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Review

Advances in the therapy of Barrett's Esophagus

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Abstract

The rising incidence of esophageal adenocarcinoma(EAC) in the world has led to continued interest in its precursor lesion, Barrett's esophagus(BE). This review endeavors to summarize the recent advances in the therapy of BE with an emphasis on novel endoscopic therapies.

Key words: Barrett's esophagus; Endoscopic therapy; Endoscopic

INTRODUCTION

A dramatic increase in the incidence of esophageal adenocarcinoma(EAC) in recent years[1] has led to unprecedented attention being focused on its precursor lesion, namely Barrett's esophagus(BE). In China, a developing country, the morbidity of EAC is increasing[2]. BE is now a well recognized premalignant condition associated with a 30-50-fold increased risk of EAC[3]. Untreated, this can become Barrett's with dysplasia, in which cells start to transform to cancer cells[4-6].The current review article endeavors to summarize the recent advances in the therapy of BE with an emphasis on novel endoscopic therapies.

Medications

The most common cause of BE is longstanding acid reflux disease, called gastroesophageal reflux disease (GERD)[7]. In people with GERD, the esophagus is repeatedly exposed to excessive amounts of stomach acid and pepsin. In order to reduce the amount of acid produced by the stomach, a class of medications called proton pump inhibitors(PPI) is commonly recommended. Although multiple studies have proved that PPI therapy is associated with a significant reduction in the risk of developing dysplasia with BE[8,9], it cannot alter the progression of genetically unstable cells(e.g. p53 positive)[10,11].

In the Chemoprevention for Barrett's Esophagus (CBET) trial, Heath and colleagues[12] evaluated the efficacy of celecoxib in decreasing cancer incidence when administered to patients with BE and dysplasia (n = 100). This phase IIb randomized, multicenter, placebo-controlled trial was based on the premise that esophageal tumorigenesis is associated with COX2 overexpression and that selective COX2 blockade could decrease progression to cancer. After 48 weeks, no statistically significant difference was noted between the celecoxib and placebo groups in the proportion of biopsy samples containing dysplasia or cancer. Thus, celecoxib at a dose of 200 mg twice daily had no impact in preventing progression.

Reflux is the predominant risk factor for BE, and proximal gastric colonization by *H. pylori* seems to amplify this risk[13].A few studies indicated that eradicating *H pylori* can help reduce the morbidity of BE, but some more recent studies suggested that *H pylori* infection may be protective against BE[14]. The most likely reason is that *H pylori* infection can lead to gastric atrophy, in particular with the more virulent strains(CagA+). Gastric atrophy and reduced acid secretion should, in turn, lessen GERD risk. The link between *H pylori* infection and BE is complex and it is debated whether or not eradicating *H pylori* can help in the therapy of BE. Future research is required to

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resolve this question.

ENDOSCOPIC THERAPIES

Photodynamic therapy(PDT)

Photodynamic therapy(PDT) is a treatment that uses chemical agents, known as photosensitizers, to kill certain types of cells(such as Barrett's cells) when the cells are exposed to a laser of specific wavelength and power[15]. Patients are administered the photosensitizer medication intravenously, and then they undergo endoscopy. During the endoscopy, a laser light is used to activate the photosensitizer and destroy the Barrett's tissue. A recent study by Overholt *et al*[16] evaluated the long-term efficacy of PDT ablation for BE patients with high-grade dysplasia(HGD). This multicenter study randomized 208 patients in a 2:1 ratio to PDT with omeprazole versus omeprazole alone. As the primary outcome, patients in the PDT arm had a significantly higher rate of HGD elimination at 5 years of follow up (77% versus 39%, $P < 0.001$). A secondary outcome was a slower and significantly lower rate of progression to cancer in the PDT group(15% versus 29%, $P = 0.027$). Although the long-term efficacy has been confirmed, it's complication(e.g. narrowing of the esophagus) and the possibility of the untreated buried lesion should always be considered. A study by Mino-Kenudson *et al*[17] indicated that buried neoplasms are not common after PDT.

Argon plasma coagulation(APC)

Argon plasma coagulation(APC) is one of the several endoscopic approaches that have been proposed in order to reverse BE and to induce squamous re-epithelialization. This technique allows ablation of large areas of specialized intestinal metaplasia with a limited depth of injury that minimizes the risk of stricture and perforation[18]. Pereira *et al*[19]used argon beam coagulation at a power setting of 65-70 W for the therapy of BE. In their study, complete restoration of squamous mucosa took place in all 33 cases(mean age: 55.2 yr, range:21-84 yr; 21 men and 12 women) after a mean of 1.96 sessions(range, 1-4). Endoscopic results were histologically confirmed. After a mean follow-up of 10.6 months there was one endoscopic, as well as histological, recurrence of Barrett's mucosa in a patient with an ineffective laparoscopic fundoplication. A study by Pedrazzani and colleagues[18] evaluated the effectiveness of 90 W APC for the ablation of BE. The ablation treatment was completed in all 25 patients but one(96%). The mean number of APC sessions needed to complete ablation was 1.6(total number: 40). Successful eradication was obtained in the majority of cases(60%) with only one session. Two sessions were required in 24% of the cases, and three or more in 16%. The follow-up was accomplished in all the patients for a mean period of 26.3 mo, and in 20 patients(84%) with a follow-up period longer than 24 mo. Only one patient relapsed with a recurrence of metaplastic mucosa 12 mo after the completion of ablation. The most frequent symptoms after APC was retrosternal pain(22.5%) and fever (17.5%).

Surprisingly, there has been a recent report of a high recurrence rate(14/21) of Barrett's epithelium in a long-term follow-up after APC[20]. The different recurrence rates reported in published studies may be due to technical differences and different PPI schedules. We suggest that optimal conditions for this procedure must be defined before further studies are undertaken.

Endoscopic mucosal resection(EMR)

Endoscopic mucosal resection(EMR) can be considered therapeutic when the lesion is confined to the mucosa and clear margins of resection are obtained[21]. A recent non-blinded and nonrandomized, single-center, prospective study by Ell and colleagues[22] evaluated the efficacy of EMR in a total of 100 consecutive patients with low risk EAC(defined as macroscopic types Ⅰ, Ⅱa, Ⅱb and Ⅱc with lesion diameter < 20 mm, absence of lymphatic invasion and early histologic grade G1 and G2). Complete local remission was achieved in 99 of the 100 patients after 1.9 months and a maximum of three resections. Although 11% developed metachronous lesions during a mean follow-up period of 36.7 months, all could be successfully re-treated endoscopically. No major complications were reported and the 5-year survival rate was 98%. The most common complications of EMR are bleeding, perforation and stenosis[23].

EMR provides large tissue specimens that can be examined by the pathologist to determine the character and extent of the lesion and these can also be used to determine if an adequate amount of tissue was removed. Therefore, this procedure can both help confirm the initial diagnosis and completely treat the abnormality (if the abnormal tissue is removed completely).

Radiofrequency ablation(RFA)

Radiofrequency ablation(RFA) is an exciting new tool that has proven to be effective in eliminating intestinal metaplasia[24]. Its role in ablating dysplasia is currently being evaluated. The radiofrequency balloon(Halo 360 system) contains a tightly packed array of multipolar electrodes capable of delivering a high-energy pulse in a fraction of a second. In a recent report of 70 patients with nondysplastic BE who underwent circumferential balloon based ablation at 10 J/cm^2, repeat post-treatment endoscopies with biopsies were performed at 1, 3, 6 and 12 months. A second ablation was performed if BE was detected at 1 or 3 months. At 1 year, the

group reported complete elimination of BE in 70%(n = 48) of patients with no major complications[25].

Carroll and colleagues of Georgetown University Hospital are planning to begin a study to see if it is worth doing Barrx treatment even earlier-in patients whose BE has not yet developed precancerous spots.

Cryoablation therapy

A new method, called cryoablation therapy, is available to damage cells in the esophagus by freezing them, preventing them from turning cancerous. The FDA has recently approved using this technique in the treatment of BE. The method employs a special catheter and liquid nitrogen to freeze the damaged tissue in the superficial lining of the esophagus. The treated tissue eventually sloughs off, allowing normal cell replacement in about six to eight weeks. This is the same technology that has been in place and used by dermatologists for years to treat skin irregularities. However, it is a completely novel technique for treating BE, and more studies are required to confirm its efficacy and safety.

Others

Many more techniques for destroying the Barrett's lining have been studied, including lasers[26], a heat probe[27] and combination therapy with chemicals and others[28,29]. As of yet, it is not clear which patients would benefit from these approaches, particularly since they may be associated with side-effects(such as narrowing of the esophagus or creation of a penetrating lesion in the esophagus during treatment).

Surgery

Prior to the development of the potent acid-reducing medications described above, surgery was used for severe cases of GERD that were not resolved with medical treatment. Because of the effectiveness of medical therapy, the role of surgery has become more limited. In general, anti-reflux surgery involves repairing a hiatal hernia and strengthening the lower esophageal sphincter.

The most common surgical treatment is the laparoscopic Nissen fundoplication. This procedure involves wrapping the upper part of the stomach around the lower end of the esophagus, thus minimizing reflux. Patients in whom surgery is being considered typically require esophageal manometry and endoscopy to confirm the diagnosis and decide which surgical treatment will be most effective. Although the outcome of surgery is usually good, complications can occur. Examples include persistent difficulty swallowing (occurring in about 5 percent of patients), a sense of bloating and gas (known as "gas-bloat syndrome"), breakdown of the repair(1 to 2 percent of patients per year), and uncommonly, diarrhea due to inadvertent injury to the nerves leading to the stomach and intestines. Comparably, endoscopic therapies result in fewer complications.

The question remains, is esophagectomy inferior to endoscopic therapies for the treatment of HGD? There are no prospective studies addressing this issue but a recent single center retrospective cohort study compared survival among 129 BE patients treated with PDT plus EMR with 70 patients treated with esophagectomy[6]. Despite the fact that patients in the endoscopic therapy group were older and had a higher comorbidity index, the overall survival as well as tumor-free survival were comparable in both groups (mortality in the PDT group was 9%[11/129] and in the surgery group was 8.5%[6/70] over a median follow-up period of 59±2.7 months for the PDT group and 61 ± 5.8 months for the surgery group). Survival was comparable when adjusted for age, BE length and comorbidity index. This review[6] makes a powerful argument in favor of endoscopic therapy, but obviously results from this high-volume center with broad expertise in endoscopy, pathology and surgery cannot be generalized to all clinical settings. While esophagectomy might not be the best procedure, we believe it can be an alternate choice available for the treatment of BE.

CONCLUSION

The rising incidence of EAC in the world has led to continued interest in its precursor lesion, BE. Significant advances have been seen in the past years and we now have many treatment options available, as indicated above. But, which is the best one? When should we use these approaches? These is no certain answer, because none of the approaches above is perfect enough to treat every patient without some risk of complications. Despite the uncertainties surrounding the therapy of BE, there is consensus on one matter: The available options should be tailored to the individual patient.

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Research paper

Research on the effects of CD137 signaling on the function of $CD3^-CD56^+$NK cells☆

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Abstract

Objective: To investigate the effects of CD137 signaling on the regulation of $CD3^-CD56^+$NK cells function. **Methods:** $CD3^-CD56^+$NK cells were treated with CD137 mAb or mouse IgG1 isotype control to study the effects of CD137 signaling on the function of $CD3^-CD56^+$NK cells. Cytotoxicity was measured by LDH activity in the supernatants of cell cultures; NKG2D and LFA-1 expression on $CD3^-CD56^+$NK cells were analyzed by flow cytometry. **Results:** CD137 was expressed on activated $CD3^-CD56^+$NK cells. The CD137 mAb enhanced the ability of $CD3^-CD56^+$NK cells to kill lung cancer cells(A549); Further studies revealed that the expression of NKG2D and LFA-1 was significantly increased in activated cells, and blockade of NKG2D and LFA-1 dramatically attenuated $CD3^-CD56^+$NK cytolysis of A549 cancer cells. **Conclusion:** CD137 signaling increases the ability of $CD3^-CD56^+$NK cells to kill cancer cells via up-regulating the expression of NKG2D and LFA-1.

Key words: CD137; $CD3^-CD56^+$NK cells; signal

INTRODUCTION

$CD3^-CD56^+$natural killer(NK) cells are innate immune cells that control certain microbial infections and tumors. $CD3^-CD56^+$NK cells form a first line of defence against pathogens or host cells that are stressed and/or cancerous. These NK cells express surface receptors that receive signals from the environment that determine their response to foreign or malignant cells[1]. $CD3^-CD56^+$NK cells have also been shown to connect the innate and adaptive immune systems. Interaction between dendritic cells(DC) and $CD3^-CD56^+$NK cells is an important $CD4^+$ T-cell-independent pathway for antitumor cytotoxic T lymphocyte(CTL) induction[2,3]. CD137(4-1BB) is a TNFR superfamily member that is expressed by activated $CD3^-CD56^+$NK cells, T cells and DCs[4,5]. The natural ligand for CD137(CD137L) is found on activated B cells, macrophages and DCs. CD137L binding to CD137 mediates the costimulatory signal that results in T cell and $CD3^-CD56^+$NK cell proliferation and cytokine production. In the present study we showed that CD137 signaling mediated by CD137 mAb could enhance anti-tumor activity of $CD3^-CD56^+$NK cells. We further investigated the mechanism by which CD137 signaling regulated the function of $CD3^-CD56^+$NK cells.

MATERIALS AND METHODS

Cell isolation

Peripheral blood mononuclear cells(PBMCs) were isolated by Ficoll density gradient centrifugation from healthy human peripheral blood. Magnetic cell sorting was then used to purify $CD3^-CD56^+$NK cells from PBMCs. After washing once in buffer, PBMCs were resuspended in 80 μl buffer per 10^7 cells, and these were added to 20 μl CD56 Microbeads(Miltenyi Biotec, Germany). The mixture was incubated for 15 min at 4℃, then washed in buffer and subjected to magnetic separation with a MS Column(Miltenyi Biotec). CD56 Microbeads were removed using MultiSort Release and Stop Reagent(Miltenyi Biotec) from the cells of the CD56 positive fraction, then CD3 MicroBeads(Miltenyi

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Biotec) were developed for isolation of $CD3^-$ cells subpopulations from $CD56^+$ cells as above. Using analysis by flow cytometry, the purity of $CD3^-CD56^+$NK cells was found to be above 95%. $CD3^-CD56^+$NK cells were collected and cultured in RPMI 1640 medium (GIBCO, USA) supplemented with 10% fetal calf serum (FCS) (GIBCO), streptomycin(100 μg/ml), penicillin (100 U/ml) and interleukin-2(IL-2)(500 U/ml). At the same time, 1 μg/ml of anti-CD137 mAb(4B4-1, BD Bioscience, USA) or mouse IgG1 isotype control(BD Bioscience) were added to the culture medium. All cells were maintained at 37℃ in a humidified 5% CO_2 atmosphere.

Cell lines

The A549 lung adenocarcinoma cell line was obtained from American Type Culture Collection(CCL no. 185) and maintained in HEPES-buffered RPMI 1640 with 10% FCS and antibiotics. A549 cells were inoculated into 25cm^2 culture flasks(Corning Costar, USA). The medium was replaced every other day and cells were passaged when they reached 80% confluence.

Cytotoxicity assay

Cytotoxicity was estimated by quantification of LDH activity in the culture medium[6]. Purified $CD3^-$ $CD56^+$NK cells were cultured with IL-2 and CD137 mAb(1 μg/ml) or IgG1(1 μg/ml) for three days, and then CD137-stimulated $CD3^-CD56^+$NK cells were collected and incubated with medium containing anti-NKG2D or anti-LFA-1(R&D Systems, USA)(10 μg/mL) for another 4 h. Cytotoxicity assays were carried out in flat-bottomed 96-well plates(Greiner Bio-one, Germany) with a final sample volume of 100 μl/well. A549(2×10^5/ml cells) in 50 μl/well were co-cultured with effector cells($CD3^-CD56^+$NK cells, treated or untreated) at 10:1 effector-to-target cell ratio. All samples were run in triplicate. The LDH activity was quantified by measuring the absorbance at 490 nm.

The following formula was used to compute percent cytotoxicity:

$$\frac{(LDH_{experimental} - LDH_{effector\ cells} - LDH_{spontaneous})}{(LDH_{maximal} - LDH_{spontaneous}) \times 100\%}$$

$LDH_{experimental}$: Release resulting from the co-culturing of effector cells and target cells.

$LDH_{effector\ cells}$: Release resulting from the culturing effector cells alone.

$LDH_{spontaneous}$: Release resulting from the culturing A549 cells alone (low control).

$LDH_{maximal}$: Release after the addition of 10 μl of lysis solution(10 ×) to 100 μl/wells of target cells(high control).

Flow cytometric analysis

Fresh PBMCs were cultured in RPMI 1640 with 10% FCS and antibiotics and IL-2(500 U/ml). At day 3, cells were collected and labeled with fluorescent-conjuncted mAbs, including PC5-labeled mouse anti-human CD56, ECD-conjugated mouse anti-human CD3, and PE-conjugated mouse anti-human CD137(BD Bioscience). The cells were then stained with the indicated mAb or the control antibody(BD Bioscience) at 4℃ for 15 min, and washed three times. The data were acquired by flow cytometry (Coulter, USA).

Fresh PBMCs were cultured in medium with CD137 mAb(1 μg/ml) or control IgG1. At day 3, single-cell suspensions were prepared and stained for 15 minutes at 4℃ with optimal dilutions of anti-CD3-ECD, anti-CD56-PC5, anti-NKG2D-PE, anti-LFA-1-PE, anti-CD95L-PE, anti-B7-H1-PE or anti-NKG2A-PE or isotype-matched control IgG1(BD Bioscience). Surface molecules on $CD3^-CD56^+$NK cells were analyzed by flow cytometer.

Statistical analysis

The statistical analysis of the data was performed using SPSS 11.5 soft ware, comparing the means between two groups using the Student's *t*-test.

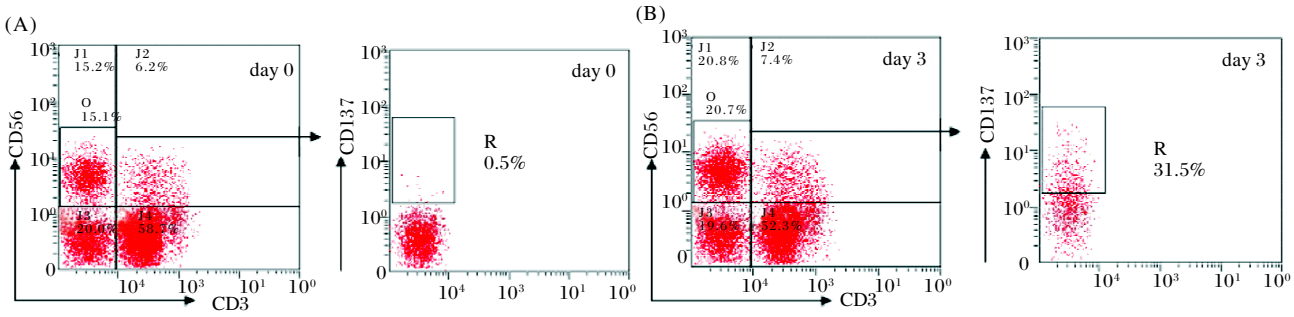
RESULTS

Expression of CD137 on $CD3^-CD56^+$ NK cells

Earlier findings reported that CD137 was expressed on activated $CD3^-CD56^+$NK cells, T cells and DCs. Here, we demonstrated that CD137 expression on $CD3^-$ $CD56^+$NK cells was increased when the cells were activated by IL-2. Flow cytometric analysis showed that resting $CD3^-CD56^+$ NK cells in PBMCs expressed CD137 at a low level[day 0,(1.60 ± 1.05)%] and the CD137 expression increased markedly upon stimulation by IL-2[day 3,(29.37 ± 4.20)%]($P < 0.05$)(***Fig. 1***).

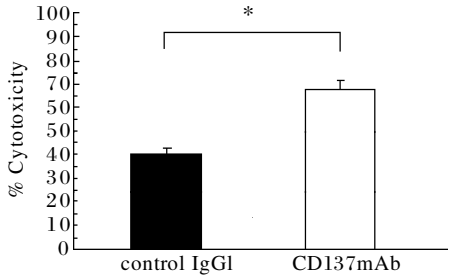
CD137 mAb enhance cytotoxicity of $CD3^-$ $CD56^+$NK cells to kill A549 tumor cells

Purified $CD3^-CD56^+$NK cells were cultured with IL-2 and CD137 mAb(1 μg/ml) or IgG1(1 μg/ml) for three days and the cytotoxicity of $CD3^-CD56^+$NK cells was evaluated by the LDH release assay. At 10:1 effector-to-target cell ratio, $CD3^-CD56^+$NK cells cultured with CD137 mAb(CD137-NK cells) killed significantly ($P < 0.05$) more A549 cells than those treated with control IgG1(control IgG1-NK cells), (65.97 ± 3.85) % and(39.93 ± 4.20)%, respectively(***Fig. 2***).



On days 0,3, PBMCs cultured with IL-2 were harvested and stained with anti-CD3-ECD, anti-CD56-PC5 and anti-CD137-PE or their isotype control mAbs. Flow cytometry analysis was gated on the $CD3^-CD56^+$NK cells for detecting the CD137 expression. (A)The expression of CD137 on $CD3^-CD56^+$NK cells on day 0. (B)The expression of CD137 on $CD3^-CD56^+$NK cells on day 3.

Fig. 1 The expression of CD137 on $CD3^-CD56^+$NK cell

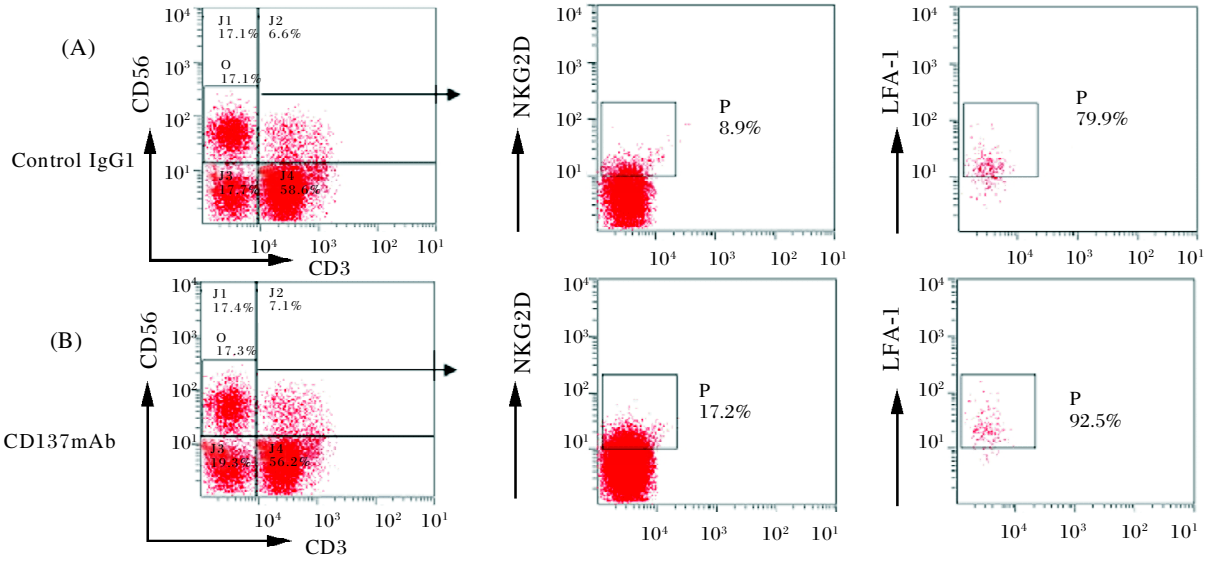


The cytotoxicity was evaluated by LDH release assay. The Purified $CD3^-CD56^+$NK cells cultured with CD137 mAb(CD137-NK cells) or control IgG1(control IgG1-NK cells) were collected on day 3 as effector cells as described in "Materials and Methods" A549 cells were gathered in the logarithmic growth stage. At the effector-to-target ratio of 10:1, the cytotoxicity of CD137-NK cells was significantly greater than control IgG1-NK cells($*P < 0.05$).

Fig. 2 The cytotoxicity of $CD3^-CD56^+$NK cells against A549 cells(10:1 effector: target cell ratio)

CD137 mAb enhance expression of NKG2D and LFA-1 on $CD3^-CD56^+$ NK cells

To gain an insight into the mechanisms leading to the enhancement of anti-tumor effects of $CD3^-CD56^+$ NK cells induced by CD137 signaling, we analyzed the expression of NKG2D, LFA-1, CD95L, B7-H1 and NKG2A surface molecules on $CD3^-CD56^+$NK cells by flow cytometry. Fresh PBMCs were cultured with CD137 mAb or control IgG1 for three days. Flow cytometric analysis showed that the expression of NKG2D and LFA-1 on CD137-NK cells was(17.37 ± 2.75)% and (89.17 ± 3.57)% , respectively, and(8.30 ± 2.16)% and(74.63 ± 5.06)% on control IgG1-NK cells, respectively. These differences between the two groups were statistically significant($P < 0.05$)(***Fig. 3***). However, there was no difference in the expression of CD95L,B7-H1 or NKG2A between the cells of the different treatment groups(data not shown, $P > 0.05$).

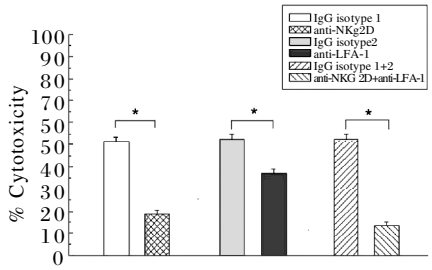


*Expression of NKG2D and LFA-1 on $CD3^-$*$CD56^+$NK cells. On days 3, PBMCs cultured with control IgG1(A) or CD137 mAb (B)were harvested and stained with anti-CD3-ECD, anti-CD56-PC5, anti-NKG2D-PE and anti-LFA-1-PE or their isotype control mAbs. Flow cytometry analysis was gated on the $CD3^-CD56^+$NK cells for detecting the NKG2D and LFA-1 expression.

Fig. 3 CD137 mAb enhanced the expression of NKG2D and LFA-1 on $CD3^-CD56^+$NK cells surface

Blockade of NKG2D and LFA-1 attenuate CD3⁻CD56⁺NK cytolysis of A549 cells

Purified CD3⁻CD56⁺NK cells were cultured with CD137 mAb(1 μg/ml) for three days, and the cells were then harvested. Anti-NKG2D or anti-LFA-1 or their IgG isotype control were added and incubated for another 4h. Cytotoxicity assays were carried out at 10:1 effector-to-target ratio. As expected, blockade of NKG2D alone and blockade of LFA-1 alone significantly inhibited the up-regulation effects of CD137 signaling on CD3⁻CD56⁺NK cytolysis compared with their isotype-matched control groups, (21.40 ± 3.56)% vs (59.55 ± 4.00)% and(36.23 ± 3.72)% vs(60.85±4.03)%, respectively($P < 0.05$). Meanwhile, both blockades dramatically attenuated the up-regulation effects of CD137 signaling on CD3⁻CD56⁺NK cytolysis of A549 cells[(15.48 ± 2.89)% vs (60.55 ± 4.69)%]($P < 0.05$). These results demonstrated that the up-regulatory effect of CD137 signaling on CD3⁻CD56⁺NK cytolysis is at least partly dependent of the interaction of NKG2D or LFA-1 and their ligands(***Fig. 4***).



Purified CD3⁻CD56⁺NK Cells were cultured with CD137 mAb for three days. Anti-NKG2D or anti-LFA-1 or their IgG isotype control were added and incubated for another 4 h. Cytotoxicity assays were carried out at a 10:1 effector-to-target ratio(*$P < 0.05$).

Fig. 4 Blockade of NKG2D and LFA-1 attenuated CD3⁻CD56⁺NK cytolysis of A549 cells

DISCUSSION

CD3⁻CD56⁺NK cells have been shown to have a dominant role in the early phase of the immune response against microbial infections and tumors. CD3⁻CD56⁺NK cells form a first line of defence against pathogens or host cells that are stressed and/or cancerous. CD3⁻CD56⁺NK cells also act as a bridge between innate immunity and adaptive immunity. CD137 is a member of the tumor necrosis factor receptor superfamily[7,8] that binds to a high-affinity CD137 ligand expressed on antigen-presenting cells(APCs), such as dendritic cells, macrophages, and activated B cells[9,10]. Upon interaction with the CD137 ligand, CD137 provides a strong signal for expansion of the CD3⁻CD56⁺NK cells[11]. Here, we investigate the effects of CD137 signaling on regulation of CD3⁻CD56⁺NK cell function.

It was reported that activated CD3⁻CD56⁺NK cells express CD137 on their surface. Expression of CD137 is restricted to IL-2-activated CD3⁻CD56⁺NK cells[12]. We confirmed that there was almost no expression of CD137 on resting CD3⁻CD56⁺NK cells, and the expression of CD137 significantly increased when CD3⁻CD56⁺NK cells were activated by IL-2(***Fig. 1***). Further, we found CD137 signaling mediated by anti-CD137 mAb could enhance the cytotoxicity of CD3⁻CD56⁺NK cells to kill A549 cells in vitro(***Fig. 2***). In order to better understand the mechanisms leading to the enhancement of the anti-tumor effects of CD3⁻CD56⁺NK cells induced by CD137 signaling, we used flow cytometery to analyze some surface molecules (NKG2D, LFA-1, CD95L, B7-H1 and NKG2A) associated with the cytotoxicity of CD3⁻CD56⁺NK cells. The results showed that the expression of NKG2D and LFA-1 in CD3⁻CD56⁺NK were induced by CD137 signaling (***Fig. 3***). The expression of either CD95L, B7-H1 or NKG2A was not affected by CD137(data not shown).

Activating CD3⁻CD56⁺NK cell receptor, natural killer group 2D(NKG2D), is a type II disulphide-linked dimer with a lectin-like extracellular domain[13]. It is encoded by a gene in the CD3⁻CD56⁺NK complex on mouse chromosome 6 and on human chromosome 12[14]. NKG2D is a unique stimulatory molecule that is found on natural killer cells, T cells and activated macrophages. It has been further validated by showing that mouse CD3⁻CD56⁺NK cells can kill syngeneic class-I-bearing RMA cells in vitro when these tumor cells are transfected with ligands for the NKG2D receptor[15,16]. The attractiveness of NKG2D as a sentinel system of the innate immune system stems from three features. First, NKG2D binds to diverse ligands expressed by "stressed or altered self", thereby complementing the missing self-recognition strategy of CD3⁻CD56⁺NK cells[17]. Secondly, these ligands generally are not expressed on healthy cells, but they are induced by an insult, such as infection or transformation[18]. Thirdly, NKG2D ligation can drive CD3⁻CD56⁺NK cell activation despite the inhibitory influence of MHC class I molecules expressed on the target cells[16].

Lymphocyte function associated antigen-1(LFA-1, CD11a/CD18) is a member of the integrin family of cell surface receptors. LFA-1 has been shown to be important in the effector function of CD3⁻CD56⁺NK cells[19]. Adhesion to target cells through LFA-1 is required for efficient lysis by CTLs and CD3⁻CD56⁺NK cells[20]. Furthermore, the activation of LFA-1 alone is sufficient to initiate CD3⁻CD56⁺NK cells[21,22]. Therefore, we speculated that CD137 signaling promoting the cytolytic ability of CD3⁻CD56⁺NK cells might be correlated with increasing the expression of NKG2D

and LFA-1 on $CD3^-CD56^+$NK cells. In support of this we showed that blockade of NKG2D alone or blockade of LFA-1 alone significantly inhibited $CD3^-CD56^+$NK cytolysis, while simultaneous blockade of both NKG2D and LFA-1 with monoclonal antibodies dramatically attenuated the up-regulatory effects of CD137 signaling on $CD3^-CD56^+$NK cytolysis.

In summary, we conclude that the CD137 signaling strengthens the ability of $CD3^-CD56^+$NK cells to kill cancer cells, probably via up-regulating the expression of NKG2D and LFA-1.

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