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The inhibitory effects of synthetic short peptides, mimicking MICA and targeting at NKG2D receptors, on function of NK cells

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Abstract

NKG2D is an activating receptor expressed on most of human NK cells, one of whose ligands is MICA. Based on the crystal structure of NKG2D–MICA complex, we synthesized three short peptides (P1, P2 and P3), mimicking functional α1 and α2 domain of MICA. The inhibitory effects of three peptides on NK-92 cells, a human NK cell line against Hela cells were observed and the inhibitory percentage was 38% at maximum for P1 + P2 + P3 in concentration of 1 nM. The same peptides had no effect on NK-92 cell against target cells lacking MICA (K562 cells line). The unrelated peptides as controls had no effect on the system. Two peptides (P2 and P3) were prolonged at one or both ends, and the longer forms of peptides exerted stronger inhibitory effects than their shorter forms. Each combination of two peptides exerted a stronger function than single peptide (P1, P2, P3), indicating that shedding of longer amino acid sequence of α1 domain or more domain sites of MICA are better than shorter sequence and fewer sites. P1 + P2 + P3 revealed the almost same inhibitory rate as the soluble MICA (sMICA). P1 + P2 + P3 were also able to alleviate the concanavalin A-induced murine autoimmune hepatitis in vivo, conforming the similarity of NKG2D between human and mice. The results demonstrate that MICA-mimicking peptides will be useful to search the specific functional sites for NKG2D–MICA interaction, but also promising in explaining NKG2D-related autoimmunity.

Keywords: NK cells; Natural cytotoxicity; NKG2D; MICA; Peptide; Autoimmune liver injury

1. Introduction

NK cells constitute an important component of the innate immune system, providing the first line of defense against certain viruses, intracellular bacteria and transformed cells. NK cells exert cell-mediated cytotoxicity and act to regulate innate and acquired immune responses through the release of various cytokines (such as IFNγ, GM-CSF and TNF-β) and chemokines. Unlike T cells, NK cells killing of virus-infected or malignant transformed cells does not need pre-sensitization and is independent of a MHC restricted manner. NK cell activity is regulated by an array of membrane-bond inhibitory and activating receptors mediated signals engaged upon interaction with target cell surface ligands [8,15,25,26]. Two inhibitory receptor families have been discovered in human: the killer cell-Ig-like receptors (KIR) and the CD94/NKG2A lectin-like receptors. Owing to the fact that KIRs are not expressed on all NK cells and that the expression of KIRs may be later than that of CD94/NKG2A during the development and differentiation of NK cells, CD94/NKG2A receptor, whose ligand is the non-classical class I molecules HLA-E, may be an important inhibitory receptor for exerting functions of NK cells [19]. It is believed that inhibitory receptors on NK cells discriminate healthy cells from diseased cells by surveying the surface expression of self-MHC class I molecules. However, evidence has accumulated that activating receptors also play a major role in activation and regulation of NK cells [8]. A well-characterized example of activating NK cell receptors to date is the NKG2D receptor, which was first recognized on

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NK cells, but was subsequently found on CD8+ T cells, γδT cells and macrophages, making it one of the most widely distributed NK receptors currently described [2,4,22,41]. In human, the NKG2D receptor binds to the stress-inducible polymorphic non-classical MHC molecules MICA (MHC class I chain-related A), MICB and the MHC class I-related UL16-binding protein-1, 2, 3 (ULBP-1, 2, 3), which are strongly upregulated in many types of tumor cells [14,37]. Recent studies reveal that the expression of MIC and ULBP on human tumor cells is sufficient to overcome the inhibitory effects of MHC class I expression on NK cell killing [31]. These observations indicate that NKG2D provides first line surveillance against stressed or abnormal cells which have been induced to express one of its ligands.

MIC has a restricted tissue distribution in intestinal epithelium, being frequently expressed in epithelial tumors, and is induced by viral and mycobacterial infections [6,9,10]. The structure of MICA is similar to the protein fold of MHC class I, with a α1α2 platform domain and a membrane-proximal Ig-like α3 domain, which neither associates with β-microglobulin nor binds peptides but combines with homodimer of NKG2D to initiate the activation of human NK cells after engagement of NKG2D by MICA [17,27]. MICA and its close relative MICB, which also serves as a ligand for NKG2D, are both polymorphic and the polymorphism has been shown to affect the affinity for NKG2D. More recently, the UL16-binding proteins (ULBPs) have been identified as additional ligands of NKG2D [5,32]. The crystal structure of the NKG2D–MICA complex revealed that a NKG2D homodimer binds to a MICA monomer in an interaction that is analogous to that seen for T cell receptor-MHC class I protein complexes [40]. Recent research revealed that soluble MICA could inhibit NKG2D–MICA interaction, by which the tumor escaped from CTL attack [11]. On the other hand, the cytotoxicity of NK cells decreased when the receptor was blocked by anti-NKG2D mAb [2,18]. In this study, we use peptides mimicking MICA functional domains to block the NKG2D–MICA interaction. Based on the structure of the NKG2D–MICA protein complex, three short peptides located at α1 and α2 domain of human MICA were synthesized as the blockades of NKG2D–MICA contacting. Different combinations of the peptides were used to incubate with the NK cells and the cytotoxicity of the NK cells against target Hela cells was specifically inhibited. The inhibitory effects of the synthetic peptides were also confirmed in alleviation of liver injury of T cell-mediated murine hepatitis.

2. Materials and methods

2.1. Peptide design

Detailed information about the association of MICA with NKG2D has been available already. The sequence of the protein (PDB code: 1HYR) was from the NCBI database at http://www.ncbi.nlm.nih.gov/Entrez [17]. A helical element of ten residues (residues 152–161) in the α2 domain of MICA, which corresponds to helix 2a in MHC class I proteins, was disordered and presumed to form an extended flexible loop. This element containing the residues that interact with NKG2D-B becomes ordered during complex formation [17], and it was used as one of the core templates for the peptide design. On the other hand, a similar element in the α1 domain (residue 70–80) containing the residues that interact with NKG2D-A was used as the second template. Because the interactions within the complex of NKG2D–MICA relate to the loop between β strands 1 and 2 (residues 13–21) of MICA that curls upward NKG2D [17], the third template was selected at the β1–β2 loop.

2.2. Peptide synthesis

The peptides were synthesized by solid phase synthesis and purified by HPLC to more than 90% purity. For easier synthesis and better dissolubility, the sequences of the three peptides were different from the templates at the ends. Moreover, two longer peptides were synthesized from templates in the α1 and α2 domains. The five peptides were (i) residues 16–20 (DGSVQ), from the template in the β1–β2 loop in the α1 domain, named as P1; (ii) residues 72–82 (KDLRMTLAHIK), from the template in the α1 domain, named as P2; (iii) residues 156–167 (THYHAMHADCLQ), from the template in the α2 domain, named as P3; (iv) residues 58–84 (KTWDRETRDLTGNGKDLRMTLAHIKDQ), longer form of P2, named as P2L; (v) residues 147–173 (EDAMKTKTHYHAMHADCLQELRRYLKS), longer form of P3, named as P3L. Additionally, we synthesized an unrelated peptide based on the similar functional domain of the HLA-E, called Px (RDTAQIFRVNLRT) as unrelated control [38].

2.3. Reagents and cells

MICA protein was a gift from Professor Roland K. Strong of U.S.A. (Division of Basic Sciences Fred Hutchinson Cancer Research Center, Seattle, WA), and it had been widely used in their published research [3,17,18,24]. Concanavalin A type IV (Con A) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and separately dissolved in pyrogen-free phosphate-buffered saline (PBS) at a final concentration of 1 mg/ml. Human NK cell line, NK-92 cells [23], was obtained from ATCC of USA. Target cells Hela, K562 and YAC-1 cells line were kept in our laboratory which were all cultured in RPMI medium 1640 supplemented with 10% bovine calf serum, at 37 °C under 5% CO_2. NK-92 cells were cultured in minimum essential medium (MEM) α medium supplemented with 10% bovine calf serum, 10% EQUINE serum, and 100 units/ml rhIL-2 at 37 °C under 5% CO_2.

2.4. Concanavalin A-induced murine hepatitis and alleviation of liver injury with the small peptides in vivo

Male C57BL/6 (B6) mice (8–10 weeks old) were obtained from Shanghai Experimental Animal Center of Chinese Sci-

ence Academy, and maintained under specific pathogen-free (SPF) and controlled conditions (22 °C, 55% humidity, and 12 h day/night rhythm) in animal facility of University of Science and Technology of China. Regarding concanavalin A (Con A)-induced hepatitis model [1,21,28], the mice were intravenously injected with Con A (15 μg/g wt. in total volume of 100 μl); sera of the mice were harvested at 12 and 24 h, respectively, after Con A injection for ALT/AST measurements. Moreover livers were excised for the histological analysis and hepatic MNC were prepared for cytotoxicity assay at the same time. Equally the small peptides were intravenously injected peptide (50 μg/g wt.) together with Con A and then examined their inhibitory effect on liver injury and natural cytotoxicity of liver MNC.

2.5. Assay for the serum ALT and AST

For assay of serum ALT and AST levels, mice were anesthetized with ether and bled via the eye. Sera (50 μl/test) were mixed with 0.5 ml of ALT or AST assay solution (Shanghai Rongsheng Company, China) and then measured in a spectrophotometer following the supplier's protocol.

2.6. Isolation of mononuclear cells (MNCs) from liver

Under deep ether anesthesia, mice were euthanized by exsanguinations from the subclavian artery and vein, and then the livers were removed. Liver mononuclear cells (LMNC) were obtained as previously described [42]. In brief, the liver was passed through a 200-gauge stainless steel mesh and then suspended in RPMI 1640 medium containing 2% FBS. After washing once, the cells were resuspended in 40% Percoll solution containing 100 U/ml heparin and centrifuged at 500 g for 10 min at room temperature. The pellet was resuspended in RBC lysis buffer that consists of 155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, and 170 mM Tris, pH 7.3. After incubation on the ice for 7 min, cells were harvested by centrifugation and washed twice in HBSS containing 5% FCS before use. The degree of contamination by Kupffer cells or hepatocytes was minimal.

2.7. Cytotoxicity assay

Four-hour ^{51}Cr release assay was processed as described previously [35]. Briefly, target cells were incubated with 40 μl ^{51}Cr (5 mCi/ml) (Perkin-Elmer Life Sciences, Inc., Boston, MA) per 10^6 cells to the final volume of 100 μl for 1 h at 37 °C under 5% CO_2, and then washed three times. All assays were performed in triplicate in 96-well plates at a predefined effector–target ratio. Spontaneous release of ^{51}Cr was determined by incubating the target cells with medium alone and was always less than 10%. Maximal release was determined by adding SDS to a final concentration of 5%. Based on the predefined effector–target ratio, effector cells were added to ^{51}Cr-labeled target cells in RPMI medium1640 to a total volume of 200 μl, and the mixture was incubated for 4 h at 37 °C in 5% CO_2. An amount of 100 μl of supernatants was taken from each well and monitored with a γ counter. Specific cytotoxicity was determined as following: % specific release = (experimental counts − spontaneous counts)/(maximal counts − spontaneous counts) × 100.

2.8. Statistical analyses of data

Student's *t*-test was used to interpret the significance of differences between experimental groups.

3. Results

3.1. Design, synthesis and property of the peptides of MICA mimicry

Based on the crystal structure of MICA and MICA–NKG2D complex that have been described [17,38], three shorter peptides mimicking functional α1 and α2 domain of MICA (residues 16–20 named as P1, residues 72–82 as P2 and residues 156–167 as P3) were synthesized, among which two peptides (P2 and P3) were lengthened at one or both ends (residues 58–84 named as P2L and residues 147–173 as P3L). The positions of the five peptides (P1, P2, P2L, P3, P3L) were shown in Fig. 1B. P1 consisted of five amino acids (DGSVQ) mimicking a part of α1 domain of MICA (position 16–20) (β1–β2 loop). P2 was composed of 11 amino acids (KDLRMTLAHIK), mimicking another part of α1 domain of MICA (position 72–82). P3 consisted of 12 amino acids (THYHAMHADCLQ), mimicking a part of α2 domain of MICA (position 156–167). The percentage of hydrophobic amino acids of the three small peptides was not more than 50%, and not at the ends. The value of PI is not far away from 7.0 except peptide 1 because it is too short (Table 1). So, all the peptides were easy to be dissolved. To calculate whether the sequences would stabilize the α-helical conformation for the peptides, we used amino acid sequence analysis software by two different methods (ANTHEPROT 4.3-Garnier and ANTHEPROT 4.3-Gibrat). The results were almost the same, indicating that the 2D structures of the synthesized peptides were nearly similar as those of corresponding elements, which was chosen as the templates in MICA. The main probability profile of each amino acid was shown in Table 2. Moreover, an unrelated peptide, Px (RDTAQIFRVNLRT) was synthesized. Px was in the α1 domain of HLA-E and had the similar position and structure to P2.

3.2. Inhibitory effect of the small MICA-mimicking peptides on cytotoxicity of NK-92 cells

The inhibitory effects of the small peptides were determined by using ^{51}Cr release cytotoxicity assay on human NK cell line (NK-92) against target tumor cells (Hela). At the effector/target ratio of 10:1, NK-92 cells (10^5 cells/well) were

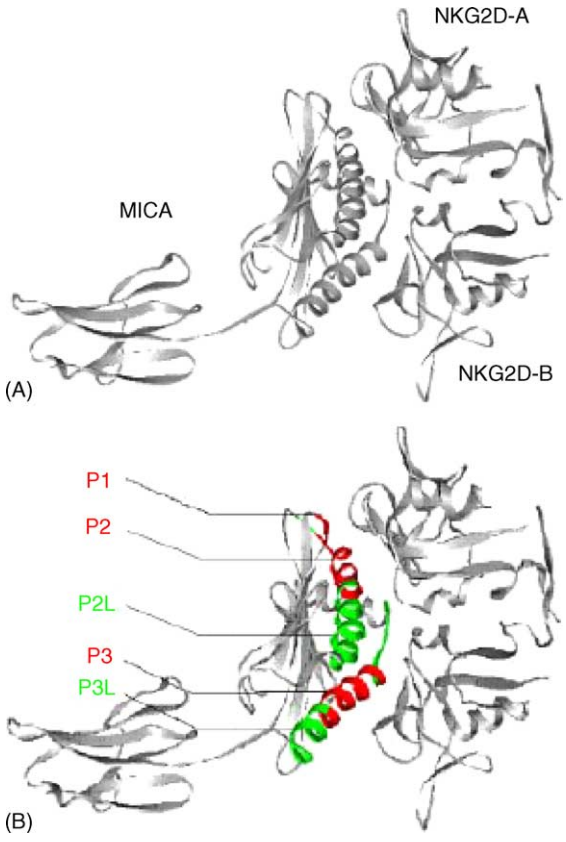

Fig. 1. The simulative position of the five synthetic peptides. (A) Three-dimensional modeling of the structure of NKG2D–MICA complex. Homodimer molecules of NKG2D are divided into molecules A and B (NKG2D-A and NKG2D-B), and single molecule MICA contains α1 domain, which interact with NKG2D-A, and α2 domain, which interact with NKG2D-B. (B) The simulative positions of the five synthetic peptides on MICA molecules and their relation to NKG2D. The red line means the normal-length synthesized peptides (P1, P2, P3), the green line means the two lengthened peptides (P2L, P3L).

first incubated in proper medium containing 1 nM peptide for 30 min at room temperature, and then added with ^{51}Cr-labeled target cells (10^4 cells/well). All three peptides, P1, P2 and P3 inhibited the cytotoxicity of NK-92 cells against Hela cells (Fig. 2A). In contrast, the unrelated peptide Px (10 folds of concentration, 10 nM) had no effect on the system of NK-92–Hela (Fig. 2A). We also examined the effects of the peptides on cytotoxicity of NK-92 cells against another target cell line, K562, which lacked MICA, and found that the peptides did not exert any effects on this NK-92/K562 system (Fig. 2B). These results demonstrated that the peptides we used were specifically acting on the NKG2D/MICA interaction. The dose response of the inhibitory effects of the small peptides was also examined. As indicated in Fig. 2C, the peptide dose-dependently inhibited the NK-92 cytotoxicity against Hela cells in the concentration of 1 nM^{-1} × 10^{-6} nM.

3.3. Increased inhibitory effect of the longer form or more binding sites of peptides

We also tested the inhibitory effects of the five peptides in different combinations. The results showed that each combination with two peptides (P1 + P2, P1 + P3, P2 + P3, each in 1 nM) exerted a stronger function than single peptide (Fig. 3A), the inhibitory percent (reduction of the percentage of specific lysis/the percentage of specific lysis of PBS group) was increased from 18% (P3) to 32% (P2 + P3). When three peptides (P1 + P2 + P3) were added together, the inhibitory effect reached 38% at maximum. The inhibitory capacity of combination with all three peptides (P1 + P2 + P3) was close to that of soluble MICA (sMICA) in the experiments (Fig. 3C), while the control peptides did not show any effects on this system (data not shown).

In addition, P2 and P3 were substituted by their longer form P2L and P3L, respectively. We found that longer form of peptide exerted a stronger inhibitory effect on NK-92 cytotoxicity, either alone or in combination with other peptides

Table 1
The polarity of the peptides

No.	Position	Sequence and polarity	PHI
P1	16–20	D (+) G (*) S (+) V (−) Q (+)	3.0
P2	72–82	K (+) D (+) L (−) R (+) M (−) T (+) L (−) A (−) H (+) I (−) K (+)	10.6
P3	156–167	T (+) H (+) Y (−) H (+) A (−) M (−) H (+) A (−) D (+) C (*) L (−) Q (+)	6.3
P2L	58–84	K (+) T (+) W (−) D (+) R (+) E (+) T (+) R (+) D (+) L (−) T (+) G (*) K (*) D (*) L (+) R (+) M (−) T (+) L (−) A (−) H (+) I (−) K (+) D (+) Q (+)	9.8
P3L	147–173	E (+) D (+) A (−) M (−) K (+) T (+) K (+) T (+) H (+) Y (−) H (+) A (−) M (−) H (+) A (−) D (+) C (*) L (−) Q (+) E (+) L (−) R (+) R (+) Y (−) L (−) K (+) S (+)	8.8

"+" means that the amino acid is hydrophilic; "−" means that the amino acid is hydrophobic;"*" means that the amino acid's polarity is not clear. The hydrophobic amino acids are not more than 50%, and not at the ends. The value of PHI is not far away from 7.0 except peptide 1 (it is too short). So the peptides are easy to be dissolved.

Table 2
The 2D simulative structures of the peptides

No.	Position	Simulation	2D sequence
P1	16–20		D G S V Q
		A	*T T C E E*
		B	C C C E E
P2	72–82		K D L R M T L A H I K
		A	*H H H H H H H H H H H*
		B	*H H H H H H H H H H H*
P3	156–167		T H Y H A M H A D C L Q
		A	*H H H H H H H H H H H H*
		B	*H H H H H H H H H H H H*
P2L	58–84		K T W D R E T R D L T G K D L R M T L A H I K D Q
		A	*C C C C T E T T H T C T T C C C H H H H H H H H H*
		B	*C C H H H C H H H C C C C H H H H H H H H H H H H*
P3L	147–173		E D A M K T K T H Y H A M H A D C L Q E L R R Y L K S
		A	*H H H H H H H H H H H H H H H H H H H H H H H H H*
		B	*H H H H H H H H H H H H H H H H H H H H H H H T T T H*

A: The peptides 2D structure was simulated by Garnier method; B: the peptides 2D structure was simulated by Gibrat method; *H*: helix; *E*: *sheet*; *T*: turn; *C*: coil.

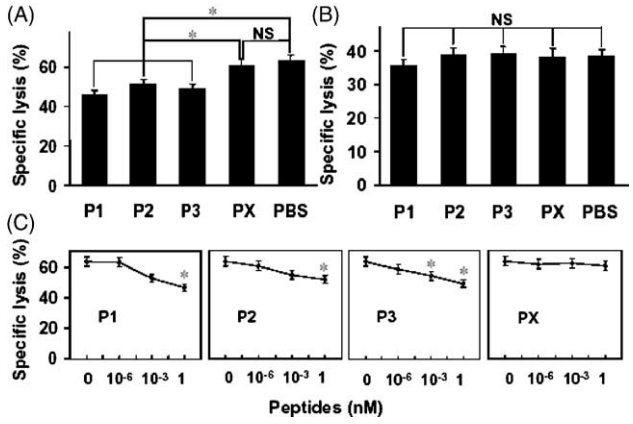

Fig. 2. The inhibitory effects of the synthetic MICA-mimicking peptides on cytotoxicity of NK-92 cells. (A) The inhibitory effects of the synthetic peptides on NK-92 (effector): Hela (target) system. The dosages of the peptides were 1 nM. The effector-to-target ratio was 10:1. Hela cells were MICA positive and sensitive to NK-92 killing. The peptides inhibited the killing of Hela cell exerted by NK-92 cells. (B) The inhibitory effects of the synthetic peptides on NK-92 (effector): K562 (target) system. The dosages of the peptides were 1 nM. The effector-to-target ratio was 5:1. K562 cells were NK-92-sensitive but MICA negative. The peptides did not exert any inhibitory effects on this E:T system. (C) The dose response of the synthetic peptides on NK-92:Hela system. The dosages of the peptides were 1 nM, 10^{-3} and 10^{-6} nM, respectively. The data were the mean ± S.E. of triplicate determinants (*$P < 0.05$). This experiment was repeated five times with similar results.

or other form of peptides (Fig. 3B), but the control peptides corresponding to the longer form peptides did not show any effects on this system (data not shown). It suggested that shedding of more amino acid sequence of α2 domain (for example, P3L > P3) or more domain sites of MICA [for example, P2 + P3 (α1 + α2) > P2 (α1) or P3 (α2)] is better than fewer sites binding. And the maximal inhibitory effect of P1 + P2L + P3L reached to the same level as that of sMICA (Fig. 3C).

3.4. Inhibitory effect of the MICA-mimicking peptides in vivo

Rodent homologues of MICA and MICB have not been identified, but other molecules, such as RAE-1 and H-60, serve as ligands of mouse NKG2D [16,27,39,43]. Murine NKG2D molecule is characterized as the similar primary amino acid sequence and global structure to human NKG2D [27,39]. So we tried to use these peptides targeting at human NKG2D functional domain in murine model. First, we found that P1 + P2 + P3 combination (1 nM for each peptide) could inhibit the natural cytotoxicity of murine splenocytes against murine NK target YAC-1 cells, which is RAE-1 positive [7] in vitro. Meanwhile, the Px peptide did not exert any effects on this system (Fig. 4A), revealing the possibility that these peptides mimicking MICA and targeting human NKG2D may act on murine NKG2D through the similar surface structure. The combination mixture of P1 + P2 + P3 was then intravenously injected into C57BL/6 mice 4 h earlier before harvesting of liver lymphocytes. The cytotoxicty of liver lymphocytes from peptides-treated mice against YAC-1 cells was decreased by 36.67% (Fig. 4B), suggesting that natural cytotoxicty in vivo was partly blocked by the MICA-mimicking peptides.

Furthermore, we used concanavalin A (Con A)-induced murine hepatitis, a well-recognized T cell-mediated liver injury model [1,21,28] to demonstrate the in vivo effect of the synthetic peptides on NKG2D-mediated autoimmune injury. After Con A intravenous injection (15 μg/g wt.), C57BL/6 mice developed distinguished liver injury as assessed by enhanced activities of circulating liver enzymes, i.e., plasma alanine aminotransferase (ALT, 5433 ± 692 IU/ml) at 6 h (Fig. 4C, PBS group). If injection of Con A and peptides (P1 + P2 + P3, 50 μg/g wt.) together, the ALT level was down to 3195 ± 461 IU/ml (41.19% inhibiting rate), and the unrelated peptides (Px) did not exerted any effects on liver injury, suggesting that MICA-mimicking peptide specifically

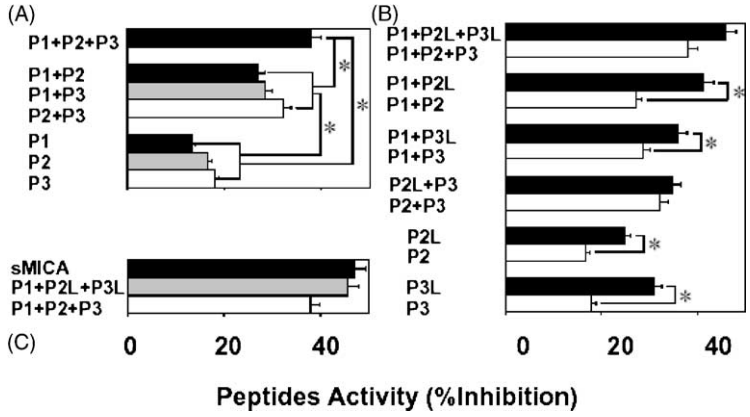

Fig. 3. The synergistic inhibitory effects of the peptide combinations on NK-92 cytotoxicity. The target of NK-92 cells was Hela cells (E:T = 10:1). The peptides were 1 nm. (A) The combination of P1, P2 and P3. The inhibitory effects of single peptide were weaker than any combination (combinations with two different peptides or three). (B) The combination of long form peptides. P2 or P3 were replaced by P2L or P3L (the long form peptide) in all combinations. The data are the mean ± S.E. of triplicate determinants (*$P < 0.05$). Similar results were obtained in three additional experiments.

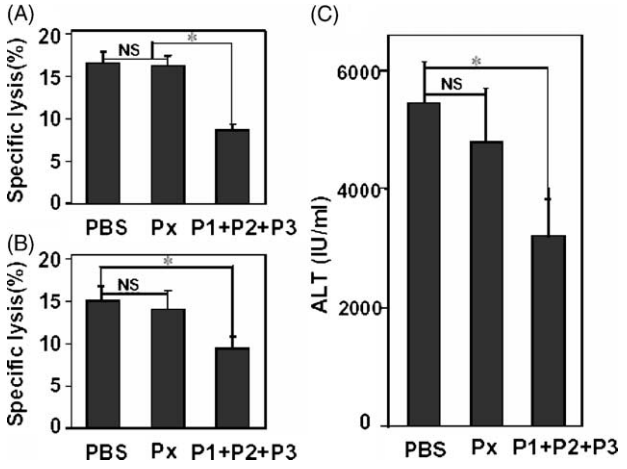

Fig. 4. The inhibitory effects of synthetic peptides on Con A-induced liver injury in vivo. (A) The direct inhibitory effects of peptides (P1 + P2 + P3, 1 nm each) on cytotoxicty of murine hepatic mononuclear cells (MNC) against sensitive target cells, YAC-1 cells, using 4 h ^{51}Cr release assay in vitro. The E:T ratio was 100:1. (B) The decreased cytotoxicity of liver MNC against YAC-1 cells after injection of mice with the peptides. Mice were injected i.p. with P1 + P2 + P3 (400 μl of 1 mM) 3 h before they were killed and the hepatic MNCs were prepared. The cytotoxicty assay was the same as in Fig. 4A. (C) The alleviation of liver injury by injection of the peptides into Con A-induced murine hepatitis model. After intravenous injection of peptides (P1 + P2 + P3, total 400 μl of each 1 mM) and Con A (15 μg/g wt.), the level of serum ALT was examined at 12 h. The data are the mean ± S.E. from five mice (*$P < 0.05$).

alleviated the Con A-induced, T lymphocytes-mediated liver injury.

4. Discussion

NK cells are innate effector cells playing a critical role in the defense against certain viral infections and tumors. Recent studies have indicated that the function of NK cells is regulated by the integration of signals from inhibitory and activating receptors. Inhibitory receptors specific for MHC class I molecules can provide protection for target cells that express normal levels of class I molecules on their surface. Although the inhibitory receptors play a key role in regulating NK cells, activating receptors are believed to be necessary for the initial activating of NK cells. Of particular interest is the lectin-like NKG2D receptor expressed on NK cells, TCR$\gamma\delta^+$ T cells, CD8$^+$TCR$\alpha\beta^+$ T cells, and activated macrophages. MICA, one of the ligands of NKG2D in human, displays up-regulated surface expression on stressed cells and is over-expressed by tumors. Recent studies show that the membrane expression of NKG2D ligands (MICA and MICB), in several tumor cell lines resulted in the rejection of the tumor cells, even when the tumor cells expressed normal levels of MHC class I molecules [11,18,32,40]. On contrary, over-expression of MICA, especially over-secretion of the soluble MICA molecules from tumor cells might alleviate the anti-tumor immune response by blocking interaction between NKG2D with membrane MICA on tumor [34], revealing the importance of NKG2D–MICA interaction in tumor surveillance or tumor escape. In this study, the soluble small peptides mimicking MICA molecules were the first observed to display a similar inhibitory effect on NK-92 cells against their target tumor, Hela cells, which further confirmed the published observation. On the other hand, it revealed that the small peptides designed by us may replace, at least partly, the function of full soluble MICA molecules, indicating that the peptides mimicking sites on MICA are important functionally domain.

The inhibitory effects of the soluble small peptides were also observed in a mouse hepatitis model, a Con A-induced, NKT cell-mediated and almost all lymphocytes including NK cells-involved liver injury [13]. The homologues of human MICA and MICB to mice have not been identified, but other molecules, such as RAE-1 and H-60, were identified to serve as ligands of murine NKG2D [16,27,39,43]. Moreover, murine NKG2D molecule is characterized with the similar amino acid sequence and global structure as human NKG2D [27,39] and murine NKG2D were constitutively expressed

on murine lymphocytes including T cells, NK cells and NKT cells [7,12,20,29,30,33]. Blockade of NKG2D recognition alleviated the pancreatic injury in murine autoimmune diabetes (NOD mice), indicating that NKG2D may be considered as a target molecule against autoimmune diseases. So, we tried to use peptides targeting on human NKG2D functional domains in murine model. It is the first time to indicate that NKG2D molecules may participate in the autoimmune process. Since NKG2D was not the only activating receptor expressed on NK cells and other lymphocytes, the cytotoxicity of NK-92 cells could not be completely blocked by these small peptides targeting at NKG2D molecules, similar as anti-NKG2D monoclonal antibody (mAb) or full length of soluble MICA molecules (sMICA). Because the molecular weights of these small peptides were much less than the anti-NKG2D mAb or sMICA protein, the small peptides targeting at NKG2D could be promising in explaining the interaction structure of MICA with NKG2D, and also utilizing in experimental therapy of NKG2D-related diseases.

We synthesized these peptides under the assumption that the peptides would bind to NKG2D. The active structures of the regions in MICA bound to NKG2D were both α-helix [17], and the computerized results showed that the 2D simulative structures of P2 and P3 were similar to the template regions of MICA molecules. Although the structure of P1 was not α-helix, it should form various structures in the solution due to its really short length, and the peptides of suitable structure (similar to the template regions) would not be absent. We believed that all the peptides formed suitable structures in the solution. Meanwhile, the amino acid sequences of the peptides were from the regions of MICA bound to NKG2D directly. So, based on the same sequence and similar structure, the peptides should have the similar surfaces to the templates, which might raise the possibility that the binding of surface NKG2D molecules with membrane MICA molecules on the target cells should be blocked by the peptides in the solution. Although we did not provide the data on direct biding of small peptides with surface NKG2D, our study had indicated that the inhibitory effects of these small peptides on NKG2D–MICA interaction were specific. All three peptides, P1, P2, and P3 inhibited the cytotoxic activity of NK-92 cells, the NKG2D positive cells [23] against Hela cells, which were MICA positive [44] in a dose-dependent manner (Fig. 2C). These inhibitory effects cannot be attributed to non-specific function of these small peptides, since the effect of the peptides was depended on the existence of MICA on the cell membrane. When the target cells lacked MICA, such as human haematopoietic cells – K562 cells [36], these small peptides did not exert any significant inhibitory effects (Fig. 2B). Moreover, the unrelated peptide had no effect on the same NKG2D–MICA interaction system (NK-92–Hela system). We also observed that combinational use of more peptides obviously strengthened the blocking capability. Each combination with two peptides (P1 + P2, P1 + P3, P2 + P3) exerted a stronger inhibition than single peptide (P1, P2, P3) (Fig. 3A), combination with three peptides (P1 + P2 + P3) reached maximal inhibition, replacement of P2 and P3 with their longer form P2L and P3L demonstrated enhanced inhibitory effects, suggesting that shedding of more amino acid sequence of α2 domain (for example, P3L > P3) or more domain sites [for example, P2 + P3 (α1 + α2) > P2 (α1) or P3 (α2)] are better than fewer sites binding. These data are helpful to explain the specific function of synthetic peptides on NKG2D–MICA interaction.

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