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Identification of Primary Natural Killer Cell Modulators by Chemical

Library Screening with a Luciferase Based Functional Assay

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Abstract (max 200 words)

Natural Killer (NK) cells are essential players of the innate immune response that secrete cytolytic factors and cytokines such as IFN- γ when contacting virus-infected or tumor cells. They represent prime targets in immunotherapy as defects in NK cell functions are hallmarks of many pathological conditions such as cancer and chronic infections. The functional screening of chemical libraries or biologics would greatly help identify new modulators of NK cell activity, but commonly used methods such as flow cytometry are not easily scalable to high-throughput settings. Here we describe an efficient assay to measure the natural cytotoxicity of primary NK cells where the bioluminescent enzyme NanoLuc is constitutively expressed in the cytoplasm of target cells, and is released in co-culture supernatants when lysis occurs. We fully characterized this assay using either purified NK cells or total PBMCs, including some patient sample, as effector cells. A pilot screen was also performed on a library of 782 metabolites, xenobiotics and common drugs, which identified dextrometorphan and diphenhydramine as novel NK cell inhibitors. Finally, this assay was further improved by developing a dual reporter cell line to simultaneously measure NK cell cytotoxicity and IFN- γ secretion in a single well, extending the potential of this system.

Introduction

NK (Natural Killer) cells are essential players of the immune response against viruses and tumor cells¹. As group 1 innate cytotoxic lymphoid cells, they are characterized by a potent cytotoxicity and the ability to secrete IFN- γ when activated. In human, they represent 5-20% of peripheral blood mononuclear cells (PBMCs). NK cells express a set of membrane receptors allowing them to discriminate normal cells from abnormal, virus-infected or tumor cells. Tumorigenesis and viral infection are indeed often accompanied by the downregulation of NK cell inhibitory ligands and/or the upregulation of activator ligands at the cell surface, allowing target cell detection by NK cells. Once activated, NK cells release the pore forming protein perforin², and pro-apoptotic factors including granzymes and death ligands (FasL, TRAIL, etc). Besides, secreted IFN- γ contributes to the activation of other effector cells and the induction of an appropriate immune response. As such, NK cells are prime targets in immunotherapy and defects in their activation are hallmarks of pathological conditions including cancers, genetic disorders and viral chronic infections. Functional assays measuring NK cell cytotoxicity *in vitro* or *ex vivo*, are therefore essential to evaluate the potential impact of small molecules, antibodies or proteins on this immune cell population.

The reference method to measure the cytotoxic activity of NK cells *ex vivo* is based on the release of chromium 51 (⁵¹Cr) by target cells pre-loaded with this radioactive isotope³. When target cells are co-incubated with NK cells, the permeabilization of their membrane is accompanied by the release of ⁵¹Cr in culture supernatant, which reflects the cytolytic activity of NK cells. This method has been widely used for decades, but is progressively abandoned because of the risks and regulatory constraints associated with the manipulation of radioactive elements. A more recent and popular method is based on the use of multi-color flow cytometry to quantify the lysis of target cells in co-cultures⁴. Fluorescent dyes or specific membrane markers are used to discriminate NK cells from target cells at the time of analysis,

while the fraction of dead target cells is calculated using viability dyes. Alternatively, NK cell cytotoxicity can be indirectly quantified by surface immunostaining of the degranulation marker CD107a (LAMP1), which is correlated with NK cell-mediated lysis⁵. Although widely used, all methods based on flow cytometry require some expensive equipment, trained personnel, and are time-consuming at both the acquisition and analysis steps. For these reasons, they are not well adapted to large-scale screening projects, and alternative strategies have been developed to measure NK cells activation in high-throughput settings.

Established strategies include the quantification of secreted IFN- γ by ELISA in co-culture supernatants, which is tractable for high-throughput screenings (HTS)^{6, 7}. However, IFN- γ secretion is not always correlated with the cytotoxic activity, a limitation that adds to the significant cost and multi-step protocol inherent to ELISA. Other described tests involve the co-culture of PBMCs or NK cells with adherent target cells⁸. After incubation, effector cells and dead target cells are washed out, and the number of living target cells still attached to culture wells is quantified by different methods. Alternatively, the number of adherent target cells in co-cultures, without the need of washing-out NK cells, can be determined by electrical impedance measurements⁹. However, such assays are limited to adherent target cells, and rely on the quantification of surviving cells in culture wells rather than a direct quantification of cell lysis. Finally, non-radioactive alternatives to ⁵¹Cr have been developed such as calcein, a fluorescent molecule, that can be loaded as a pro-form (Calcein-AM) in target cells¹⁰. However, calcein staining relies on endogenous cellular esterase for processing the pro-form of the dye and was criticized for its low signal-to-noise ratio. Europium was also used as a non-radioactive substitute to ⁵¹Cr, but never became popular due to some high background observed in many target cell lines¹¹. Other cytotoxicity assays are based on the release of endogenous cytosolic enzymes such as lactate dehydrogenase¹². This enzyme can be detected in culture supernatants with synthetic substrates that are converted into colored or

fluorescent products. The sensitivity of these methods has been a matter of debates, in particular in co-cultures because when dying, not only target cells but also effectors cells potentially release such endogenous enzymes in the medium. Therefore, there are still some unmet needs for a sensitive high-throughput assay to measure the cytotoxicity of NK cells.

Along with the development of *firefly* luciferase as a reporter in biological systems, bioluminescent assays have been engineered to measure NK cell cytotoxicity. Decarboxylation of the luciferase substrate luciferin by *firefly* luciferase leads to light emission that can be quantified. Interestingly, this reaction requires ATP that is essentially present in living but not dead cells. Thus, target cells were engineered to express *firefly* luciferase, and co-cultured with NK cells. When luciferin is added to culture wells, it penetrates all cells in the co-culture but is only processed by luciferase-expressing target cells that are alive and produce ATP¹³. This method was shown to outperform the standard ⁵¹Cr cytotoxicity assay, but is dependent on intracellular ATP levels and signal-to-noise ratio can be low in common target cell lines such as K562¹⁴. In parallel, other research groups tried to develop cytotoxicity assays based on the release of *firefly* luciferase in culture supernatants when lysis occurs^{15, 16}. However, this approach was hampered by the short half-life of *firefly* luciferase in culture medium at 37°C. Recently, the development of high performance luciferases derived from marine organisms has provided a new start to this approach^{17, 18}. The best example is NanoLuc, a synthetic luciferase of 19 kDa developed by Promega from a luminescent enzyme of the sea shrimp *Oplophorus gracilirostris*¹⁹. This enzyme uses furimazine as a substrate, of which decarboxylation is ATP independent. NanoLuc is >150 times brighter than *firefly* luciferase and more importantly, is stable for days in culture medium. We took advantage of this new generation of luciferase to develop a high-throughput functional assay to measure the natural cytotoxicity of primary NK cells (Fig. 1). Two clonal cells lines expressing high levels of NanoLuc in their cytoplasm, called K562-NL and

twINNE, were engineered, and NanoLuc release in culture supernatant was determined in co-cultures with purified effector cells including primary NK cells, total PBMCs from healthy donors, treated or not with cytokines or immunomodulatory compounds, and PBMCs from a perforin deficient patient. We also established that NanoLuc release by target cells is robust enough to screen chemical libraries in high-throughput settings and to identify modulators of NK cell activity. Finally, we further improved this assay by developing the dual reporter cell line twINNE where a *firefly* luciferase reporter gene is used to monitor interferon signaling in target cells while in parallel, NanoLuc release is reporting their lysis.

Material and Methods

Cells and culture conditions

Cells were cultured at 37°C and 5% CO₂ in RPMI-1640 medium (Sigma-Aldrich; R8758) containing 10% fetal calf serum (FCS). Human K562 cells were kindly provided by Dr. T. Walzer (CIRI, Lyon). The reporter cell line STING-37, corresponding to HEK-293 cells stably transfected with the ISRE-luciferase reporter gene, was previously described ²⁰. Blood from healthy blood bank donors was obtained from “Etablissement Français du Sang” (Convention # 07/CABANEL/106; Paris; France). Human peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation with Lymphoprep medium (StemCell Technologies) from total blood or leucocytes concentrates obtained when plateletpheresis is performed. NK cells were purified from PBMC using the NK cell isolation kit from Miltenyi. Recovered NK cells were pure at >94% as assessed by their CD3⁻/CD56⁺ phenotype. A patient with a confirmed homozygous missense mutation in *PRF1* was included in this study. A blood sample was obtained upon informed consent.

Cytokines, small compounds and reagents

IL-2, IL-15, and TGF- β were obtained from Peprotec. R848 was obtained from Sigma-Aldrich, Torin2 was from Tocris, and Rapamycin was from Calbiochem. Luciferase induction in twINNE cells was determined using the Bright-Glo (Promega) or Britelite plus reagents (PerkinElmer), according to the manufacturer's recommendations. NanoLuc expression was quantified in culture supernatants as previously described¹⁹. Cellular viability was determined by quantification of ATP in culture wells using the CellTiter-Glo assay (Promega). Bioluminescence was measured for 0.1 s with a luminometer (EnSpire; PerkinElmer). Tergitol NP9 was from Sigma-Aldrich.

Establishing K562-NanoLuc and twINNE cell lines

The NanoLuc gene was introduced in target cells by transduction with the pLVX-Puro lentiviral vector (Clontech). First, the NanoLuc sequence was cloned in the pLVX-Puro vector using the Gateway cloning system (Thermo Fisher Scientific). Briefly, the pLVX-Puro plasmid was made compatible with the Gateway system by inserting the Gateway cassette C1 at the SmaI site to generate the new destination vector called pLVX-Puro-GW. A pDONR207 plasmid containing the coding sequence for the NanoLuc enzyme (Promega) was kindly provided by Dr. Y. Jacob (Institut Pasteur, Paris). The NanoLuc sequence was transferred by *in vitro* LR recombination from pDONR207 to pLVX-Puro-GW vector following manufacturer's recommendations to obtain pLVX-Puro-NanoLuc. This vector was used to produce lentiviral particles by co-transfection with packaging plasmids pVSV-G and pGag-Pol (kindly provided by Dr. S. Nisole; IRIM; Montpellier) into HEK-293T cells. Culture supernatants containing lentiviral particles were collected two days later and stored at -80°C.

K562 and STING-37 cells were transduced with the lentiviral vector expressing NanoLuc, and puromycin at 1 $\mu\text{g/ml}$ was applied for 4 weeks to enrich the cellular pool for transduced cells. Using a cell sorter, 576 and 63 cells were cloned from the transduced

populations of K562 and STING-37 cells, respectively. After 4 weeks, culture wells were tested for the expression of NanoLuc to identify best growing clones expressing high levels of the enzyme. Top candidates were amplified for establishing the K562-NL and the twINNE cell lines, which respectively correspond to K562 and STING-37 clones expressing high levels of NanoLuc.

Cell cytotoxicity assay.

PBMC from healthy donors were incubated overnight in the absence or presence of compounds to be tested. Unless specified otherwise, PBMC were plated in 96-well round-bottom plates at a concentration of 400,000 cells/well in a final volume of 100 μ L. After 16 h, 16,000 K562-NanoLuc or 100,000 twINNE cells were added to cultures wells (100 μ L of a cell suspension at 160,000 cells/ml or 1.10^6 cells/ml, respectively). Total volume in culture wells was 200 μ L, and plates were centrifuged briefly for 2 min at 1,200 rpm. Unless specified otherwise, 20 μ L of culture supernatants were collected after 4 h or 7 h of co-culture for K562-NL or twINNE, respectively, and NanoLuc activity was determined by adding 40 μ L of RPMI-1640 and 30 μ L of NanoLuc reagent (as described in ¹⁹; Promega) in black, flat-bottom, 96 $\frac{1}{2}$ -well plates. Bioluminescence was measured during 0.1 s with a luminometer (Enspire; PerkinElmer).

To quantify the death of target cells by flow cytometry, K562-NL cells were labeled with the VDP450 dye at 1 μ M in PBS (BD Biosciences). After 10 min of incubation at 37°C, cells were washed thoroughly in culture medium, en co-cultured with PBMCs as described above. After 4 h, cells were collected and dead cells were labeled using the LIVE/DEAD fixable green stain reagent from Invitrogen following manufacturer's recommendations. Cells were analyzed by flow cytometry, and the percentage of dead K562-NL cells was determined by gating on the VDP450⁺ cells.

Screening of the TMIC Human Metabolome Library.

The TMIC Human Metabolome Library was obtained from The Metabolomics Innovation Center in Canada (TMIC; www.metabolomicscentre.ca). It is a comprehensive library of compounds representing a broad range of metabolites, xenobiotics and drugs commonly found in body tissues. Briefly, 5 mg of each compound was dissolved in 2 ml of either pure water or a 50% mix of water and DMSO to achieve a final stock concentration of 2.5 mg/ml. Compounds were rearranged in deep-well plates, and daughter plates were prepared by spotting 2 μ L in round-bottom 96-well plates for screening. Culture wells were filled with PBMC at 400,000 cells/well in a final volume of 100 μ L as described above, so that test compounds were at 50 μ g/ml. For each screening plate, negative control wells were filled with culture medium alone, whereas positive control wells were filled with PBMC plus 2 μ L of a 50% water:DMSO mixture. After 16 h, 16,000 K562-NanoLuc cells were added to all cultures wells (100 μ L of a cell suspension at 160,000 cells/ml). NanoLuc activity in culture supernatants was determined after 4 h of co-culture as described above, and results were expressed as a percentage relative to positive control wells. The Z' factor that reflects the robustness and quality of the assay was calculated from means (μ^+ and μ^-) and standard deviations (σ^+ and σ^-) of negative and positive controls such as $Z' = 1 - 3 * (\sigma^+ + \sigma^-) / (\mu^+ - \mu^-)$ as described by Zhang JH & al ²¹. Very good assays have Z' factors that are close or superior to 0.5. The signal-to-background ratio (S/B ratio) corresponds to μ^+ / μ^- , and the signal-to-noise ratio (S/N ratio) to $(\mu^+ - \mu^-) / \sigma^-$ as previously defined ²¹.

Results

Development and assessment of the K562-NL reporter cell line

Myeloid leukemia cells K562 were transduced with a lentiviral vector to express NanoLuc, and cell clones were carefully screened to select those expressing this bioluminescent enzyme at high levels. The best cellular clone was amplified to establish the K562-NL reporter cell line. First, we tested if NanoLuc is released in culture supernatants when membrane permeabilization occurs. K562-NL cells were treated with 1% Tergitol as a detergent to lyse cells and culture supernatants were harvested at different time-points. Cell lysis was confirmed by standard light microscopy (data not shown). As presented in Fig. 2A, high levels of NanoLuc activity were detected in culture supernatants and were maximal after only 3 min. As expected, ATP in culture wells, which is a common biomarker of cellular metabolic activity and used to determine viability, was gradually degraded in the 0-30 min time-window showing that NanoLuc release is concomitant with cell death.

Since our goal was to determine whether K562-NL cells can be used as target cells to measure the cytotoxic activity of NK cells in PBMCs, we verified that NanoLuc activity in culture supernatants is not impaired by the addition of a large number of PBMCs in the culture wells. Increasing numbers of K562-NL cells - ranging from 2,500 to 80,000 cells - were dispensed in culture wells of a 96-well plate and killed by freezing-and-thawing. Then, 400,000 PBMCs were added or not to culture wells and after centrifugation, culture supernatants were immediately collected to quantify NanoLuc activity. As expected, the NanoLuc activity dramatically increased in culture supernatants when K562-NL cells were killed and more importantly, the signal was not affected by the addition of PBMCs to culture wells (Fig. 2B). We also verified that the NanoLuc signal in culture supernatants of killed K562-NL cells mixed with PBMCs was stable in time, without any significant loss over 24 h (data not shown). Altogether, this demonstrated that NanoLuc release in culture supernatants of K562-NL cells is suitable to monitor their lysis by NK cells.

Measuring NK cell cytotoxicity with K562-NL target cells

A fixed number of K562-NL cells – herein referred as the target cells – were co-cultured with PBMCs – the effector cells – at different effector/target (E/T) ratios ranging from 0.8 to 50. After 4 h of incubation, culture supernatants were harvested and tested for NanoLuc activity. Results were expressed as the percentage of NanoLuc released by K562-NL cells permeabilized with 1% Tergitol. As shown in Fig. 3A, NanoLuc activity increased proportionally with E/T ratios. We also performed the same experiment quantifying the number of dead K562-NL cells in co-cultures by chemical staining (LIVE/DEAD reagent) and conventional flow cytometry analysis. NanoLuc accumulation in culture supernatants appeared to be more sensitive than flow cytometry, as the killing of K562-NL cells was detectable at lower E/T ratios (Fig. 3A). We also performed the same co-culture experiment at a 25/1 ratio and quantified NanoLuc in supernatants at different time-points (Fig. 3B). As expected, the signal continuously increased over 8 h of co-culture. After 24 h of co-culture, 100% lysis was reached in these co-culture conditions (data not shown). However, 4 h were sufficient to obtain a strong signal that is statistically distinct from K562-NL cells alone. Finally, donor-to-donor variation was assessed by testing PBMCs from 13 different donors at an E/T ratio of 25/1, and measuring lysis of target cells after 4 h of co-culture (Fig. 3C). Altogether, this demonstrated the efficiency and robustness of this assay to detect the cytotoxic activity of PBMCs.

The percentage of NK cells in PBMCs varies from 5 to 20%. We thus performed the same assay with NK cells purified from PBMCs. As expected, NanoLuc activity increased in culture supernatants of K562-NL cells when co-cultured with purified NK cells (Fig. 3D) and reached high levels at lower E/T ratios than previously reported with total PBMCs. Furthermore, we showed that when PBMCs were depleted of NK cells, NanoLuc did not accumulate anymore in co-culture supernatants (Fig. 3E). Reconstitution with purified NK

cells restored the cytotoxic activity of PBMCs and the accumulation of NanoLuc in culture supernatants. Altogether, this data demonstrated the key role of NK cells in the cytotoxicity of PBMCs towards K562-NL cells and the accumulation of NanoLuc in culture supernatants.

Testing the effects of known modulators of NK cell cytotoxicity

We then tested in this assay the effect of small compounds or cytokines that are known to modulate NK cell cytotoxicity, either directly or indirectly. First, PBMCs were stimulated with R848 (or resiquimod), a small agonist of TLR7 and TLR8. R848 mimics the immunostimulatory effects of single-stranded viral RNA molecules and stimulates NK cell cytotoxicity through the activation of antigen-presenting cells. PBMCs were pretreated overnight with R848, and K562-NL target cells were added. After 4 h of co-culture, NanoLuc activity in culture supernatants was measured. As shown in Fig. 4A, R848 increased NanoLuc levels in co-culture supernatants as a consequence of NK cell stimulation. Similar results were obtained when pretreating PBMCs with IL-15, a cytokine known to increase the cytotoxicity of NK cells (Fig. 4B)²². Alternatively, PBMCs were pretreated overnight with TGF- β , a cytokine known to inhibit NK cell functions²³. As expected, NanoLuc accumulation in co-culture supernatants was reduced (Fig. 4B). Similar results were obtained with Torin2, a potent inhibitor of the mTOR pathway that is essential to NK cell cytotoxicity (Fig. 4B)²². Altogether, these results demonstrated that our assay is suitable to screen small compounds or cytokines to identify new inhibitors or activators of NK cells.

Familial histiolymplocytosis (FHL) is a severe inflammatory condition characterized by abnormally activated macrophages infiltrating multiple tissues, hepato-splenomegaly, non-remitting high fever, cytopenia, hypertriglyceridemia and hyperferritinemia. It is caused by gene mutations that impair NK and CD8 T cell cytotoxic function. In this group of diseases, perforin (PRF1) deficiency is responsible for FHL type 2 (FHL2). Quick diagnosis of FHL

and potent treatment are essential to prevent fatal evolution. To determine if our assay could help in the diagnosis of such pathological condition, PBMCs from a perforin deficient patient were tested. Because of technical constraints, PBMCs from this patient and the matching healthy control were conserved for overnight at 4°C before the assay could be performed. This probably accounts for the low cytotoxic activity detected with control PBMCs even at a high E/T ratio. Nevertheless, results obtained clearly showed that PBMCs from the perforin deficient patient have completely lost their cytotoxic activity as assessed by the absence NanoLuc accumulation in culture supernatants (Fig. 4C). Therefore, this assay can be used to monitor *ex vivo* the cytotoxic activity of NK cells from patients while starting from a blood sample of only few milliliters and could be used as a screening test in the diagnosis of diseases caused by defects in lymphocyte cytotoxicity.

Screening a chemical library of metabolites, xenobiotics and common drugs

Our next objective was to test if our NanoLuc-based functional assay was suitable to rapidly screen hundreds of molecules using fresh PBMCs. To establish a proof of concept, we selected a chemical library from The Metabolomics Innovation Center (TMIC, Canada) that aggregates 782 metabolites, xenobiotics, and frequently used over-the-counter medications: the TMIC Human Metabolome Library. Our objective was to determine if some of these molecules might impact the function of NK cells, with potential consequences on the organism's ability to fight against virus infections or tumors.

PBMCs from two healthy donors were incubated overnight with compounds at 50 µg/ml, and K562-NL target cells were added to evaluate the cytotoxic activity of NK cells. Each screening plate included either 4 (Donor #1) or 7 (Donor #2) positive and negative controls that corresponded to PBMCs + K562-NL cells or K562-NL cells alone, respectively. After 4 h of co-culture, NanoLuc activity was measured in culture supernatants. On average,

the signal-to-background (S/B) ratio, which corresponds to the mean value of positive controls (μ^+) divided by the mean value of negative controls (μ^-), was 8.6 for Donor #1, and 19.2 for Donor #2. The signal-to-noise (S/N) ratio, which corresponds to the difference of μ^+ minus μ^- relative to the standard deviation of negative controls (σ^-), was 22.2 for Donor #1, and 66.5 for Donor #2. The Z' factor, which reflects the overall quality of the assay and is calculated with the formula $1-3*(\sigma^+ + \sigma^-)/(\mu^+ - \mu^-)$, was 0.49 for Donor #1 and 0.61 for Donor #2. Altogether, these statistical parameters qualify this assay for HTS²¹. In parallel, PBMCs were incubated in the same conditions with test compounds, and ATP levels in culture wells were determined to identify toxic molecules (Fig. 5A). 80 compounds reduced ATP levels by more than 25%, thus supporting some significant impact on cellular metabolic activity and viability. Of the remaining 702 compounds, 12 compounds were found to inhibit the lysis of target cells by >75% in the two screens, supporting the inhibition of NK cell cytotoxicity towards target cells (Fig. 5A).

Interestingly, 6 of these compounds were previously reported to inhibit NK cells (Fig. 5B), thus confirming the robustness of our screening pipeline²⁴⁻²⁹. We also identified 6 compounds as new inhibitors, including 3 metabolites (7-dehydrocholesterol, oleanolic acid, and tryptamine), and 3 medications (dextrometorphan, diphenhydramine, brompheniramine). These compounds were retested in dose-response experiments using PBMCs from at least three healthy donors to estimate the half maximal inhibitory concentrations, which ranges from 10 to 126 μ M (Fig. 5C). Dextrometorphan and diphenhydramine were particularly interesting because of the potent inhibition of target lysis, and a limited impact on the viability of PBMCs.

Development of a dual reporter assay to measure both target cell lysis and IFN secretion

NK cell activation is associated to the production of type II interferon (IFN- γ), which is released through vesicles and secretory pathways that are distinct from cytotoxic granules³⁰. In order to further improve our screening assay, we developed new target cells for monitoring both the cytotoxic activity and the production of interferons by NK cells. We previously developed a HEK-293 cell line stably transfected with a *firefly* luciferase reporter gene controlled by Interferon-Stimulated Response Elements (ISRE) in its promoter (STING-37 cell line)²⁰. These reporter cells not only respond to type I IFNs but also to IFN- γ , which efficiently induced the expression of *firefly* luciferase (Fig 6A), making them suitable to detect the secretion of this cytokine by activated NK cells. Thus, STING-37 cells were engineered to express NanoLuc, so that NK cell cytotoxicity can be concomitantly monitored in the same culture wells. Best clones were amplified and carefully selected to establish a reporter cell line that we called twINNE because of its dual reporter system based on *firefly* and NanoLuc luciferases. When these cells were co-cultured with PBMCs for 7 h at relatively low E/T ratios ranging from 0.5:1 to 4:1, NanoLuc expression in culture supernatants increased as a consequence of target cell lysis (Fig. 6B). In parallel, the cellular expression of *firefly* luciferase increased as the ISRE-luciferase reporter was induced in co-cultures (Fig. 6B). When the same experiment was performed at higher E/T ratios, the expression of *firefly* luciferase did not increase (data not shown), probably because target cells were lysed before the induction of the ISRE-luciferase reporter. Therefore, this dual reporter system is allowing the quantification of both target cell lysis and interferon signaling from a single well, provided that experiments are performed at low E/T ratios. Finally, we tested Torin2, diphenhydramine, and dextromethorphan in this new assay. As expected, all three compounds were found to inhibit the lysis of target cells but interestingly (Fig. 6C, upper panel), only Torin2 completely blocked the expression of the ISRE-luciferase reporter (Fig. 6C, lower panel). The situation was intermediate with dextromethorphan, while the ISRE-luciferase

reporter was barely inhibited by diphenhydramine (Fig. 6C, lower panel). These profiles demonstrate the inhibitory properties of these drugs, but also support distinct modes of action for Torin2, dextrometorphan and diphenhydramine and different consequences on NK cell functions. Altogether, this validates our assay and highlights the interest of such a dual reporter system.

Discussion

NK cells play a central role in the innate immune response against intracellular pathogens and tumor cells, are considered as prime targets for innovative immunotherapies, and functional defects are associated to pathological conditions. Both natural and artificial molecules, including cytokines, drugs and natural products, have been defined as modulators of NK cell activity, and some of them are developed for medical purposes. The identification of novel modulators requires both target-based approaches and functional screenings that are complementary. But the latter is complicated since current NK cell assays are not easily adapted to the high-throughput screening of large libraries of compounds. Besides, quick functional assays, ideally usable at the patient's bedside, are needed for the quick diagnosis of pathological conditions such as FHL where NK cell cytotoxicity is altered.

Luciferase-based functional assays have become increasingly popular because of the exceptional performances of this family of bioluminescent enzymes that emit a strong signal and are fully compatible with biological systems. Some attempts have been made to develop cell cytotoxicity assays based on firefly luciferase, but the poor stability of this enzyme after release in the extracellular medium greatly limited their performances^{15, 16, 31}. This analytical issue was solved with the development of brighter, smaller and much more stable luciferases of marine origins such as NanoLuc that use coelenterazine or its chemical analog furimazine as a substrate¹⁷⁻¹⁹. As we were developing our system, two research groups recently

developed with success their own cytotoxicity assays by expressing these enzymes in target cells. They were used to measure Antibody-Dependent Cellular Cytotoxicity (ADCC), target cell lysis by Chimeric Antigen Receptor (CAR) expressing T cells, and complement-dependent cytotoxicity^{32, 33}. Performances were found to be at least similar to ⁵¹Cr release that is considered as the gold standard. Here, we established our own assay to measure the natural cytotoxicity of primary NK cells using appropriate targets, *i.e.* K562 or HEK293. Using PBMCs as a source of NK cells, this assay can be used to measure the inhibitory or stimulatory properties of small compounds or cytokines, but also to detect functional defects in PBMCs from patients (Fig. 4C). In the future, it will be interesting to further miniaturize this assay using 384-well plates as blood samples from patients, especially when testing babies, are limited in volume. Furthermore, we demonstrate the scalability of this assay as assessed by excellent statistical parameters. As proof of concept, we used the following settings to identify inhibitors of NK cells: $0.4 \cdot 10^6$ PBMCs/well at a 25/1 E/T ratio in a 96-well format. If we consider that $1 \cdot 10^9$ PBMCs are usually purified from one standard blood bag (500 ml), cells from a single donor are sufficient to screen 25 collection plates including 80 compounds and control wells, which corresponds to 2,000 different molecules. This makes possible the screening of medium-scale libraries on primary effector cells, a key point when looking for relevant cytotoxicity modulators.

From the screen of the TMIC Human Metabolome Library, we identified 6 molecules that were previously described in literature for their inhibitory properties on NK cells²⁴⁻²⁹, and 6 molecules that were not reported for this activity before. Interestingly, several of these molecules fall into pharmacological classes of drugs previously identified by Theorell & al.³⁴ as modulators of antibody-dependent NK cytotoxicity, including adrenergic receptor ligands (propranolol), Ca^{2+} channel blocker (verapamil, diltiazem), H1R-antagonist (diphenhydramine, brompheniramine), serotonergic, dopaminergic, and muscarinergic

antagonists (diphenhydramine, brompheniramine, dextrometorphan) —, and with sometimes opposite effects (17 α -Ethinylestradiol). However, all these drugs have multiple side targets, and mechanisms responsible for their biological activity towards PBMCs, and NK cells in particular, remain elusive without further investigations. Among the new NK cell inhibitors identified in the screen, dextrometorphan and diphenhydramine were the most interesting, showing some potent effects with a limited cytotoxicity on PBMCs. Dextrometorphan (*d*-3-methoxy-17-methylmorphinan) belongs to the morphinian family, and is widely prescribed as a cough suppressant but is also used in the treatment of specific neurological disorders. It shows no affinity for the opioid receptor, but was characterized as a nanomolar to low micromolar range antagonist of NMDA receptor, an agonist of the σ 1 receptor (σ 1R), a serotonin–norepinephrine reuptake inhibitor, an antagonist of nicotinic receptors, and an inhibitor of voltage-gated calcium and potassium channels³⁵. Interestingly, it was previously reported to have immunosuppressive properties at higher concentrations, for example inhibiting the maturation of conventional dendritic cells³⁶, but also disease progression in experimental mouse models of autoimmune disorders^{37, 38}, or inflammation and oxidative stress in heavy smokers³⁹. Mechanisms involved are still poorly understood, but could also control the activation of NK cells. One hypothesis is that dextrometorphan, through interactions with σ 1R or voltage-gated channels, perturbs Ca²⁺ mobilization that is essential for NK cell activation and degranulation. A second inhibitor of interest identified in the screen was diphenhydramine, a common first generation H1-antihistamine, which is closely related to brompheniramine that was also among selected hits. The IC₅₀ of diphenhydramine for the H1 receptor is in the nM range, which is incompatible with the higher concentrations required for inhibiting NK cells (Fig. 5C). Diphenhydramine has pharmacological interactions with multiple secondary targets, including muscarinic and α -adrenergic receptors, but also organic cation, serotonin, dopamine and norepinephrine transporters⁴⁰. Several of these

receptors are expressed by immune cells, and are potentially involved the inhibitory effects of diphenhydramine on NK cells. This is supported by a report showing that diphenhydramine is suppressing the innate immune response in a mouse model of septic peritonitis, and these effects are not mediated by H1R⁴¹. Altogether, our data suggest that diphenhydramine, dextrometorphan, and related drugs that are widely used to treat the symptoms of common cold may have unwanted effects on the innate immune response. This adds elements to the current debate on the low benefit/risk ratio of these molecules. Finally, it should be noticed that we did not identify NK cell activators in the screen, probably because immunostimulatory molecules, that potentially induce inflammation, are relatively rare amongst prevalent metabolites or frequently used drug.

Finally, we further improved our screening system by developing an original dual-reporter cell line to monitor both the secretion of IFN- γ by NK cells and their cytotoxic activity. Results obtained showed a good correlation between NK cell cytotoxicity and IFN- γ expression as expected (Fig. 6B). Interestingly, when testing NK cell inhibitors in this dual reporter system, we found that Torin2 efficiently blocked target cell lysis and the IFN response, whereas the effects of dextromethorphan and diphenhydramine were skewed for the inhibition of NK cell cytotoxicity (Fig. 6C). This suggests that these drugs have different modes of action. Cytotoxic granules with perforin/granzymes and vesicles containing IFN- γ are separate entities, which are delivered to the surface of NK cells through different secretory pathways³⁰. While the secretion of IFN- γ vesicles is not polarized, cytotoxic granules are released at the contact interface with target cells and this pathway could be selectively inhibited, in particular by diphenhydramine. Further experiments using purified NK cells and cellular imaging should be performed to test this hypothesis. Finally, it should be stressed that the dual-reporter assay we developed should be performed at a relatively low E/T ratio to let the expression of IFN- γ induce the ISRE-luc reporter before target cells are killed. Provided

that this rule is properly followed, this dual reporter system will provide much more complete functional data on the activation of NK cells when screening libraries of compounds or biologics in high-throughput settings. In the future, this should help identify modulators of NK cells of interest for developing innovative therapies.

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Figure Legends

Figure 1: *Principle of the luciferase-based cytotoxicity assay.* PBMCs are co-cultured with target cells expressing high levels of NanoLuc in the intracellular compartment. After few hours of incubation, NK cells from PBMCs mediate the lysis of target cells, thus releasing NanoLuc in culture supernatant. NanoLuc activity in culture supernatant reflects target cell lysis.

Figure 2: *Nanoluc accumulates in culture supernatants of K562-NL cells when killed by chemical or physical treatments.* (A) K562-NL cells were treated with 1% Tergitol, and NanoLuc released in culture supernatants was determined at different time-points. Alternatively, ATP in culture wells was quantified as a measure of cellular viability. Raw luminescence values were normalized to the signal obtained with living K562-NL cells alone. (B) K562-NL cells were dispensed at different concentrations in culture wells, and maintained alive or killed by freezing-and-thawing. When indicated, 400.000 PBMCs were added to culture wells containing dead K562-NL cells. Cell cultures were immediately centrifuged, and NanoLuc activity quantified in culture supernatants. Raw luminescence values were normalized to the signal obtained with 80.000 living K562-NL cells alone. All data correspond to means $\pm$ SD of three independent experiments. “ns” is for non-significant as determined by two-way ANOVA with Bonferroni post-hoc test.

Figure 3: *Measuring NK cell cytotoxicity with K562-NL target cells.* (A) PBMCs were co-cultured with K562-NL cells at different effector/target (E/T) ratios. NanoLuc activity in culture supernatants was measured after 4 h of incubation. Results were expressed as a percentage of NanoLuc released by K562-NL cells permeabilized with 1% Tergitol. In parallel experiments, the number of dead K562-NL cells in co-cultures was determined by

LIVE/DEAD viability staining and flow cytometry analysis. (B) PBMCs were co-cultured with K562-NL at a 25/1 ratio, and Nanoluc activity in culture supernatants was determined at different time-points. (C) PBMCs from 13 different donors were co-cultured with K562-NL at a 25/1 ratio, and Nanoluc activity in culture supernatants was determined after 4 h of co-culture. Each circle corresponds to one specific donor, and the mean $\pm$ SD is represented (D) NK cells were co-cultured with K562-NL cells at different E/T ratios, and NanoLuc activity in culture supernatants was determined after 4 h of incubation. (E) Total PBMCs or PBMCs depleted of NK cells (w/oNK) were co-cultured with K562-NL cells at a 25/1 ratio, and NanoLuc activity in supernatants was determined after 4 h of incubation. The “w/oNK+NK” condition corresponds to PBMCs depleted of NK cells that were reconstituted with 8% of purified NK cells. (A-D) Results were expressed as a percentage of NanoLuc released by K562-NL cells permeabilized with 1% Tergitol. All data correspond to means $\pm$ SD of at least three donors. (*) $p < 0.05$, (**) $p < 0.01$ (***) $p < 0.001$ as determined by one-way ANOVA with Bonferroni post-hoc test. “ns” is for non-significant.

Figure 4: Testing the effects of known modulators of NK cell cytotoxicity. (A) PBMCs were pretreated overnight with R848 (15 μ M) to stimulate the cytotoxicity of NK cells. K562-NL target cells were added, and NanoLuc activity in culture supernatants was determined after 4 h of co-culture. Data correspond to means $\pm$ SD of one representative experiment of three performed in triplicate. (B) PBMCs were pretreated overnight with IL-15 (100 ng/ml), TGF- β (10 ng/ml) or Torin2 (250 nM), and then K562-NL target cells were added. Experiment was performed at an E/T ratio of 30, and cells were co-cultured for 4 h at 37°C before NanoLuc activity was determined in culture supernatants. Data correspond to means $\pm$ SD of three donors. (C) The cytotoxicity of PBMCs from a perforin $-/-$ patient was compared to a healthy donor. PBMCs were co-cultured with K562-NL at different E/T ratio and after 7 h at

37°C, NanoLuc activity in culture supernatants was determined. Results were expressed as a percentage of NanoLuc released by K562-NL cells when permeabilized with 1% Tergitol (A and C) or co-cultured with IL2-stimulated PBMCs at a 100:1 E/T ratio (B). Data correspond to means $\pm$ SD of one experiment performed in duplicate. (*) $p < 0.05$, (**) $p < 0.01$ (***) $p < 0.001$ as determined by two-way ANOVA (A and C) or one-way ANOVA (B) with Bonferroni post-hoc test. “ns” is for non-significant.

Figure 5: *Screening a chemical library of metabolites, xenobiotics and common drugs.*

PBMCs from two healthy donors were used to screen the TMIC Human Metabolome Library. (A) Screening pipeline showing the selection of non-toxic inhibitors of NK cell cytotoxicity. The viability of PBMCs was determined by ATP quantification in culture wells. Measuring NanoLuc levels in culture supernatants assessed the inhibition of target cell lysis. (B) Twelve compounds were found to inhibit target cell lysis in two independent screens. pIC_{50} s correspond to $-\log_{10}$ values of the IC_{50} s as determined on PBMCs from ≥ 3 healthy donors. References correspond to former studies already showing the inhibition of NK cells. (C) Dose-response experiments showing the viability of PBMCs and the inhibition of target cell lysis. (A-C) Results were expressed as a percentage of NanoLuc released by K562-NL cells when co-cultured with untreated PBMCs. Data correspond to means $\pm$ SD of ≥ 3 healthy donors.

Figure 6: *Measuring NK cell cytotoxicity and IFN secretion with dual-reporter cells.*

(A) Recombinant human IFN- γ is inducing the ISRE-Luciferase reporter gene in twINNE cells. Firefly luciferase activity was determined after 24 h of incubation. (B) PBMCs were co-cultured with twINNE cells at different effector/target (E/T) ratios. NanoLuc activity in culture supernatants was measured after 7 h of incubation. Results were expressed as a

percentage of NanoLuc released by twINNE cells permeabilized with 1% Tergitol. In parallel, ISRE-Luc induction was determined in cell cultures at the same time point. (C) Same experiment as above at an E/T ratio of 4 with PBMCs pre-treated overnight with Torin2 (500 nM), dextromethorphan (Dex.; 140 μ M) or diphenhydramine (Diph.; 170 μ M). Upper and lower panels correspond to NanoLuc activity in supernatants and ISRE-Luc induction in co-cultures, respectively. (A-C) Data correspond to means $\pm$ SD of ≥ 3 experiments performed in duplicate. (*) $p < 0.05$, (**) $p < 0.01$ (***) $p < 0.001$ as determined by one-way ANOVA with Bonferroni post-hoc test. “ns” is for non-significant.

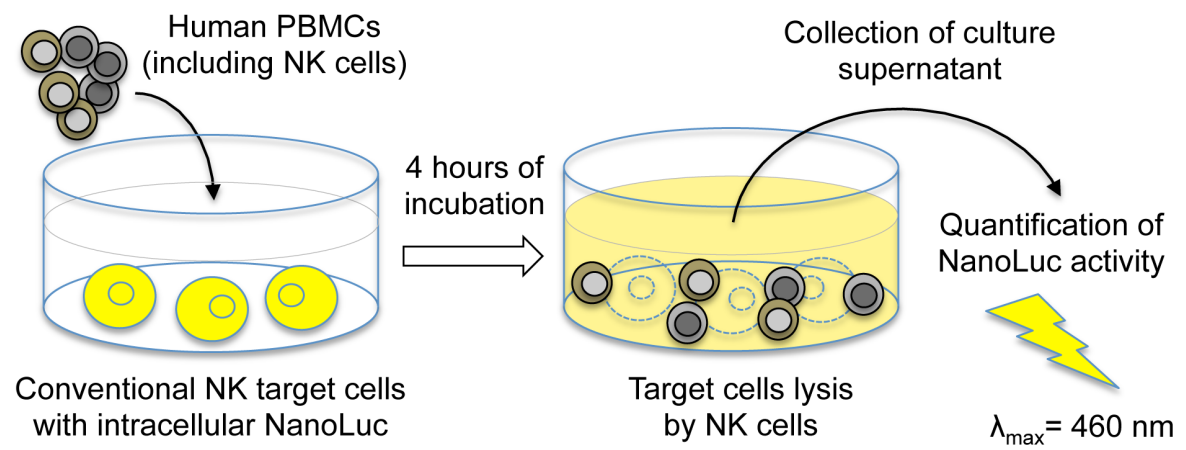


Figure 1

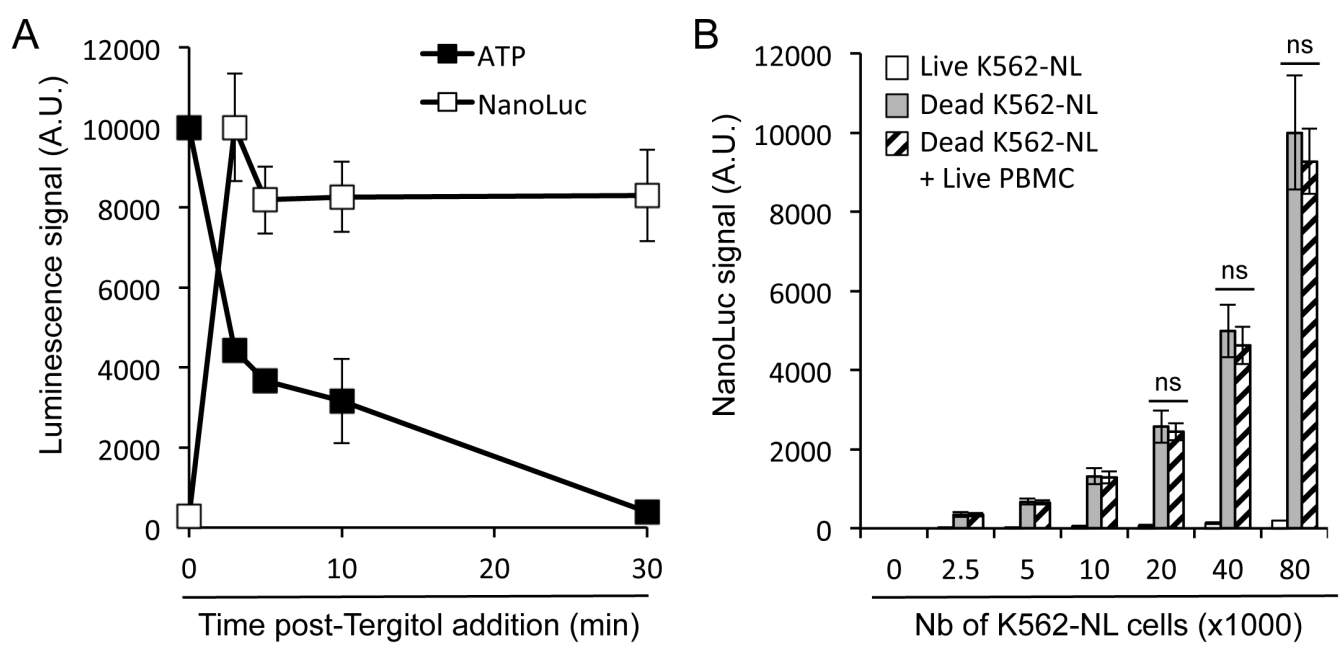


Figure 2

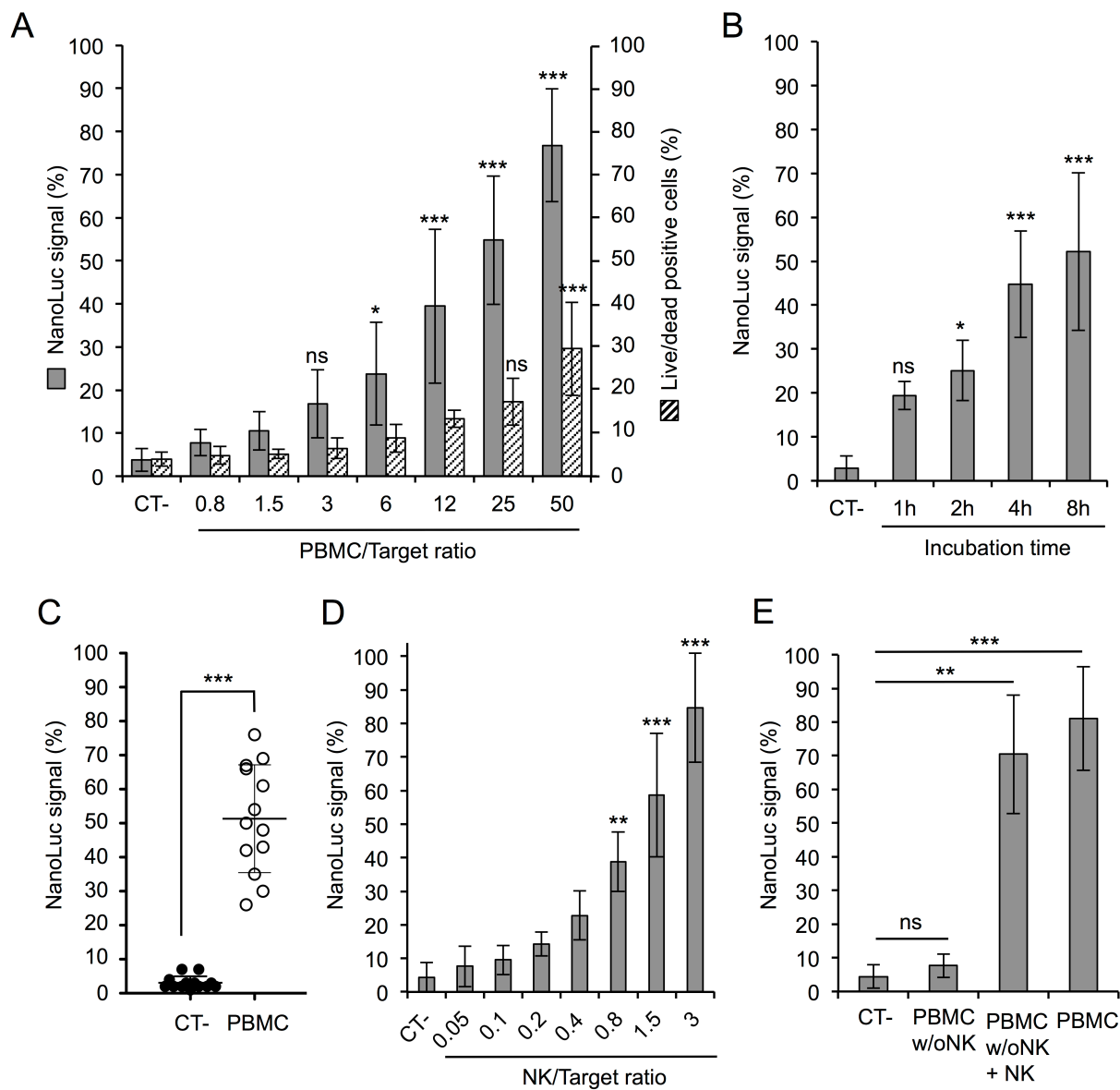


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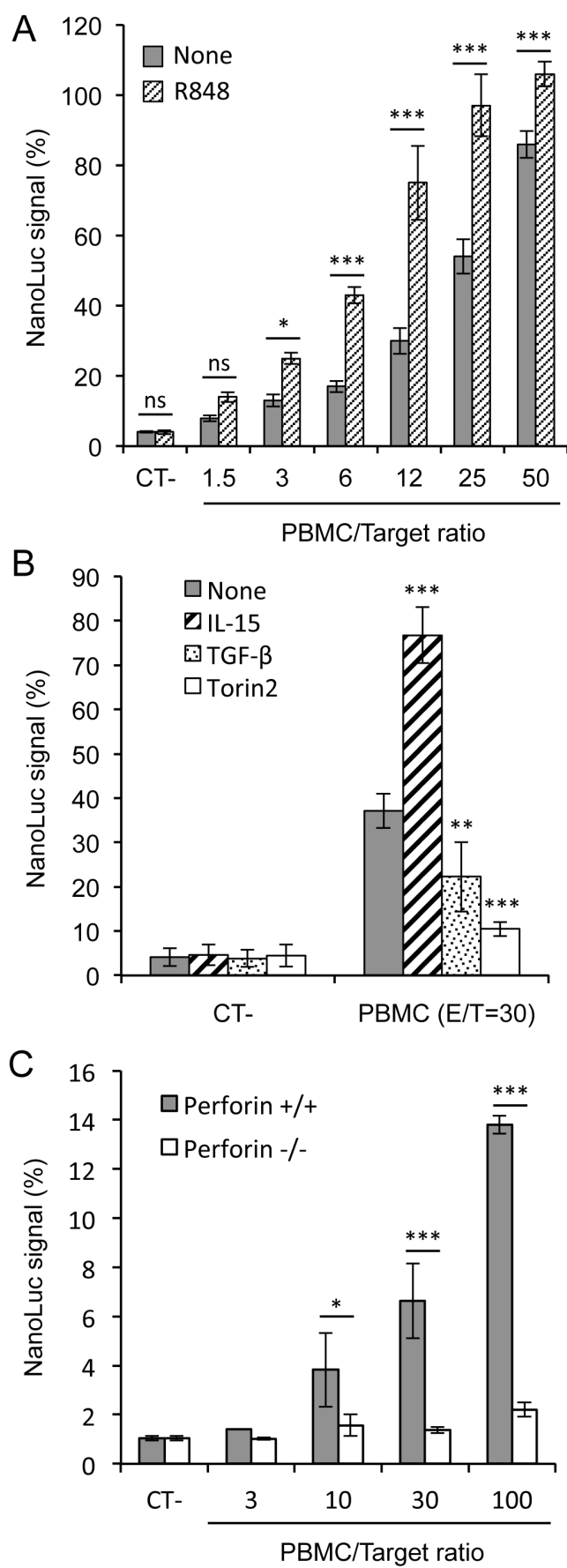


Figure 4

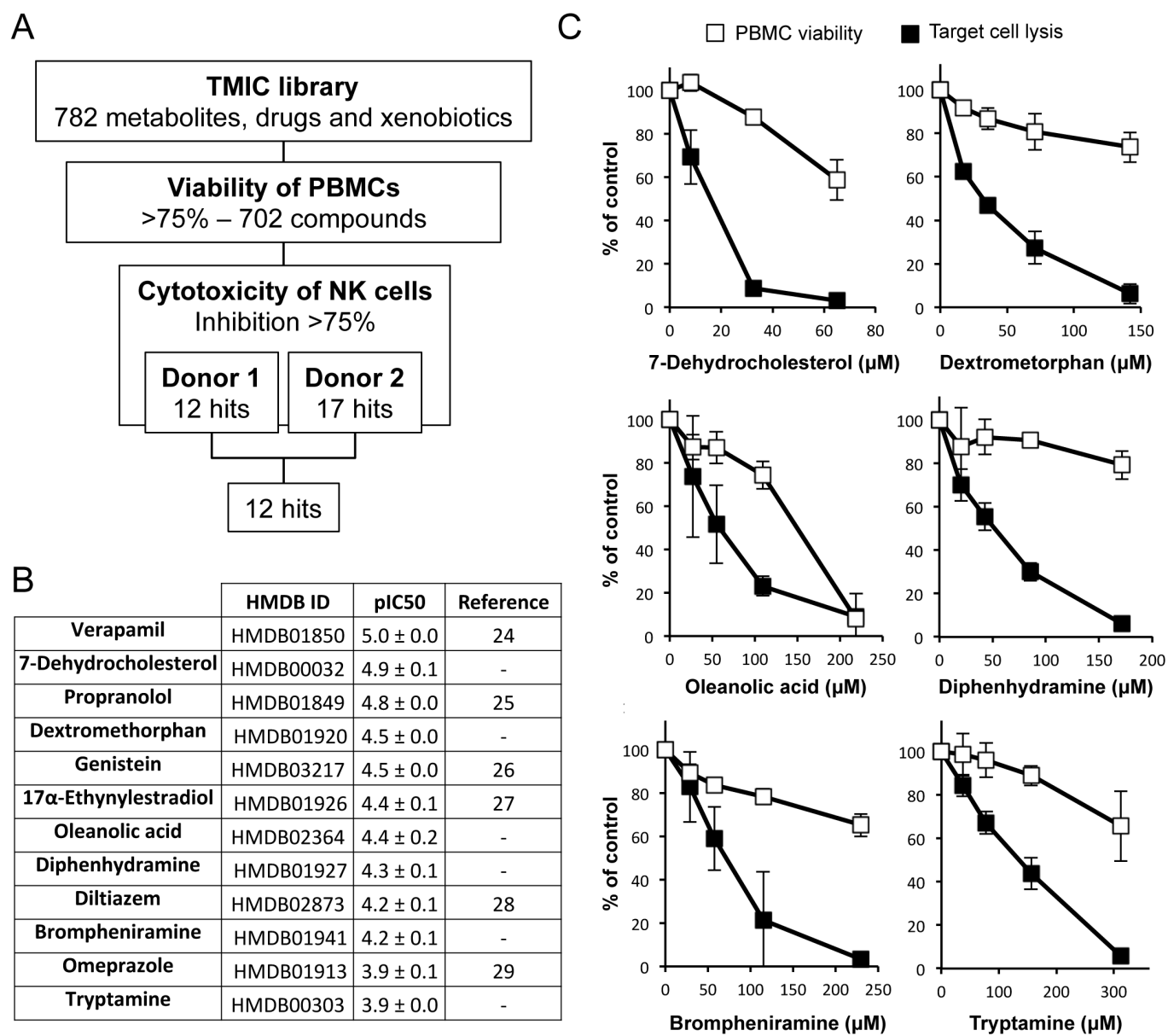


Figure 5

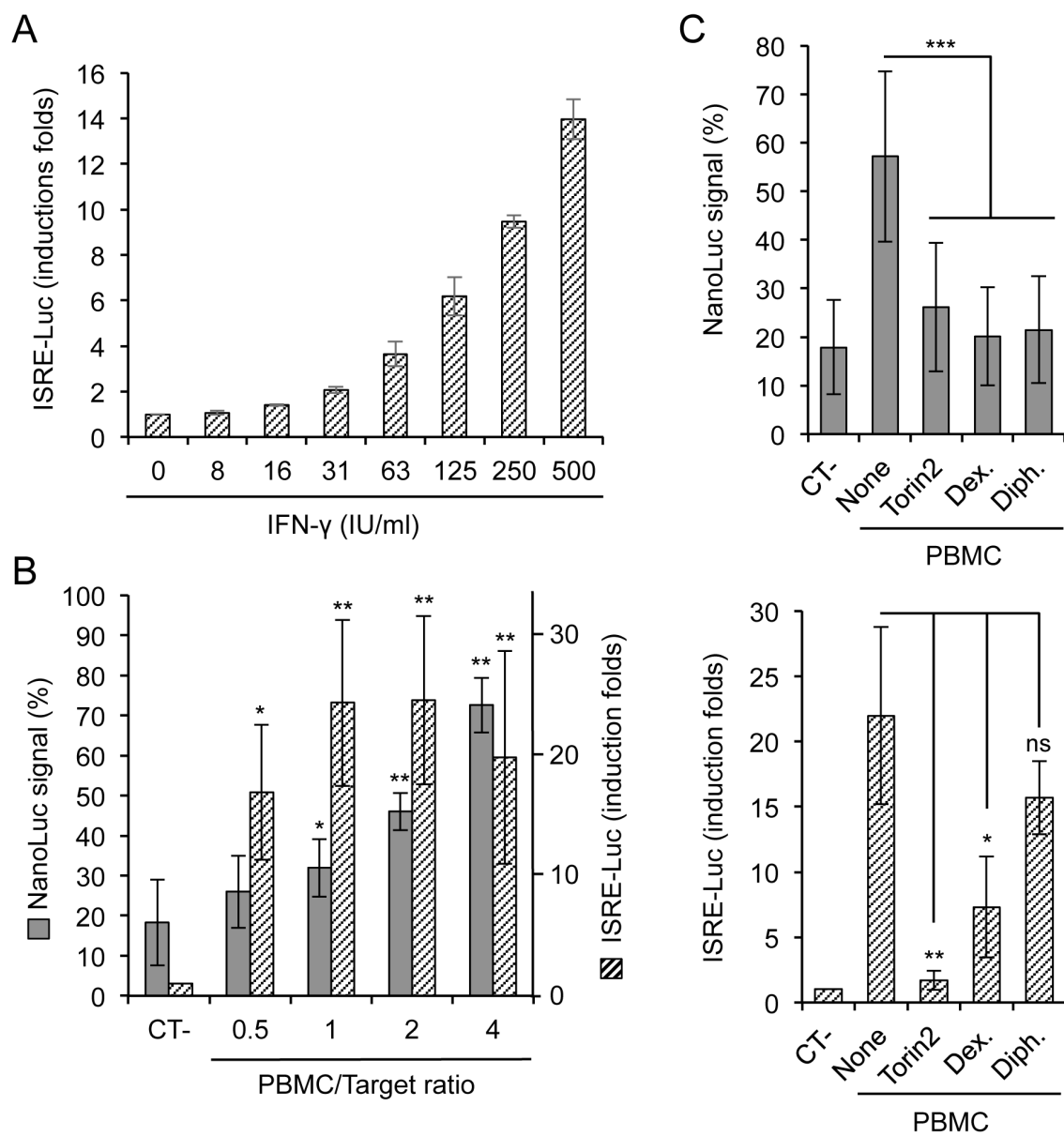


Figure 6