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A potential role of hyporesponsive NK cells in cancer defence and mice model to study nk cell education

NK cells education is an intensively studied field of immunology. In mice Ly49 receptor-H-2 class I interactions and KIR-MHC I in human mediate this process. NK cells bearing self-MHC specific inhibitory receptors are “licensed (L)” and lacking them are “hyporesponsive (HR)”. HR state can be reversible upon certain conditions. The role of HR NK cells in defence against cancer stem cells (CSC) was not investigated. HR NK cells have features making them more potent for this purpose compared to licensed counterparts. A mouse model to study NK cell education *in vivo* is described here, where Ly49 ligand binding ability and the influence on target cell lysis is unified. At least four types of mice can be a basis for many experimental designs. Mouse model is proposed to be applied to study the processes of HR NK cell involvement in CSC eradication.

Keywords: Natural killer cells, licensing, hyporesponsiveness, cytotoxicity, mice model, cancer stem cells.

В.А. Абрамова, Н.Н. Беляев

Ісіке қарсы қорғаныстағы гипореактивті NK жасушаларының потенциалды рөлі және NK жасушаларының «үйренуін» зерттеуге арналған тышқан үлгісі

NK – жасушалық оқыту иммунологияның өте кеңінен қолданылатын бөлімі болып табылады. Тышқандардағы Ly49 NK- жасушалық рецептордың H-2 I классының антигендермен және адамдардағы HLA I классының антигендерімен KIR-ң өзара әрекеттесуі бұл процестің ортақтайды. MHC-I -тің спецификалық ингибиторлық рецепторларын таситын NK – жасушалар «лицензиялы» болып табылады,яғни литикалық функцияға қабілетті. Олардың жоқтығы «гипожауапкершілікке» әкеледі. Лицензиялы NK - жасушалармен салыстырғанда HR NK- жасушалардың ісік жасушаларға қарсы қолданылуы MHC-I таситын нысананың лизисіне әкелетін қабілеті болса да бұрын қарастырылмаған. Ly49 лигандалардың унифицерлинген in vivo жағдайында NK- жасушалық оқытылу үшін тышқан моделі ұсынылды. Ең болмағанда 4 түрі эксперименталды протоколдар үшін қолданылады. CSC өлтіруі үшін бағытталған HR NK процесстерді зерттеуде осы ұсынылатын модель қолданылатын болады.

Түйін сөздер: табиғи киллер жасушасы, лицензиялау, гипореактивтілік, цитотоксинділік, тышқан үлгісі, ісік бағаналы жасушалары

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Потенциальная роль гипореактивных NK-клеток в противоопухолевой защите и мышиная модель для изучения «обучения» NK-клеток

NK-клеточное «обучение» является интенсивно изучаемой областью иммунологии. Взаимодействие NK-клеточного рецептора Ly49 с антигенами H-2 I класса у мышей и KIR с HLA I класса у людей опосредуют этот процесс. NK-клетки, несущие специфические ингибиторные рецепторы к собственным MHC-I, являются «лицензированными» (L), и обладают способностью лизировать неэкспрессирующие MHC-I клетки. Отсутствие этих рецепторов приводит к «гипореактивности» (HR). HR состояние может быть обратимо при некоторых условиях. Роль HR NK-клеток в защите против раковых стволовых клеток (CSC) не рассматривалась ранее. Предлагается мышиная модель для изучения NK-клеточного обучения in vivo, где Ly49 лиганд-связывающая способность и влияние на лизис клеток-мишеней унифицированы. По крайней мере, 4 типа мышей могут служить основой для многочисленных экспериментальных протоколов. Предлагаемая мышиная модель будет пригодна для применения к исследованиям процессов вовлечения HR NK в уничтожение CSC.

Ключевые слова: натуральные киллерные клетки, лицензирование, гипореактивность, цитотоксичность, мышиная модель, раковые стволовые клетки

NK cells play an important role in a host defence against tumor and virus-infected cells [1]. NK cells bear various activating and inhibitory receptors interacting with Major Histocompatibility Complex Class I molecules (MHC I). Human Killer Immunoglobulin-like receptor (KIR) interact with Human Leukocyte antigen (HLA-I). Analogous by function and not by structure Ly49 receptor family (C-type lectins, type-II transmembrane receptors) in mice use H-2 I class molecules as ligands. Remaining NK cell receptors use other ligands [1,2]. NK cells can be classified as licensed (L) and hyporesponsive (HR). L NK cells include NK expressing self-MHC- I specific inhibitory receptors, and NK cells not bearing them are HR [3,4]. NK cells acquire licenced state during development in bone marrow upon interactions with bone marrow stromal cells expressing MHC-I. L NK cells can recognize and kill MHC-I deficient targets (tumor cells, virus infected cells) and in contrast to HR [4]. In other relations L and HR NK are similar [5]. HR state in NK cells is flexible [6]. They can acquire killing capacity in certain circumstancies. The involvement of HR NK cells in cancer stem cells (CSC) eradication is proposed here.

Klra gene (encoding Ly49) content and its expression patterns vary in different mice strains [7,8]. As far as Ly49 receptor diversity make different contribution to target cell lysis depending on bind affinities to H-2 molecules [7], it is hardly to obtain a suitable mouse *in vivo* model based on H-2 and Ly49 interaction. Here a scheme of development of such a system is provided. We begin with mouse model description and then describe its application in studying anti-CSC response.

The mouse model. The proposed mouse model in general is dedicated to study NK cell education processes. Currently, Ly49-deficient [5], -transgenic or humanized (HLA-Cw3-KIR expressing [9]), various H-2 gene expressing [10] or non-expressing mice (β2m-, TAP1- or H-2K- H-2D- deficient [4]) exist. Undoubtely these methodological tools provide a basis for studying NK cell education process.

In the simplest case, each NK cell can bear Ly49 self-MHC-I specific inhibitory (IS);-self-MHC-I specific activatory (AS); non-self-MHC-I specific inhibitory (INS) and non-self-MHC-I-specific activating (ANS) (only one type). Also, each NK cell can bear at least binary combinations of these receptor types (Table 1).

Table 1 - Various combinations of Ly49 types on NK cells

Types of receptors	AS	IS	ANS	INS
AS	2	1	2	2
IS	1	1	1	1
ANS	2	1	2	2
INS	2	1	2	2
1 – L NK cells, 2 – HR NK cells				

So, in the settings of NK cell education, the minimal receptor set involved in this process, which encompass all possible interactions with single H-2 molecules, consists of four types of receptors and their combinations.

The mice will be choosen on C57BL/6 background, because in terms of NK cell licensing studies this strain is the best studied [8]. At least four variants of mice will be created. Eache of them express one of the following type of receptor (AS, IS, ANS or INS) with a uniformed affinity to the corresponding H-2 ligand

and the uniformed ability to influence target cell lysis according to their activating or inhibitory nature. Crosses between them can be made to investigate complicated receptor combinations (each NK cell will coexpress selected receptors). Bone marrow cells can be used in mixed bone marrow chimeras. In this case NK cells will bear Ly49 receptor types separately. Potentially, this system allows performing multiple experimental designs with different complexity to investigate processes during L or HR state establishment or maintenance.

The order of steps proposed for development of mice model. The mice resulting from sequential cross-breeding of Ly49 deficient [5], SV40TL- [11] and HLA-Cw3-KIR^{+} transgenic mice [9] to the state of homozygosity on C57BL/6 mice background ($K^{b-}D^{b-}$) will represent a basis for manipulations. It is anticipated that NK cells from this cross-breeding will be in licensed state.

1. NK cell line will be obtained from these mice (further designated as NKCL). NKCL will serve as a basis for transfection with AS, IS, ANS, INS Ly49 cDNAs. For the "self MHC" D^{d} will be considered. This molecule was shown to represent the second, by the NK educational force [10], H-2 ligand after K^{b}. K^{b} is not chosen, because of lack of defined AS Ly49 receptors [8].
2. In table 2 selected ligand-receptor combinations are shown. For the reference point, NKCL transfected with particular Ly49 cDNA can be used. Its binding to cognate H-2 ligand will be used as a binding strength parameter. The lysis of YAC-1 cells (MHC deficient) by Ly49 deficient NKCL will represent a basic level of lysis. Unification of Ly49 by these parameters will be done as follows: binding strength equal to reference receptor, influence on target cell lysis (increase or decrease, for activating or inhibitory Ly49, respectively) on a defined percent. In case of Ly49H, binding to m157 [12] may be lost.

In order to achieve such unification, NKCL should be transfected separately with Ly49 cDNAs. Chemical mutagen can be applied to NKCL bearing Ly49 constructs in order to increase the speed of generation and the diversity of Ly49 modified states. cDNA of altered Ly49 receptors will be isolated from selected NKCL clones and used to generate transgenic mice on the Ly49 deficient D^{d} (K^{b-} D^{b-}) transgenic background (as a starting point).

Table 2 - Proposed Ly49 receptors for unification (not all specificities are indicated in parentheses), according to [8]

AS	IS	ANS	INS
Ly49D (D^{d})	Ly49G2 (D^{d})	Ly49H (D^{b})	Ly49D (K^{b})

Role of Hyporesponsive NK cells in CSC lysis. Tumor represents a hierarchy of cell types, with CSC, representing the root of the tumor [13]. CSC are chemo- and radioresistant, and targeted therapy represents an urgent task [14]. Tumor derived CSC and normal SC express a little amount of MHC I, which can be upregulated upon IFNγ administration [15, 16]. Brain tumor derived stem cells express significant levels of HLA-A, -B, -C, possess an antigen processing machinery and are susceptible to CD8 mediated lysis [16].

HR NK cells are good effectors of immune response upon infection with MCMV [12]. HR state is not rigid in some experimental settings [6]. The following hypothesis is proposed. As L NK cells express self-MHC-specific inhibitory receptors, when CSC upregulate MHC I, they may avoid NK cell mediated lysis. The most critical for NK cell target discrimination is HLA-C expression, while HLA-A, -B are more critical for CTL-mediated lysis [17]. HR NK cells, bearing no self-MHC class I inhibitory Ly49 [4] can be indispensable, as MHC class I receptors do not represent an obstacle for them as potent inhibitory ligands. The question is by what mechanism the hyporesponsiveness can be relieved, as the molecular basis for HR state is poorly understood [3, 9]. HR should be relieved transiently, only for the period of time which is needed to implement lysis, any prolongations can be potentially dangerous to the host in terms of autoimmunity.

KIR expression is enriched on cytotoxic $CD56^{dim}$ subset in human [1], but cytotoxic subsets are decreased in cancer patients [18]. This can represent the strategy of tumor to avoid NK cell lysis in terms of KIR-bearing cells elimination.

Evaluation of the hypothesis using proposed mice model. The experimental model proposed here can be used in analyzing the role of NK cells HR state plasticity. An analysis will be performed using NK cells from obtained mice, bearing Ly49 receptors calibrated in binding strength and the influence on target cell lysis. Tumor recipients will be D^{d+} $K^{b-}D^{b-}$ Ly49 deficient mice (self NK cells are HR), transferred with IS^{+} (from D^{d+} $K^{b-}D^{b-}$ mice, licensed, congenic) or AS^{+} IS^{-} Ly49 NK cells (from D^{d+} or D^{d-} mice, HR, congenic).

Tumor should be arisen in D^{d+} $K^{b-}D^{b}$ mice by chemical carcinogenesis. The possibility of AS^{+} IS^{-} HR NK cells *de novo* acquisition of MHC I deficient cell lysis, involvement of AS^{+} NK cell in the tumor eradication will be assessed. Also, it will be clear, whether NK cell tolerance (hyporesponsiveness breakdown) can occur in conditions of tumor growth and not only during infection [12] or in artificial system with changed MHC-I environments [6].

For positive control of HR relief, NK obtained from Ly49 AS^{+} IS^{+}; AS^{-} IS^{+} or AS^{+} IS^{-} (all from D^{d} or $\beta2m^{-}$ mice, all HR) mice transferred to D^{d+} mice can be used. Each variant (by Ly49 receptors) will allow investigating the necessarity for certain kind of receptor to *de novo* cytotoxicity acquisition.

Questions to address are at least the following. If the *de nov*o licensing acquisition event does not occur in tumor bearing mice, whether there CSC by themselves or other components of tumor represent the culprits? If the induction of cytotoxicity occurs, is it acquired at the proximity to CSC? Whether HR NK cells "scan" for altered SC? Are HR relieved NK cells lie at the intermediate position between CD8+ cells, which require high MHC class I expression by the target [15], and L NK cells which recognize target with ever visible MHC levels? Whether HR loss is prohibited at the latest stages of tumor development and what factors contribute to this? Is HR subversion necessarily results in the acquisition of phenotypical changes similar to L-state acquired during development? This question is better to address in HLA-Cw3-KIR mice [9] as they express Ly49.

Cancer can arise at sites where chronic inflammation persists [18]. Whether in condition of experimental chronic inflammation cytotoxicity acquired NK cells pertain their function?

NK cell education process underneath such fundamental NK cell functions as lysis of virus-infected or transformed cells which have altered MHC expression or loss of it. Analogous processes take place in human [3, 17]. The contribution of particular KIR – donor HLA combinations in some malignancies have been studied [20]. Most research evaluating NK cells anti-tumor response is directed toward CD56 and CD16 expression profile of NK cells or on the bulk of NK cells [21]. Normal SC as well as CSC are susceptible targets for NK cell lysis and NK cells become anergic after this contact [22]. The studies were performed on the bulk NK cell population and *in vitro*.

Cytotoxicity acquired HR NK cells in theory are powerful tools for defence against CSC as they lack self-MHC-specific KIR or Ly49 receptors, in contrast to L NK cells. L NK can potentially lose their lysing ability after CSC acquire a certain level of MHC class I expression, only by this reason. HR relief should be transient in order to avoid autoimmunity, and possibly, at the late stages of cancer progression (equilibrium, escape [23]), HR subversion is hampered. Mechanisms, underlying L or HR establishement behind receptor binding are poorly defined [3, 4, 9] and are not discussed here.

To evaluate the possibility of this phenomenon, the proposed mouse model can be used. Possible drawbacks of model should be noted. Will the *in vitro* obtained unifications in ligand binding and target lysis be the same as *in vivo*? The full *Klra* locus in Ly49 deficient mice is not deleted physically, Ly49 robust downregulation occurred by another mechanism [5]. Will NKCL preserve the general trend of Ly49 expression (i.e. lack of it) during mutagenesis? The expression of Ly49 specific for self-MHC is adjusted to the level of ligand. In MHC I deficient mice the level of expression of particular Ly49 is higher than in mice with cognate MHC I gene expression [24]. Presumably, in this model constructs with promoters conferring rigid expression will prevent it.

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Оценка стимуляции антительного иммуного ответа под действием иммуностимуляторов различного происхождения в опытах на мышах

Установлена чёткая зависимость активности продукции вирусспецифических антител от вида применяемого иммуностимулятора: наиболее высокий уровень IgG был в группах иммунизированных субъединичной гриппозной вакциной в сочетании с иммуностимуляторами Ключевые слова: Хитозан, Квил А и Иммувир.

Ключевые слова: Иммуностимулятор, гуморальный иммунитет, вирус гриппа.

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Иммунды ынталандырушы антиденеңің иммундық жауабының әртүрлі әсер етуі арқылы, тышқандарға тәжірибе жүргізу

Анықталған анық тәуелді белсенді өнімді вирусөзіндік антиденелер қолданылатын иммунды ынталандырғыш: көпшілік жоғары деңгейі IgG сол топтағы иммунды екпеде суббірлікті тұмау вирусы тіркесімі иммунды ынталандырғыш Хитозан, Квил А және Иммувир.

Түйін сөздер: Иммунды ынталандырғыш, гуморалды иммунитет, тұмау вирусы.

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