric cancer cells as a model in which to study the cancer-modulating potential of capsaicin. The gastric cancer cell lines AGS, MKN45 and Hs746T were incubated with capsaicin for 5 or 24 h, and apoptosis was measured by annexin-V/PI staining. MKN45 and Hs746T cells underwent apoptosis upon exposure to 100 μM capsaicin for 24 h, were only slightly apoptotic upon exposure to 50 μM capsaicin and not apoptotic at low concentrations (10–20 μM) (Supplementary Figure S3, available at Carcinogenesis Online). AGS cells were resistant to capsaicin and did not undergo apoptosis. No increase in apoptosis was detected in gastric cancer cells upon exposure to capsaicin for 5 h, which is the time frame of the NK cell cytotoxicity assay. In control experiments, apoptosis was detected after staurosporine treatment of gastric cancer cells.

Next, we investigated whether capsaicin affects the susceptibility of gastric cancer cells to NK cells independently of its ability to induce apoptosis. As shown in Figure 3, NK cells responded to all gastric cancer cells by triggering cytotoxic degranulation, which was significantly attenuated in the presence of AGS and Hs746T cells by 50 μM capsaicin but not by lower concentrations. To dissect the effect of capsaicin on NK and cancer cells, gastric cancer cells were directly treated with 10–50 μM capsaicin and then incubated with NK cells without capsaicin. Unlike the results from capsaicin-treated NK cells, the susceptibility of gastric cancer cells to NK cells was not affected by capsaicin treatment of cancer cells (Supplementary Figure S4A, available at Carcinogenesis Online) despite some cocarcinogenic signaling such as EGFR-dependent ERK activation in capsaicin-treated AGS cells (Supplementary Figure S4B and C, available at Carcinogenesis Online). Thus, capsaicin-induced NK cell dysfunction appears to be primarily attributed to the direct effect of capsaicin on NK cells.

In conclusion, although capsaicin induces apoptosis in a subset of gastric cancer cells, the effect requires a higher concentration and longer exposure time than what is required to trigger NK cell dysfunction. These results indicate that capsaicin has contrasting effects on cancer development: capsaicin is directly cytotoxic to cancer cells but inhibits cancer surveillance by NK cells.

NK cells express functional TRPV1 receptor

TRPV1 is a cognate receptor for capsaicin, and its expression was detected in some immune cell types but not in NK cells (3,27,28). To determine whether TRPV1 is involved in capsaicin-induced NK cell dysfunction, we assessed the expression of TRPV1 in NK cells. Western blot analysis was performed using specific anti-TRPV1
Capsaicin-induced NK cell dysfunction

Results revealed a band with a molecular weight of ~95 kDa corresponding to the size of the TRPV1 protein (Figure 4A). To compare the expression level of TRPV1 in NK cells to that observed in other cell types reported to express TRPV1 protein, we included PBMCs, U87 glioma cells and mouse brain tissues (stem and spinal cord) (27,29,30). The expression level of TRPV1 in NK cells was comparable with that observed in PBMCs and U87 glioma cells but was much lower than that observed in mouse brain tissues. The expression of TRPV1 in primary NK and NKL cells was also assessed at the messenger RNA level and found to be comparable with that of PBMCs but lower than that of U87 glioma cells (Figure 4B). We next assessed TRPV1 functionality. Given that stimulation of TRPV1 by capsaicin leads to Ca\(^{2+}\) mobilization in neuronal cells, we conducted calcium imaging studies. Compared with vehicle control, 10 μM capsaicin evoked a weak but clear increase in intracellular Ca\(^{2+}\) in NK cells loaded with fluo-4 (Figure 4C). There was no further effect of capsaicin at higher concentration (50 μM) on Ca\(^{2+}\) response, suggesting an already maximal response; however, the capsaicin-induced Ca\(^{2+}\) response could be blocked by prior treatment with the TRPV1 antagonist SB (10 μM) or CPZ (1 μM) (Supplementary Figure S5, available at Carcinogenesis Online), suggesting an involvement of TRPV1 in the response. Treatment with SB or CPZ alone did not evoke Ca\(^{2+}\) response. To characterize the capsaicin-induced Ca\(^{2+}\) response in NK cells, similar experiments were performed in U87 glioma cells and mouse DRG neurons. Compared with NK cells, the capsaicin-induced
Ca\textsuperscript{2+} influx was slightly larger in U87 glioma cells (Figure 4D) and, as expected, was robust in DRG neurons (Figure 4E). In comparison, the Ca\textsuperscript{2+} ionophore ionomycin induced robust Ca\textsuperscript{2+} mobilization. These results suggest functional expression of TRPV1 in NK cells and the association of capsaicin-induced Ca\textsuperscript{2+} response with TRPV1 expression level.

**Capsaicin-induced NK cell dysfunction is largely independent of TRPV1**

The functional expression of TRPV1 in NK cells prompted us to evaluate the requirement of TRPV1 in capsaicin-induced NK cell dysfunction. We investigated whether the impairment of NK cell function by capsaicin could be prevented by simultaneous exposure to specific TRPV1 antagonists, SB or CPZ. As shown in Figure 5A and B, 50 μM capsaicin significantly impaired the degranulation of NK cells against K562 cells; this effect was not prevented by prior treatment with increasing concentrations of SB up to 10 μM. Although we observed some additive effects of SB with capsaicin on NK cell degranulation, the statistical significance was not consistent. Treatment with SB alone did not significantly affect the degranulation of NK cells. Similar NK cell degranulation against K562 cells was observed using CPZ and against 221 cells using both CPZ and SB (Figure 5C and D). Thus, blockade of TRPV1 receptor by specific antagonists fails to prevent capsaicin-induced NK cell dysfunction.

To determine directly whether TRPV1 is required for capsaicin-induced NK cell dysfunction, we employed a mouse strain with a genetic deficiency in TRPV1 (Supplementary Figure S6, available at Carcinogenesis Online). Splenocytes were prepared and incubated with YAC-1 cells for 4 h after a 1-h pretreatment with 10–100 μM capsaicin. Spleen NK cells were identified as CD3-NKp46+ among size-gated lymphocytes. Compared with vehicle control, capsaicin caused a concentration-dependent reduction in the degranulation of WT NK cells against YAC-1 cells, similar to that observed with human NK cells (Supplementary Figure S7, available at Carcinogenesis Online), supporting the relevance of the mouse NK cell results to our study. This capsaicin-induced NK cell dysfunction was observed in TRPV1 KO NK cells exposed to 50–100 μM capsaicin (Figure 5E and F). Statistical analysis revealed significant NK cell dysfunction in both WT and TRPV1 KO NK cells exposed to capsaicin at 50–100 μM but not at 10–20 μM compared with their respective vehicle control, suggesting marginal contribution of TRPV1 to capsaicin-induced dysfunction of mouse NK cells.

**Capsaicin-induced NK cell dysfunction relies on defective Ca\textsuperscript{2+} mobilization**

To gain further insight into the mechanism of action of capsaicin on NK cells, we investigated the involvement of the cyclic adenosine 3',5'-monophosphate (cAMP) pathway since elevation of cAMP level is observed upon exposure to capsaicin (31,32) and is associated with NK cell dysfunction (33,34). We used Rp-8Br-cAMP, a cell-permeable cAMP analog that blocks the activation of signaling downstream of cAMP by competing with cAMP (35). Simultaneous exposure to Rp-8Br-cAMP up to 1000 μM failed to prevent the capsaicin-induced (50 μM) impairment in degranulation of human NK cells after stimulation with target cells (Figure 6A–D). Instead, Rp-8Br-cAMP marginally reversed the capsaicin-induced NK cell defects against target cells (Figure 6A–E). In contrast, Rp-8Br-cAMP as low as 100 μM was very effective in reversing the inhibitory effects of PGD\textsubscript{2} on NK cell degranulation (data not shown), confirming the effectiveness of Rp-8Br-cAMP in blocking the cAMP pathway. Since capsaicin at high concentrations inhibits the store-operated Ca\textsuperscript{2+} entry (36,37) that is crucial to NK cell effector functions upon target cell recognition (38), we investigated the effect of capsaicin on Ca\textsuperscript{2+} influx triggered by NK cell activation receptor. Stimulation of NK cells through a combination of NKG2D and 2B4 receptor evoked a robust Ca\textsuperscript{2+} mobilization, which was diminished modestly by 10 μM capsaicin and markedly by 50 μM capsaicin (Figure 6F). Thus, results suggest that capsaicin-induced NK cell dysfunction is largely independent of TRPV1 receptor and cAMP but relies on a mechanism involving defective Ca\textsuperscript{2+} mobilization.

**Discussion**

We showed that capsaicin dampens NK cell effector functions, such as cytotoxic degranulation and cytokine production, and thereby impairs the susceptibility of gastric cancer cells to NK cells independently of its ability to induce apoptosis in the cancer cells. Thus, our results may support the view of capsaicin as a ‘double-edged sword’ with both carcinogenic and chemopreventive properties (2). Gastric cancer cells were chosen as a model in which to evaluate the cancer-modulating
Capsaicin-induced NK cell dysfunction

Potential of capsaicin in NK cells. The chemopreventive activity of capsaicin has been recognized in numerous in vitro studies in which capsaicin triggered apoptosis or growth arrest in cancer cells (8); however, accumulating epidemiological and animal studies suggest that capsaicin may also play a role in promoting cancer (7). These conflicting results raise the possibility that the toxicity of capsaicin in cancer cells might be compromised by adverse effects on other cell types involved in the suppression of cancer growth. It became relevant to study the effect of capsaicin on the function of immune cells that mediate the antitumor response, given that few such studies exist. The immune system eliminates cancer cells and its dysfunction often leads to cancer development and poor prognosis (39,40). NK cells are major effectors of cancer surveillance through their ability to rapidly recognize and eliminate cells undergoing carcinogenesis (41).

Fig. 5. TRPV1 blockade did not antagonize the inhibitory effect of capsaicin. PBMCs were pretreated (30 min) with the TRPV1 antagonists SB (A, B) and CPZ (C) before treatment with 50 μM capsaicin. Thereafter, degranulation of NK cells was assessed after incubation with K562 cells as in Figure 1. (D) Lysis of 221 cells by NKL cells (10:1 E:T ratio) pretreated with 50 μM capsaicin in combination with CPZ or SB. (E, F) Splenocytes from WT and TRPV1 KO mouse were pretreated (1 h) ± capsaicin and incubated with YAC-1 cells (4 h) (E:T ratio 1:2). Degranulation was measured by surface expression of CD107a on CD3-NKp46+ NK cells. (E) Representative FACS profiles showing percentage of CD107a+ NK cells. (F) Summary graphs of statistical line charts showing expression of CD107a by NK cells. Error bars represent standard error the mean. Data are representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 by Student’s t-test.
In our experimental conditions, we observed that a subset of gastric cancer cells could be rendered apoptotic, which is consistent with the recognized ability of capsaicin to induce apoptosis in cancer cells; however, such direct anticancer effects were manifest at a higher concentration (~100 μM) and longer exposure time (~24 h) than those (~50 μM and ~3 h) required to trigger significant NK cell dysfunction. Supporting our results, numerous studies showed that the anticancer activity of capsaicin in vitro becomes evident at capsaicin concentrations >100 μM with an exposure time >1 day in various types of cancer cells (8). Although 10–20 μM capsaicin failed to induce apoptosis in gastric cancer cells, it could suppress NK cell effector functions. In addition to NK cells, activation of a T-cell line through T-cell receptor stimulation was suppressed by capsaicin (IC_{50} ≅ 18 μM) (42). In this respect, capsaicin may promote cancer by impairing cancer cell susceptibility to NK cells unless the cancer cells are highly susceptible to apoptosis by capsaicin at concentrations that do not significantly affect NK cell function. Given differential conditions for capsaicin to trigger NK cell dysfunction and apoptosis of gastric cancer cells tested, caution would be required for the consideration of capsaicin as a cancer preventive agent.

A partial recovery of capsaicin-induced NK cell dysfunction was observed by washing out capsaicin from the culture medium after pretreatment. This phenomenon was more evident when NK cells were pretreated with low (10–20 μM) but not high (50–100 μM) concentrations of capsaicin, suggesting the requirement for continued presence of capsaicin to maintain the effect on NK cells. Unlike the results from capsaicin-treated NK cells, the susceptibility of gastric cancer cells to NK cells was independent of capsaicin treatment of cancer cells (Supplementary Figure S4, available at Carcinogenesis Online). Thus, despite some cocarcinogenic signaling (9) in capsaicin-treated gastric cancer cells, capsaicin-induced NK cell dysfunction appears to be associated with the direct effect of capsaicin on NK cells. However, the possibility that prolonged exposure of cancer cells to capsaicin affects their susceptibility to NK cells cannot be excluded.

A mechanistic study indicated that capsaicin-induced NK cell dysfunction was largely independent of its cognate receptor TRPV1 and of the cAMP pathway but could be attributed to a defect in Ca^{2+} mobilization triggered by NK activation receptors. TRPV1 is a non-selective cation channel initially discovered as a thermosensitive molecule expressed in primary sensory neurons. Recent studies revealed...
that TRPV1 is widely expressed in various non-neuronal cell types, including keratinocytes, hepatocytes, gastric mucosa and some cancer cells (2,3,8). Although the biological role of TRPV1 in non-neuronal cells remains unclear, the wide distribution of TRPV1 suggests a diverse function beyond pain perception. TRPV1 was observed in immune cells, such as neutrophils (37), platelets (43), macrophages (28) and dendritic cells (44), and capsaicin had a direct effect on the function of these cells. Nonetheless, no study of TRPV1 expression in NK cells or of the effect of its ligand, capsaicin, on NK cell activity has been performed to date despite evidence showing an indirect effect of capsaicin on rat NK cell cytotoxicity (45). In this study, we demonstrated that human NK cells express detectable functional TRPV1 and that capsaicin inhibits NK cell function directly in a concentration-dependent manner. In subsequent studies dissecting the role of TRPV1 in the suppression of NK cell function by capsaicin, we observed a marginal contribution of TRPV1 to NK cell function using antagonists, cells deficient in TRPV1 and blockade of the downstream cAMP pathway. Thus, the suppression of NK cell function by capsaicin, particularly at high concentrations, is largely independent of TRPV1. Corroborating this, capsaicin-induced cancer cell death relies on TRPV1 at low concentrations but is independent of TRPV1 at high concentrations (4).

Given the crucial role of store-operated Ca\(^{2+}\) entry in NK cell effector function (38) and the ability of high concentrations of capsaicin to inhibit store-operated Ca\(^{2+}\) entry in a TRPV1-independent manner (36,37), we investigated whether a similar mechanism of action applied to capsaicin-induced NK cell dysfunction. Results revealed that 50 µM capsaicin, but not 10 µM, diminished Ca\(^{2+}\) influx upon stimulation of NK activation receptors, which correlated with NK cell effector function, suggesting the inhibition of Ca\(^{2+}\) mobilization as a mechanism underlying capsaicin-induced NK cell dysfunction.

In contrast to direct inhibition of immune effector cells, such as NK cells, capsaicin promotes the anticancer immune response by selective targeting of cancer cells (46). Intratumoral administration of capsaicin led to tumor regression by apoptosis and to T-cell-mediated anticancer immune response (46). Capsaicin-induced apoptosis of cancer cells may not be immunologically quiescent but may lead to antitumor T-cell response via sensitization of antigen-presenting cells by providing maturation signal and tumor antigen. As dying cells are not simply disposed of but are exposed to the immune system, they are able to expose or release cellular components that can affect the immune response upon their removal or uptake by antigen-presenting cells (47). Supporting this, late apoptotic cells and types of apoptosis induced by certain therapies, called immunogenic cell death, were found to involve priming of antigen-specific T-cell responses (47,48).

A recent study also suggested that capsaicin may elicit a cell death with immunogenic properties in bladder cancer cells since it induced immunogenic cell death hallmarks (early apoptotic cell surface exposure of calreticulin and heat shock proteins, and late apoptotic cell release of high-mobility group box 1 and heat shock proteins) (49). Thus, given contrasting results on the direct effect of capsaicin on antigen-presenting cells (44,50,51), it may be possible to design therapies that harness the immunogenic cytotoxic potential of capsaicin while maintaining the function of immune effector cells involved in cancer surveillance.

In summary, capsaicin has a direct inhibitory effect on NK cell effector function. Although NK cells have functional expression of TRPV1, capsaicin-induced NK cell dysfunction was largely independent of TRPV1. Since NK cell effector functions tolerated low concentrations of capsaicin, NK-based immunotherapy may be feasible selectively in cancers with high apoptotic sensitivity to capsaicin. Alternatively, given potent apoptotic and immunogenic death associated with high concentrations of capsaicin in some cancer cells, selective targeting of capsaicin to cancer cells by local delivery may be possible. Our study provides important insights into the effects of capsaicin in cancer and may contribute to designing effective therapies that harness the chemopreventive potential of capsaicin.

Supplementary material

Supplementary Figures S1–S8 can be found at http://carcin.oxfordjournals.org/

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