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4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) regulates CTL activation and memory programming

Zhifeng Sun, Zhengguo Xiao *

Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742, USA

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ABSTRACT

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the major carcinogens in tobacco. NNK has been associated with various cancers in tobacco users, especially lung cancer. However, the effects of NNK on cytotoxic T lymphocytes (CTLs), the cells responsible for destrcution of maligant and pathogen-infected cells, has not been elucidated. Using transgenic CTLs *in vitro* and *in vivo*, we show that NNK can directly affect CTL activation. NNK can enhance the expression of adhesion molecule CD62L in CTLs during their activation *in vitro*, but has no effects on their expansion and production of effector molecules such as IFN and granzyme B. After transferred into recipient mice, however, the NNK pretreated CTLs suffer an early loss in expansion. The percentage of memory precursors is higher in NNK pretreated CTLs, but the total amount of memory precursors is similar to controls. The final memory CTL population from NNK pretreated CTLs is reduced, but sustains a more central memory phenotype. In conclusion, NNK can affect CTL activation by modulating adhension molecule expression and reducing memory programming.

1. Introduction

Cigarette smoking causes about 5 million deaths worldwide every year, and close to half a million deaths in the US [1–3]. Compared to nonsmokers, smokers have a 13 to 14-year shorter lifespan [1]. It has been well established that many diseases are related to cigarette smoking, such as a variety of cancers and increased susceptibility to infectious diseases [2,3]. There are about 60 carcinogens identified in cigarette smoke or tobacco products, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of most important [4,5]. NNK induces lung tumor in rats [6] over a wide range of doses [7], and is a likely candidate for smoke-associated lung, nasal, oesophageal and liver tumors [4,8,9]. The mechanisms for the carcinogenesis of these compounds are related to DNA adducts, which lead to mutations when DNA repairment is incomplete [4]. In addition, NNK can bind to nicotinic receptors, inhibiting apoptosis and enhancing transformation [10]. However, there is little information published about how the immune system, specifically Cytotoxic T lymphocytes (CTLs), is affected by these carcinogens.

CTLs can directly destroy malignant or pathogen-infected cells, but they have to be activated appropriately [11]. Full activation of CTLs requires three signals: antigen, costimulation and inflammatory cytokines such as IL-12 and type I interferon [11–13]. In addition, the brief activation with three signals can lead to memory programming, which leads to the formation of functional memory CTLs that provide protection against pathogen challenge [12]. More importantly, this programming can be affected by other signaling pathways such as mTOR [14,15]. It is unknown if carcinogens can affect CTLs, thus indirectly contributing to the increased cancer incidence and disease susceptibility in cigarette smokers [2,3].

In this report, we investigated how NNK, one of the major carcinogens in tobacco, acutely affects CTL activation. CTLs can sense NNK by upregulation of adhesion molecule CD62L, but NNK has no effects on the production of effector molecul. After transfer, NNK-pretreated CTLs suffered an early loss in expansion, but sustained a higher percentage of memory precursor population. The final memory population was reduced in NNK pretreated CTLs, but maintained a more central memory phenotype. Therefore, NNK can affect CTL activation by modulation of adhension molecule expression and reduction of memory programming. These data suggest that carcinogens may be able to modulate immune responses, thus affecting carcinogenesis and disease resistance.

2. Results

2.1. NNK regulates CD62L expression during in vitro CTL activation

IL-12 together with antigen and costimulation (3SI) can drive full activation of CTLs and program memory CTLs *in vitro*[11–13]. We sought to understand if this 3SI-driven memory programming could be affected by NNK. Briefly, purified naïve OT-I cells were stimulated with 3SI for 3 days [12]. NNK was added

* Corresponding author. Fax: +1 301 405 7980.
E-mail address: xiao0028@umd.edu (Z. Xiao).

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simultaneously at 3 different concentrations covering a 100-fold range from 0.01 to 1 mM as previously used in rats [5,7,16]. Activated OT-I cells were harvested at day 3 for analysis. Changes were not observed in CD25 (IL-2 receptor α subunit) (Fig. 1). Granzyme B is directly related to CTL killing ability [13], and IFNγ is closely related to CTL effector function [15]. There were no consistent changes induced by NNK at any dose (Fig. 1). CD62L is an adhesion molecule related to lymphocyte migration [17,18], and has been used as a marker for memory CTL precursors [17,19]. NNK increased CD62L expression in a dose-dependent way (Fig. 1). Interestingly, no effect of NNK was observed in CTL expansion even at high concentration (data not shown). Thus, NNK can directly regulate CD62L expression on CTLs during their activation, but seems not to affect their effector function.

2.2. NNK pretreatment causes an early loss of CTL expansion after transfer

Three-signal activated CTLs go through drastic expansion and temporary down regulation of CD62L after transfer into recipient mice [14]. To test the effects of NNK on this process, NNK pretreated CTLs were transferred into naïve mice. At the peak of expansion (day 5) [14], the CTL population in the blood of transferred mice was significantly reduced at NNK 0.01 to 0.1 mM levels, and further decreased when the level reached 1 mM (Fig. 1A). Consistent with our previous report [14], the transferred population went through a dip in expression of CD62L (Fig. 2B) compared to that in activated cells *in vitro* (Fig. 1). However, high concentration of NNK led to high CD62L expression (Fig. 2B). KLRG1 is an inhibitory receptor for T cell activation, and is associated with short-lived effectors [20,21]. KLRG1 expression was slightly enhanced when NNK was administered at 0.01 mM, whereas high doses (1 mM) caused reduced KLRG1 expression (Fig. 2C). These data indicate that NNK acute treatment at high doses may impair the expansion capacity of CTLs, and may promote an altered migratory phenotype.

CD62L High and KLRG1 Low are used as markers for memory precursors, which are correlated with the quantity of final memory CTLs [17–19,22]. Gating on these markers (Fig. 3A), NNK pretreatment was able to enhance the percentage of memory precursor at high concentration (1 mM) (Fig. 3B). Taking into account the loss of population (Fig. 2A), the memory precursor percentage of total CD8 in each of the treatments was pretty close (Fig. 3C). These data indicate that even though NNK does enhance the percentage of CD62L expressing CTLs, it does not change the total number of memory precursors.

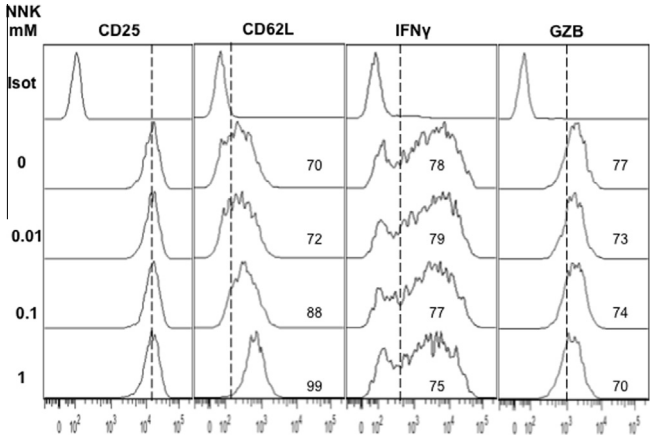

Fig. 1. NNK affects CTL activation. Purified OT-I cells were cultured for 3 days with 3SI in the presence of NNK at different concentrations. Cells were harvested for comparison of IFNγ, granzyme B (GZB) and other surface molecules. Data are representatives of at least three experiments with similar results.

2.3. NNK pretreatment results in reduced memory

To determine if acute NNK exposure had any effects on 3SI-driven memory programming, 10^6 harvested CTLs were transferred into naïve B6 mice, which sat for 30 days for CTL memory to develop [14]. Memory mice were examined for the presence of memory CTLs in the blood. Surprisingly, pretreatment of CTL with NNK led to reduced memory CTL formation at all three levels (Fig. 4A). This is different from the memory precursor prediction based on the peak of CTL expansion in (Fig. 3C). However, the expression of CD62L was significantly higher than the control (Fig. 4B) and KLRG1 was the lowest (Fig. 4C and D) at the highest NNK level in pretreatment. These data suggest that the higher expression of CD62L and low expression of KLRG1 may be the intrinsic regulatory function of NNK on CTLs, but may not be directly associated with memory CTL precursors.

To test the functionality of the memory CTLs from NNK pretreatment, memory CTLs were examined in the spleens of memory mice. Typical memory CTLs generated after 3SI treatment *in vitro* can produce IFNγ [14]. Although NNK pretreatment caused decreased memory CTL formation (Fig. 4A), the percentage of IFNγ-producing cells in memory CTLs was not affected by NNK at any concentration (Fig. 4E and data not shown). Interestingly, most of the IFNγ -producing memory CTLs also produced TNFα (Fig. 4E and data not shown). To examine the protection ability of these memory CTLs, memory mice were challenegd with LM-OVA [12,14,23]. Three days after challenge, LM was recovered from the spleen of challenged memory mice. The 3SI programmed memory CTLs controlled LM infection (Fig. 4F), and this memory protection was not affected by NNK (Fig. 4F), indicating that the memory CTLs from NNK pretreatment were functionally protective during pathogen challenge.

3. Discussion

NNK is one of the major carcinogens associated with cancers in human smokers. CTLs play an essential role in the control of malignant cells and intracellular pathogens [24–26]. It is unclear if carcinogens can affect CTL activation, which may be related to carcinogenesis and disease resistance.

Here, we present evidence that NNK can directly affect CTLs during their activation. Although their expansion and production of effector molecules are not altered, adhesion molecule CD62L was upregulated during the acute treatment *in vitro*. More importantly, the *in vitro* pretreatment caused reduced expansion capability after transfer, but CD62L expression was maintained at high levels (Fig. 2). It will be interesting to test if NNK can similarly regulate CD62L expression in other immune cells. The final memory CTL formation was decreased in the NNK-pretreated groups (Fig. 4A), which was more of central memory CTL phenotype (Fig. 4B–D). These data clearly demonstrate that CTLs can respond to NNK even after acute exposure.

Despite the reduced memory population by NNK pretreatment *in vitro* (Fig. 4A), there was no significant difference in the final protection against LM-OVA challenge (Fig. 4F). Protection is positively associated with the number of memory CTLs, but the association is not strictly linear. Thus the about 2-fold reduction of memory CTLs by NNK pretreatment may not be reflected in protection due to the high efficacy of memory CTLs in control of pathogen challenge (Fig. 4F). In addition, IFNγ and TNFα production was not affected by NNK pretreatment, suggesting that the memory CTLs are functional. However, with smoking, which exposes CTLs to low dose

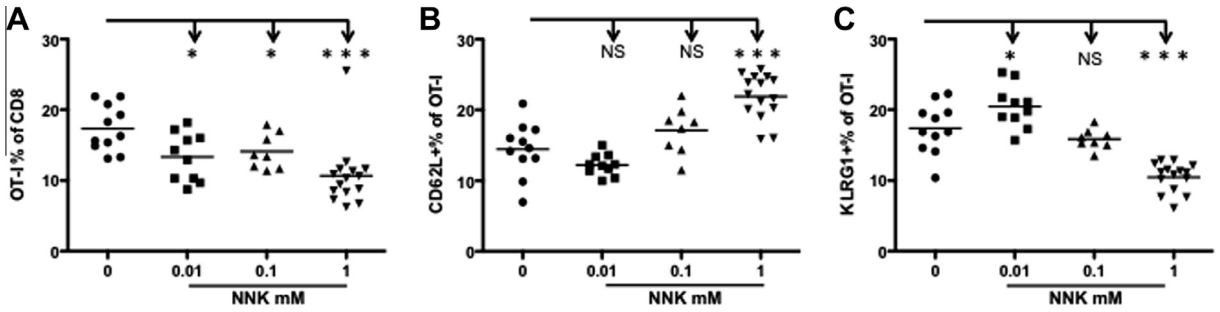

Fig. 2. NNK reduces CTL expansion after transfer into recipients. Purified OT-I cells were cultured for 3 days with 3SI in the presence of NNK at different concentrations. Three days after stimulation, CTLs were harvested and transferred into B6 recipients at 10^6 cells/mouse. (A) Comparison of percentage of OT-I cells in total CD8 cells in blood at day 5 post transfer. (B and C) Comparison of the phenotype of OT-I cells in blood from mice at day 5 post transfer. Asterisks indicate statistical significance. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. NS: no significance. This will be the same for the rest of figures.

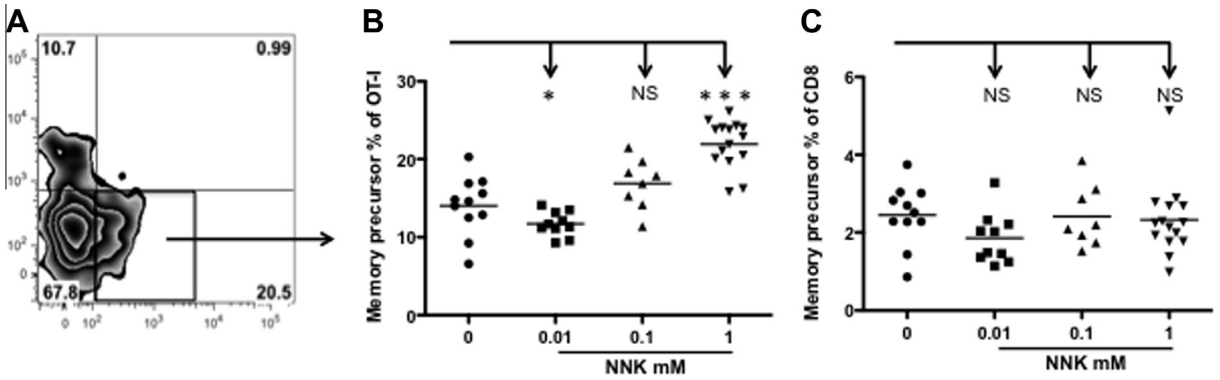

Fig. 3. NNK effects on memory precursors. Comparison of memory precursors in the blood at day 5 post transfer. (A) Gating strategy on memory precursors. (B) Comparison of the percentage of memory precursors in OT-I cells. C. Comparison of the percentage of memory precursor in CD8 cells.

NNK for decades, the effects may become accumulative and therefore different from its acute effects.

Although we have established clear evidence that NNK can directly affect CTL memory programming, elucidation ofunderlying molecular mechanisms is needed and is currently underway. NNK can cause DNA adducts in cells in the lung, pancreas, liver or oral epithelium [10,27], and CTLs should be similarly susceptible to these effects in smokers. In addition, CTLs express many types of nicotinic receptors [28], which can receive signals from nicotine and NNK [10]. It will be interesting to test if one or both of these mechanisms are involved in the sensitivity of CTLs to NNK.

There are some important implications for these observations in this report. First, this experiment used acute exposure of NNK to CTLs for 3 days, and already demonstrated significant regulation from NNK on CTLs. Although in smokers the concentration of NNK in their blood and urine is much less than these concentration we used [4,5,7], the chronic (for decades) exposure of smokers to NNK may have an even more significant impact on the immune system. There are three possible consequences for CTLs in long time smokers. First, NNK chronic exposure may alter the way of naïve CTLs to respond to pathogens or malignant cells. For example, by changing the expression of adhesion molecule CD62L (Fig. 1, Fig. 2B and Fig. 4B), activated CTLs may remain preferably in secondary lymphoid tissues instead of migrating to the site of infection or malignancy, thus leading to less efficacy in control of tumor development or pathogen infection *in situ*. Second, the induction of memory CTLs in vaccination may be impaired by NNK either directly or indirectly. As shown here, CTLs can directly sense NNK. In addition, NNK may affect other cell type, such as dendritic cells, thus altering their antigen presentation to CTLs. Third, once memory CTLs are induced after vaccination, they stay and go through slow homeostatic proliferation [17,21]. The chronic exposure of established memory CTLs to NNK may lead to accumulated mutation similar to other somatic cells [4]. Vaccine efficacy may suffer a loss in memory CTLs or malfunction after decades of chronic exposure to carcinogens. Therefore, carcinogens may modulate immune system to facilitate cancer development and increase susceptibility of smokers to pathogens, in addition to potential deleterious effects on vaccination.

4. Materials and methods

4.1. Mice and reagents

OT-I mice (a gift from Dr. Mescher, University of Minnesota) expressing a transgenic TCR specific for H-2K^b and OVA$_{257-264}$[29] were crossed with Thy1-congenic B6.PL-Thy1a/Cy (Thy1.1) mice (Jackson ImmunoResearch Laboratories, Bar Harbor ME) and bred to homozygosity. C57BL/6 mice were purchased from the National Cancer Institute. All mice were maintained under specific pathogen-free conditions at the University of Maryland, and these studies have been reviewed and approved by the Institutional Animal Care and Use Committee. NNK was purchased from Sigma (St. Louis, MO), and NNK stock was made in DMSO. All directly conjugated fluorescent antibodies were purchased from BD Biosciences, eBioscience or Biolegend.

4.2. Naive T cell purification

The naïve T cell purification was performed as previously described [12,14]. In brief, axillary, brachial, cervical, inguinal, and mesenteric lymph nodes (LNs) were harvested from WT OT-I mice, pooled, and homogenized, thus creating a single cell suspension. FITC-labeled antibodies specific for CD4, B220, I-A^b, and CD44 were incubated with the cells. The subsequent addition of Anti-FITC

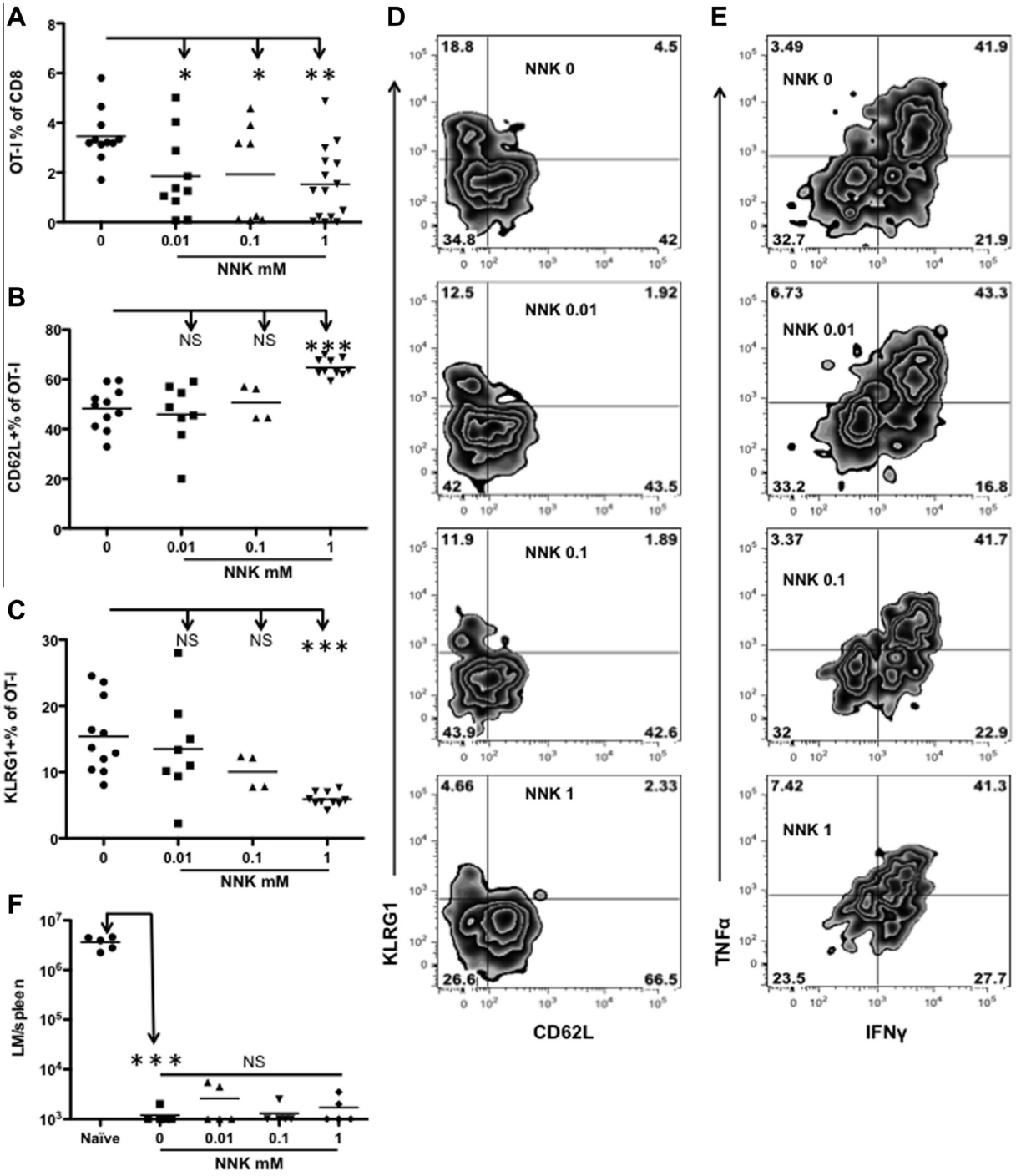

Fig. 4. NNK impairs memory CTL programming. Purified OT-I cells were cultured for 3 days with 3SI in the presence of NNK at different concentrations. Three days after stimulation, CTLs were harvested and transferred into B6 recipients at a concentration of 10^6 cells/mouse. At day 30 post-transfer, memory CTLs were examined. (A) Comparison of memory CTLs in blood. (B–E) Comparison of phenotype of memory CTLs. (F) Recipient mice were challenged with LM-OVA as previously reported [14] and protection was examined at day 3 after challenge in spleens. "Naive" control mice were transferred with 10^5/mouse naïve OT-I cells.

magnetic MicroBeads (Miltenyi Biotech) to the cells allowed filtration using separation columns attached to a MACS magnet. Cells that did not bind to the column were collected, and exhibited a purity of >95% $CD8^+$ cells and <0.5% $CD44^{hi}$ Cells. Purified naive OT-I cells were then sorted to reach close to 100% purity.

4.3. Adoptive transfer and flow cytometric analysis

In vitro activated OT-I cells identified as $CD8^+Thy1.1^+$ were adoptively transferred into normal C57BL/6NCr mice by i.v. (tail vein) injection of 10^6 cells/mouse. The analysis of memory CTLs was based on samples from spleen and/or blood obtained at indicated times. After preparation of single cell suspensions, flow cytometry was used to determine the percentage of OT-I cells present. Identical staining of cells from normal C57BL/6 mice (no adoptive transfer) was used as background for determining OT-I cell numbers. A FACSCalibur™ flow cytometer and CELLQuest™ software (BD Biosciences) were used for determining the percentage and total number of OT-I cells in the samples. Flowjo software (Tree Star Inc.) was used for data analysis.

4.4. Intracellular cytokine staining after in vitro stimulation

Spleen cells from adoptively transferred mice were incubated at a concentration of 2×10^6 cells/ml in RP-10 with 0.2 μM $OVA_{257-264}$ peptide and 1 μl Brefeldin A (Biolegend) for 3.5 h at 37 °C. Cells were then incubated in fixing buffer (Biolegend), and subsequently permeablized in Saponin-containing Perm/Wash buffer (Biolegend), each step for 15 min at 4 °C. The fixed and permeablized cells were then stained with PE-conjugated antibody to IFNγ and APC-conjugated antibody to TNFα for 30 min at 4 °C. Cells were then washed once with Perm/Wash buffer, and once with PBS containing 2% FBS.

4.5. In vitro stimulation of naïve OT-I T cells

In vitro stimulation of purified (as described above) naïve OT-I PL T cells was performed in flat bottom microtiter wells coated with antigen (DimerX H-2 Kb:Ig fusion protein loaded with $OVA_{257-264}$ peptide; BD Pharmingen) and recombinant B7–1/Fc chimeric protein (R&D Systems) as previously described [12,14]. Each well of the 24 well plate contained 3×10^5 cells in 1.5 ml of Allos media along with 2.5 U/ml of IL-2 and 2 U/ml of murine rIL-12 (R&D Systems). NNK stock was diluted with corresponding culture medium as indicated. Transferred cells were identified by staining with anti-Thy 1.1 and anti-CD8 mAbs.

4.6. Statistical analysis

Data was graphed and analyzed using a two-tailed Student's *t* test (GraphPad Prism 5.0 software). Comparisons with a *P* value of <0.05 were considered significantly different.

5. Conflict of interest

The authors have no financial conflict of interest.

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