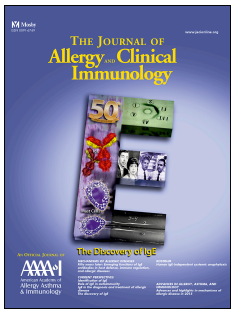
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PD-1 Blocks Lytic Granule Polarization with Concomitant Impairment of Integrin 'Outside-in' Signaling in Natural Killer Cell Immunological Synapse

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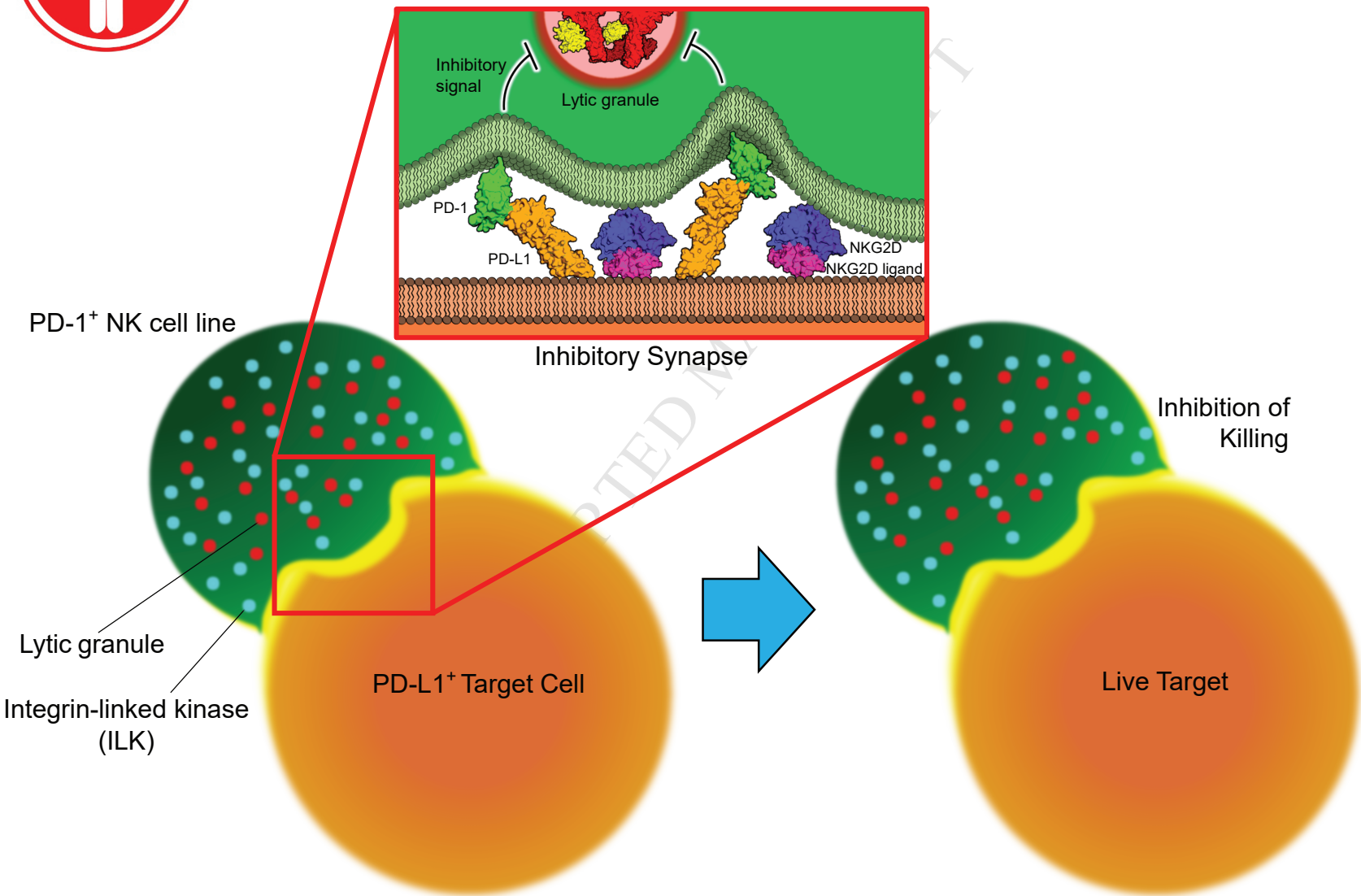
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# PD-1 Blocks Lytic Granule Polarization with Concomitant Impairment of Integrin 'Outside-in' Signaling in Natural Killer Cell Immunological Synapse

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Yu Huang and Zhiying Chen contributed equally to this work.

**Key words:** Natural killer cell, Programmed cell death protein-1, Lytic granule, LFA-1, Immunological synapse

**Abbreviations used**

ADCC: Antibody dependent cellular cytotoxicity

CsA: Cyclosporine A

CTLs: Cytotoxic T lymphocytes

HEK293T: Human embryonic kidney 293T

ICAM-1: Intercellular adhesion molecule 1

ILK: Integrin-linked kinase

IS: Immunological synapse

LFA-1: Leukocyte function–associated antigen 1

LG: Lytic granule

NK: Natural killer

NC: Natural cytotoxicity

NKG2D: NK group 2 member D

PD-1: Programmed death-1

PD-L1: Programmed death-ligand 1

TCR: T cell receptor

$T_{reg}$: Regulatory T cell

## ABSTRACT

**Background:** Inhibitory receptor programmed cell death protein-1 (PD-1) is upregulated on a variety of immune cells, including natural killer (NK) cells, during chronic virus infection and tumorigenesis. Blockade of PD-1 or its ligands produces durable clinical responses with tolerable side effects in patients with a broad spectrum of cancers. However, the underlying molecular mechanisms of how PD-1 regulates NK cell function remain poorly characterized.

**Objective:** We sought to determine what effect PD-1 signaling has on NK cells.

**Methods**: PD-1 was overexpressed in CD16-KHYG-1 (a human NK cell line with both antibody-dependent cellular cytotoxicity via CD16 and natural cytotoxicity via NKG2D) and stimulated by exposing the cells to NK-sensitive target cells expressing the physiological PD-1 ligand (PD-L1).

**Results:** PD-1 engagement by PD-L1 specifically blocked NK cell-mediated cytotoxicity without interfering with the conjugation between NK cells and target cells. Further examination showed that PD-1 signaling blocked lytic granule polarization in NK cells, which was accompanied by the failure of integrin-linked kinase (ILK), a key molecule in the integrin ‘outside-in’ signaling pathway, to accumulate in the immunological synapse after NK-target cell conjugation.

**Conclusion:** Our results suggest that NK cell cytotoxicity is inhibited by PD-1 engagement, which blocks lytic granule polarization to NK cell immunological synapse with concomitant impairment of integrin ‘outside-in’ signaling. This study provides novel mechanistic insights into how PD-1 inhibition disrupts NK cell function.


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**Capsule Summary**

The precise mechanisms of PD-1 signaling in human NK cells remain elusive. This study describes novel mechanistic insights into how PD-1 inhibition disrupts NK cell function, implicating potential ways for therapeutic manipulation in patients with exhausted NK cells.

**Key Messages**

- PD-1 signaling inhibits both natural cytotoxicity and antibody-dependent cellular cytotoxicity in NK cells.
- PD-1 signaling blocks lytic granule polarization and impairs ILK accumulation (integrin signaling) to NK cell immunological synapse.
- PD-1 signaling does not affect the conjugation between NK cells and target cells.

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## INTRODUCTION

Since programmed death 1 (PD-1, also known as CD279) was discovered in 1992[1], pathway blockade of PD-1 and its ligands, including PD-L1 (also called CD274 and B7-H1) and PD-L2 (also called CD273 and B7-DC), have been exploited in a wide variety of potential applications to treat cancers[2, 3], viral infections[4], autoimmune diseases[5], and many other diseases[6, 7]. The roles of PD-1 in T cell- and B cell-mediated immune responses have been well characterized in the field of basic immunology[8]. PD-1 signaling can beneficially protect against tissue damage and autoimmune diseases by inducing and maintaining T cell tolerance[8] and by inhibiting self-reactive T cells[5], respectively. However, PD-1 signaling can be harmful, as it is the major cause of effector cell exhaustion during long-term, chronic antigen stimulation[8]. Specifically, PD-1 signaling blocks T cell functions, including proliferation[9], cytokine (e.g., IL-2) production[10], adhesion[11], and cytotoxicity[12]. The underlying molecular basis for using PD-1 pathway blockade is that the suppression of PD-1 signaling allows activation of tumor-specific cytotoxic T cells or other effector cells (e.g., NK cells) by removing the cytotoxic lymphocyte activation “brake” and modifying the tumor microenvironment[2, 13].

PD-1 is expressed on a variety of immune cells, including activated and/or exhausted T cells and B cells, activated monocytes, dendritic cells[8], regulatory T cells (Treg) [14], and NK cells[15-18]. NK cells provide the first line of defense to cancer and virus infection[19, 20]. For effective elimination of virus infected cells or tumor cells, NK cells have to integrate both activation receptor signaling and inhibitory receptor signaling[20, 21]. However, despite the importance of NK cells in combating diseases, the precise mechanisms whereby PD-1 mediates its effects on NK cells remains poorly characterized.

Integrins play an important role in NK cell activation, which is mediated by both 'outside-in' (e.g., granule polarization) and 'inside-out' (e.g., conjugation) signals[22, 23]. In T cells, T cell receptor (TCR) signaling alone can induce lytic granule (LG) polarization[24]. TCR signaling also provides strong 'inside-out' signals to leukocyte function–associated antigen 1 (LFA-1, also called $\alpha_L\beta_2$ integrin). The co-engagement of LFA-1 and TCR further enhances the extent of LG polarization induced by the TCR alone[24]. However, in NK cells, the ligation of LFA-1 with intercellular adhesion molecule 1 (ICAM-1) alone is sufficient to trigger LG polarization[22, 23]. Thus, NK cells can serve as a good model system for the analysis of 'outside-in' signaling by LFA-1 independently of other signals[25]. A recent study shows that a signaling network centered on an integrin-linked kinase (ILK)-Pyk2-paxillin pathway is required for LG polarization (outside-in)[26]. ILK was shown to be recruited to the immunological synapse (IS) by proximity ligation assays, and silencing of ILK impaired the polarization of LGs in NK cells[26]. However, it remains unclear whether PD-1 affects integrin signaling in NK cells.

In this study, we investigated the role of PD-1 in human NK cell cytotoxicity. We found that PD-1 activation by PD-L1 blocked NK cell cytotoxicity. However, PD-1 signaling did not inhibit cell-cell conjugation between NK and target cells, an initial step for NK-mediated immune responses. In fact, NK cells that expressed PD-1 formed stable inhibitory IS with target cells that expressed PD-L1. Yet, LG polarization was blocked and the integrin 'outside-in' signaling pathway was disrupted by PD-1 engagement.

## METHODS AND MATERIALS

### Cell Culture

Human Embryonic Kidney 293T (HEK293T) cells were maintained in DMEM (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco), 2 mM L-Glutamine (Gibco), and 10 mM HEPES (Gibco). K562 and Daudi cell lines (American Type Culture Collection, ATCC) were cultured in R10 medium, which contained RPMI 1640 medium (Gibco) supplemented with 10% FBS, 2 mM L-Glutamine, 10 mM HEPES, 1 mM sodium pyruvate (Cellgro, USA), and 1% MEM non-essential amino acids solution (Gibco). CD16-KHYG-1, a human NK cell line capable of both antibody-dependent cellular cytotoxicity and natural cytotoxicity[27], was a gift from Dr. David Evans (University of Wisconsin, Madison, WI) and cultured in R10 medium supplemented with 1 µg/ml cyclosporine A (CsA, Sigma-Aldrich, USA), 50 µg/ml Primocin (Invivogene, USA) and 10 U/ml interleukin-2 (IL-2, Roche, USA). All cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$. For all experiments, the culture medium of CD16-KHYG-1 cells was replaced to remove CsA one day before.

### Plasmid constructs and transduction of CD16-KHYG-1, K562, and Daudi cell lines

The GFP, PD-1-GFP, mCherry, PD-L1-mCherry lentiviral plasmids were generated as previously described [28]. Briefly, to generate the PD-1-GFP construct, the full length of PD-1-GFP (OriGene, USA) was subcloned into the lentiviral expression vector, pCDH (System Biosciences, USA), using EcoRI and BamHI (Thermo Scientific, USA) insertion sites. To make the C-terminal mCherry-tagged PD-L1 construct, the PD-L1 cDNA (OriGene) was first subcloned into EcoRI and BamHI digested pmCherry-N1 vector (Clontech, USA). The sequence of PD-L1-mCherry was amplified by polymerase chain reaction (PCR) and inserted into EcoRI digested pCDH using the In-fusion cloning system (Clontech). The

sequence of turboGFP or mCherry was inserted into pCDH to create the construct for GFP or mCherry, respectively, for lentiviral production, and for serving as experimental control. All plasmids were verified by sequencing.

To generate polyclonal stable cell lines, the lentivirus was produced by co-transfecting HEK293T cells with the desired lentiviral vector and 3 packaging plasmids (pMLg/pRRE, pRSV-Rev, and pMD2.g) using Lipofectamine reagent (Invitrogen, USA). Briefly, 4 µl Lipofectamine was mixed with 0.8 µg total DNA (0.128 µg desired pCDH construct, 0.32 µg pMLg/pRRE, 0.16 µg pRSV-Rev, 0.192 µg pMD2.g) and added into one well of HEK293T cells cultured in a 6-well plate at approximately 70% confluence. At 48 hours after transfection, viral particles were harvested by filtering the culture medium via a 0.45 µm filter (GE Healthcare Life Sciences, USA). Then, CD16-KHYG-1 cells were infected with 4 ml viruses for GFP or PD-1-GFP, and K562 and Daudi cells were infected with 4 ml viruses for mCherry or PD-L1-mCherry, at a density of $5\times10^4$ cells/ml suspended in 4 ml R10 medium. The transduced cells were cultured for 24 hours in the presence of 8.0 µg/ml polybrene (Santa Cruz Biotechnology, USA) and subsequently sorted by an Aria II cell sorter (BD Biosciences, USA). The expression of green or red fluorescence in these cells was verified by flow cytometry using an LSR Fortessa cell analyzer (BD Biosciences).

**Chromium$^{51}$ release assay**

NK cell cytotoxicity toward K562 or Daudi target cells was examined using a $^{51}$Cr-release assay, as described[29]. Briefly, the target cells were labeled with $^{51}$Cr at 37°C for 1 hour. After washing 3 times with fresh R10 medium, cells were re-suspended in R10 medium at a density of $1\times10^5$ cells/ml. An aliquot of the target cells (0.1 ml for $1\times10^4$ cells) were mixed with a serial dilution of NK cells, and the mixture incubated at 37$^o$C for 4 hours. For

antibody-dependent cellular cytotoxicity (ADCC) assays, Daudi cells were incubated with 20 μg/ml Rituximab on ice for 30 minutes before the addition of NK cells and the Rituximab was also present during the 4-hour incubation at 37$^{o}$C. At the end of the incubation, the medium was collected and radioactivity was measured using a Topcount NXT Scintillation and Luminescence Counter (Perkin Elmer, USA). The mean percentage of specific lysis of triplicate wells was calculated as follows: [(test counts-spontaneous counts)/(maximum counts-spontaneous counts)] ×100%.

**Conjugation assays**

NK-target conjugates were measured as described previously[23]. NK cells and target cells (K562 or Daudi) were washed 3 times with phosphate-buffered saline (PBS) and allowed to mix at 37°C with the densities of $1\times10^5$ NK cells and $2\times10^5$ target cells per ml in a total volume of 200 μl for 0 to 30 min. At the end of incubation, cells were fixed by freshly prepared 0.5% paraformaldehyde in PBS for 15 min at room temperature. After a gentle vortex, cells were analyzed using the LSR Fortessa flow cytometer. The NK-target conjugates were identified as dual-color positive particles with both green and red signals.

**Immunofluorescence staining**

NK cells ($5 \times 10^5$) and target cells ($1 \times 10^6$) were mixed in 500 μl RPMI 1640 on ice and centrifuged at 4°C for 3 min at 300 rpm. Samples were placed at 37°C for various times. The reaction was stopped by gentle pipetting and the mixture was added to poly-lysine coated slide at 4°C for 10 min. After the conjugates attached, the medium was removed, and cells were fixed with 4% paraformaldehyde in PBS for 20 min. The fixed cells were washed 3 times with PBS and permeabilized with a buffer containing 0.1% Triton X-100 and 5% normal donkey serum before incubation with the primary antibodies at 4°C overnight. The

cells were washed and probed with the fluorophore-conjugated secondary antibodies (Invitrogen, USA) at room temperature for 1.5 hours. When needed, Alexa Fluor dye-conjugated phalloidin (Invitrogen, USA) was used to stain F-actin. The slide was mounted with a glass coverslip using the ProLong Gold antifade reagent containing DAPI (Life Technologies, USA).

**Laser scanning fluorescence confocal microscopy**

Images for both live and fixed cells were acquired using the LASAF software (Leica Microsystem). For live cell imaging, high-resolution images were captured by a confocal fluorescent microscope (Leica TCS SP8, Leica Microsystem, Germany) equipped with a 63× oil immersion objective lens (NA 1.47, Leica Microsystem) under 37°C. The fluorescence from GFP and mCherry and the bright field images were detected simultaneously. For fixed cells, three-dimensional (3D) images were reconstructed from acquired z-stacks of X-Y scanning of entire cells for perforin, F-actin, and ILK. ImageJ (National Institutes of Health) was used for image processing and creating projected images from 3D reconstructions.

To measure the distance between LGs (perforin staining) and IS (F-actin staining), ImageJ and Imaris (Bitplane) programs were used. From the 3D image, coordinates for the center of all "dots" (perforin, $x_1$, $y_1$, $z_1$) and that of the "surface" (accumulated F-actin, $x_2$, $y_2$, $z_2$) were obtained for each conjugate, and entered into the equation:

$D = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2}$, where *D* is the average distance of every perforin to the center of the IS for each pair of NK-target conjugate.

**Antibodies**

The antibody sources are as follows: anti-PD-1 (clone 29F.1A12, Biolegend); anti-PD-L1 (clone 10F.9G2, Biolegend); anti-NKG2D (clone 1D11, Biolegend); anti-CD16 (clone 3G8, Biolegend); anti-LFA1 (clone H155-78, Biolegend); anti-perforin (clone δG9, Thermo Fisher); anti-ILK (clone EPR1592, Abcam); anti-Actin (clone C4, Santa Cruz).

**Statistical analysis**

Unpaired or paired two-tailed t-tests were performed using Prism software (GraphPad Software, Inc.).

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## RESULTS

### PD-1 signaling attenuates NK cell cytotoxicity

As a first step to understand PD-1 signaling in NK cells, we generated a stable CD16-KHYG-1 cell line expressing PD-1-GFP (NK-PD-1-GFP). CD16-KHYG-1 is a human NK cell line that recognizes and kills target cells, such as K562 and Daudi, through two distinct mechanisms: natural cytotoxicity (NC) through activating receptors, such as NKG2D[30], and antibody-dependent cellular cytotoxicity (ADCC) through CD16[20,31]. To test NC and ADCC of NK cells in response to PD-1 signaling blockade, K562 and Daudi cell lines stably expressing the PD-1 ligand, PD-L1-mCherry (named as K562-PD-L1-mCherry and Daudi-PD-L1-mCherry, respectively), were used as target cells. CD16-KHYG-1 and K562 (or Daudi) cell lines stably expressing GFP only or mCherry only (named as NK-GFP, K562-mCherry, and Daudi-mCherry) were used as respective controls. The expression of GFP in the stable CD16-KHYG-1 cell lines and that of mCherry in the stable K562 and Daudi cell lines were verified by flow cytometry (see **Fig. E1**, *left panels,* in this article's Online Repository at www.jacionline.org). Using PD-1 and PD-L1 antibodies, we found that more than 95% cells expressed PD-1 and PD-L1 in the NK-PD-1-GFP and K562-PD-L1-mCherry (or Daudi-PD-L1-mCherry) cells, respectively. However, no PD-1 staining was observed in the parental wild-type CD16-KHYG-1 cells or NK-GFP cells, and no PD-L1 was detected in the wild-type or mCherry-expressing K562 or Daudi cells (**Fig. E1**, *right panels,* in this article's Online Repository at www.jacionline.org). Thus, we successfully established a PD-1-positive NK cell line and PD-L1-positive target cells and their control groups for testing the effects of PD-1 signaling on NK cell cytotoxicity.

We then examined the subcellular distribution of the expressed PD-1-GFP and PD-L1-mCherry in transduced cells. Prominent plasma membrane localization and

intracellular aggregation of PD-1 and PD-L1 were observed in cells expressing PD-1-GFP and PD-L1-mCherry (**Fig. E2**, *left panels,* in this article's Online Repository at www.jacionline.org), but not in cells expressing GFP only and mCherry only (**Fig. E2**, *right panels,* in this article's Online Repository at www.jacionline.org). The subcellular localization of the expressed PD-1 and PD-L1 in the stable CD16-KHYG-1 and K562 (and Daudi) cells resembled that of endogenous PD-1 in primary T cells, which also showed plasma membrane localization and intracellular aggregation, as reported previously[32].

To test the effect of PD-1 signaling on the cytotoxicity of NK cells, we examined the NC and ADCC ability of NK-PD-1-GFP cells using a chromium-51 ($^{51}$Cr) release assay. For NC, NK-GFP and NK-PD-1-GFP cells were co-incubated for 4 hours with $^{51}$Cr-loaded K562-mCherry or K562-PD-L1-mCherry cells, whereupon the amount of $^{51}$Cr in the medium was determined. The results showed that co-incubation of NK-PD-1-GFP cells with K562-PD-L1-mCherry cells inhibited the cytotoxicity of NK cells against K562 (**Fig. 1A**), but NC was unaffected without the engagement of PD-1 and PD-L1 (**Fig. 1A** and **E3A,** in this article's Online Repository at www.jacionline.org). Similarly, ADCC, as examined using Daudi-mCherry or Daudi-PD-L1-mCherry cells as NK target cells induced by rituximab (anti-CD20), was abolished only when NK-PD-1-GFP cells were co-incubated with Daudi-PD-L1-mCherry cells (**Fig. 1B**). Co-incubation of CD16-KHYG-1 cells expressing PD-1 with PD-L1-nonexpressing Daudi cells or co-incubation of Daudi cells expressing PD-L1 with PD-1 non-expressing CD16-KHYG-1 cells did not alter ADCC (**Fig. 1B** and **E3B**, in this article's Online Repository at www.jacionline.org). Thus, the engagement of PD-1 with its natural ligand is required for inhibition of NC and ADCC. PD-1 signaling attenuates NK cell cytotoxicity.

**Stable immunological synapse is formed in NK cells in the presence of PD-1 signaling**

The unaltered killing capability of NK-PD-1-GFP cells on PD-L1-negative target cells indicated that the activating receptors functioned normally in the transduced CD16-KHYG-1 cells in the absence of PD-L1. Indeed, by antibody staining, we showed that neither GFP nor PD-1-GFP affected the surface expression of NKG2D or CD16 (**Fig. E4,** in this article's Online Repository at www.jacionline.org), the major activating receptors in CD16-KHYG-1 cells that mediate NC and ADCC, respectively, indicating that PD-1 overexpression or lentivirus infection did not change the expression of these two major activating receptors in NK cells.

To further understand the underlying molecular basis of PD-1 inhibition in human NK cells, we investigated the effect of PD-1 signaling on the formation of the NK cell IS, a pivotal platform for mediating an effective immune response in both the adaptive and innate immune systems[28, 33, 34]. For cytotoxic killing, an IS forms between the lymphocyte and the target cell, where activating receptors accumulate, followed by release of LGs[35]. To determine whether PD-1 in NK cells binds to PD-L1 from its susceptible target cells at the IS, NK-GFP and NK-PD-1-GFP cells were allowed to form conjugates with K562 or Daudi cells that expressed either mCherry only or PD-L1-mCherry in a 1:1 ratio, and were then inspected by confocal microscopy using live cell imaging. As shown in **Fig. 2**, PD-1-GFP accumulated in the IS only when the NK-PD-1-GFP cell was conjugated with a K562 (for examining NC) or CD20 antibody pretreated Daudi cell (for examining ADCC) that expressed PD-L1-mCherry, which was also concentrated at the IS. The accumulation occurred within minutes and persisted for more than four hours (**Movie S1,** in this article's Online Repository at www.jacionline.org), resembling previous reports for T cells[32, 36] and inhibitory IS formation in NK cells[28]. By contrast, if the target cells expressed mCherry only instead of

PD-L1-mCherry, PD-1-GFP did not accumulate at the IS. Conversely, PD-L1-mCherry failed to concentrate at the IS without the expression of PD-1-GFP in the NK cells (**Fig. 2**). We also observed the similar phenotype by using PD-1-GFP infected primary NK cells and K562-PD-L1-mCherry (**Fig. E5,** in this article's Online Repository at www.jacionline.org). In conclusion, PD-1 only accumulates at the IS when PD-L1 is present on the target cells for both NC and ADCC.

**PD-1 signaling does not affect NK-target cell conjugation**

NK cytotoxicity involves multiple distinct steps, including conjugation with target cells, granule polarization, degranulation, and cytotoxic killing[33]. The engagement of PD-1 with PD-L1 inhibits cytotoxic killing of target cells by CD16-KHYG-1, suggesting the formation of inhibitory synapses between PD-1 expressed in the NK cells and PD-L1 expressed in the target cells. Our live imaging data shown above indeed demonstrated the close apposition between PD-1-GFP and PD-L1-mCherry expressed in NK and target cells, respectively, at the IS, which remained stable for hours (**Fig. 2** and **Movie S1**). However, whether this represents stable conjugations between NK cells and target cells and how it compares to cells that do not express the inhibitory receptor are unclear.

To address this question, we used flow cytometry to determine the percentage of CD16-KHYG-1 cells that were conjugated with target cells. The NK cells (green) were allowed to conjugate with target cells (red) for 5, 10, and 30 minutes, and then fixed for testing by FACS. Interestingly, no difference in the percentage of GFP and mCherry double-positive conjugates was observed over the period of 30 min in either the NC (**Fig. 3A**) or the ADCC (**Fig. 3B**) assays among the different combinations of NK and target cells, with or without PD-1 and PD-L1 expression (**Fig. 3**). Together, these results indicate that the

PD-1/PD-L1 engagement does not affect the conjugation of NK cells with their target cells, indicating that the PD-1 signaling may affect the downstream steps of NK cytotoxicity.

**PD-1/PD-L1 engagement blocks lytic granule polarization at inhibitory immunological synapse of the NK cell**

After conjugation with target cells, LGs in NK cells, which contain perforin, become polarized to the IS[35, 37]. This represents another key step of cytotoxic killing[38]. To examine if engagement of PD-1 and PD-L1 affected LG polarization in NK cells, we measured the distance of perforin to the IS in target-conjugated CD16-KHYG-1 cells that expressed GFP only or PD-1-GFP. Cells were co-stained for F-actin and perforin, and 3D fluorescence images of individual cell conjugates were collected by confocal microscopy. Actin accumulated in the IS of the majority of conjugates we examined with or without PD-1 signaling, indicating that PD-1 signaling does not affect actin accumulation. In these images, NK cells were identified by positive staining with perforin; the IS was indicated by the accumulated F-actin signal at the boundary between NK and the target cells. To quantify the LG polarization, we calculated the distance between perforin and the accumulated F-actin at NK IS (**Fig. 4**).

Additionally, we found that LG polarization occurred between 10 min and 30 min after conjugation in control groups; however, in the presence of PD-1 signaling, the LGs did not polarize to the IS throughout the 2-hour test period (**Fig. E6**, in this article's Online Repository at www.jacionline.org). In both the NC and ADCC models, the distance between the perforin positive LGs and the IS was significantly longer in conjugates formed between NK-PD-1-GFP cells and the target cells that expressed PD-L1-mCherry than that between NK-GFP cells and targets that expressed mCherry at 30 min after conjugation (**Fig. 4**). The

finding that LGs did not polarize to IS at later time points (60 min, 120 min) in the presence of PD-1 signaling (**Fig. E6,** in this article's Online Repository at www.jacionline.org) excluded the possibility that granule polarization could still occur during PD-1 engagement with different kinetics. PD-1/PD-L1 engagement rendered the LGs of the NK cells unable to reach the IS for a successful killing of their susceptible target cells. Thus, PD-1/PD-L1 engagement specifically blocks LG polarization at inhibitory NK IS.

**PD-1 engagement inhibits integrin signaling**

Integrin signaling pathway regulates LG polarization in NK cells[26]. In response to the activation of LFA-1 by its ligand ICAM-1, the LGs along with the microtubule-organizing center (MTOC) in the NK cell polarize towards the target cell. This process requires a signaling network consisting of ILK, Pyk2, paxillin, and several other proteins; ILK also accumulates at the IS in response to NK cell activation[26]. To gain insights into the functional relationship between PD-1/PD-L1 engagement and integrin-mediated adhesion and consequent LG polarization, we co-stained ILK and perforin in target-conjugated NK-GFP and NK-PD-1-GFP cells (**Fig. 5A**). Confocal fluorescence imaging revealed that ILK concentrated in the IS between the majority of conjugated NK-GFP and K562-mCherry cells at 30 min and 60 min after conjugation (**Fig. 5B**), indicative of accumulation at the IS. Although the accumulation subsided at 120 min in control groups, ILK appeared, at all the time points we tested, as dispersedly distributed puncta in most of the conjugated NK-PD-1-GFP and K562-PD-L1-mCherry cells (**Fig. 5B**). To rule out the possibility of decreased overall expression of integrin molecules on NK cells transduced with PD-1, we measured the mean fluorescence intensity (MFI) of LFA-1 using flow cytometry and found comparable overall expression levels of LFA-1 on NK cells transduced with GFP only and that with PD-1-GFP (**Fig. E7,** in this article's Online Repository at www.jacionline.org). We

also checked the expression of ILK by western blot. There was no difference between the NK-GFP and NK-PD-1-GFP cells, and the target cells also exhibited moderate levels of ILK expression that were unaffected by the transduction of PD-L1-mCherry (**Fig. E8,** in this article's Online Repository at www.jacionline.org). Therefore, we conclude that PD-1/PD-L1 engagement disrupts integrin signaling, known to be required for LG polarization in NK cells.

## DISCUSSION

In this study, we show that PD-1 signaling blocks NK cell-mediated immune responses through impairing LG polarization to the NK cell IS. Neither NK-target cell conjugation nor NK-target cell inhibitory immunological synapse formation was blocked by PD-1 engagement. Although the specific role of PD-1 can vary in different cell types and different disease settings[8], here we show that PD-1 indeed functions as an inhibitory receptor in NK cells, similar to its action in cytotoxic T cells and other lymphocytes during chronic viral infections and cancers[39] as well as chronic antigen stimulation[40]. The engagement of PD-1 expressed in the NK cells with its cognate, natural ligand PD-L1 expressed on NK-sensitive target cells inhibited both forms of NK-mediated cytotoxicity, NC and ADCC. Furthermore, the engagement of PD-1 blocked ILK-mediated integrin 'outside-in' signaling, which may account for the inhibition of LG polarization of NK cells and the consequent inhibition of cytotoxicity.

Our results reveal a number of unexpected features of PD-1 signaling on NK cell cytotoxicity. First, it remains controversial whether inhibitory receptors alter IS stability. Some previous studies have suggested that inhibitory receptors destabilize the NK cell IS, rendering the NK cell to disengage from its target before it can successfully initiate a cytolytic attack[41, 42]. This

441 concept also gained support from experiments with mouse $CD4^+$ T cells, where continued
442 PD-1/PD-L1 interactions destabilized adhesion of self-reactive $CD4^+$ T cells in the pancreas
443 by inhibiting the TCR-driven "stop signals"[11], and PD-1/PD-L1 binding blocked stable
444 synapse formation on the glass-supported lipid bilayers containing PD-L1 molecules[36].
445 However, these observations are in contrast to the finding of long-term (>40 min)
446 PD-1/PD-L1 interactions and promoted stable immunological synapse formation in
447 virus-specific $CD8^+$ T cells in the lymphocytic choriomeningitis virus (LCMV) clone 13(a
448 strain that establishes persistent viral infection) infected mice[43]. Nevertheless, in this study,
449 we demonstrate that NK cells can form stable IS even when the inhibitory signaling
450 dominates. The discrepancy may be explained by the differential impact of PD-1 signaling on
451 $CD8^+$ cytotoxic lymphocytes (including NK and $CD8^+$ T cells) and $CD4^+$ T cells due to
452 different downstream signaling cascades mediated by PD-1 engagement between these cell
453 types.
454
455 Indeed, inhibitory receptor signaling does not block accumulation of activating receptors[44].
456 This has been shown with 2B4 and CD2 at inhibitory synapses with target cells[44] and with
457 the minimal combination of activation ligand human IgG1 Fc (a ligand for the low affinity Fc
458 receptor FcγRIIIA, also known as CD16) and HLA-E (a ligand for inhibitory receptor
459 CD94/NKG2A)[37]. Recently, using a newly developed vertical cell pairing (VCP) system, we
460 showed the formation of a stable inhibitory IS after engaging PD-1 expressed on NK cells
461 with PD-L1 expressing target cells[28], supporting the idea that stable inhibitory IS formation
462 indeed occurs during inhibitory signaling in NK cells. These results await further validation
463 with a large sample size, but indeed bring into the question whether inhibitory receptor
464 signaling acts through destabilization of the IS in NK cells or through other unidentified
465 molecular mechanisms. Our data strongly support the notion that NK cells can form a stable

466 inhibitory synapse even when inhibitory signaling dominates. However, further studies will
467 check whether PD-1 signaling affects the accumulation of activating receptors and the
468 activating synapse in human primary NK cells.
469
470 The current study shows that PD-1 signaling does not decrease the percentage of NK-target
471 cell conjugation. Usually, cell adhesion is the initial step in immune responses[33, 45, 46]. After
472 effector-target conjugation, the immunoreceptor starts to trigger immune signaling, either
473 activation or inhibition. For initialing inhibition signaling, the current dogma is that blocking
474 early signaling (e.g., adhesion) by inhibitory receptor will be more effective than blocking the
475 late phase of immune signaling. Previous study showed that PD-1 antibody CT-011 enhanced
476 the immune complex formation between NK and target cells, suggesting that PD-1 might
477 affect NK cell adhesion[16]. Unexpectedly, our data showed that PD-1 inhibitory signaling
478 preferentially blocked integrin-mediated LG polarization, without affecting NK-target cell
479 adhesion or cell-cell conjugation. The discrepancy can be explained by different NK cell
480 types and experimental methods, as well as the different strategies to deliver PD-1 signaling.
481
482 It is well characterized that for cytotoxic killing, integrin molecules in cytotoxic lymphocytes
483 possess both 'inside-out' signaling (conjugate formation) and 'outside-in' signaling (LG
484 polarization)[47, 48]. Our data suggest that PD-1 preferentially impairs LFA-1-dependent
485 'outside-in' signaling, rather than 'inside-out' signaling pathway, in NK cells. This differs
486 from the recent studies in T cells that showed PD-1 engagement with its physiological ligand
487 inhibited T cell adhesion[49, 50]. LFA-1-dependent 'outside-in' signaling in T cells requires
488 'inside-out' to enhance LG polarization[24]. This is different from that of LFA-1 in human
489 primary, resting NK cells, in which the binding of LFA-1 on NK cells to ICAM-1 on target
490 cells is sufficient to induce the polarization of LGs to NK IS[22, 23]. The different LFA-1

signaling pathways between T and NK cells could explain this discrepancy[51].

Previous studies use CD107a assays and cytokine production assays to evaluate the effects of PD-1 signaling on NK cells,[15, 17, 18, 52], which is different from the observations in this study. Here, we used the LG polarization, an earlier event in NK cell cytotoxicity, to assess the PD-1 signaling. The fact that PD-1 engagement preferentially blocks LG polarization to the NK cell IS is reminiscent of previous studies showing that inhibitory receptors block signals for LG polarization (by integrin LFA-1) more effectively than signals for degranulation[51]. Using a *Drosophila* cell line S2 transfected with various ligands of human NK cell receptors, previous studies investigated whether inhibitory receptors could block LG polarization and degranulation. These studies showed that LG polarization was highly sensitive to inhibition mediated by killer cell immunoglobulin-like receptor (KIR) and CD94-NKG2A receptors[51]. However, the previous study did not specifically define the underlying molecular basis of this inhibition. In the current study, we discovered that PD-1 signaling specifically blocked polarization of the common integrin-signaling molecule ILK at the NK cell IS. To our knowledge, although PD-1 signaling is well characterized in T cells and SHP-2 is the only cellular molecule that directly interacts with immunoreceptor tyrosine-based switch motif (ITSM) domain of PD-1[8], ILK is a newly-identified molecule targeted by PD-1 signaling in this study (**Fig. 6**). Future studies will confirm this observation in freshly isolated primary NK cells, either infected by PD-1 lentivirus or patients' NK cells with high PD-1 expression.

In conclusion, we investigated the role of PD-1 in NK cell-mediated cytotoxicity, which reveals the underlying molecular basis of the inhibitory receptor PD-1 in NK cell immune responses. Although PD-1 expression is limited in NK cells from healthy individuals, it is up-regulated on NK cells from patients with a variety of chronic diseases[15, 17, 18, 52, 53]. We

found that PD-1 engagement by its natural ligand, PD-L1, preferentially blocks LG polarization with concomitant impairment in the polarization of a central integrin signaling molecule, ILK, at the NK cell IS. Our study uncovers a novel signaling molecule used by PD-1 inhibitory receptor in human NK cells and a relationship between PD-1 and integrin signaling, which sheds lights on harnessing the power of NK cells by targeting a downstream PD-1 signaling molecule for disease treatment in the future or modulating integrin signaling to enhance therapeutic responses to PD-1 blockade in cancer and infectious diseases.

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**Figure Legends:**

**Figure 1. PD-L1-positive target cells inhibit the cytotoxicity of PD-1-expressing NK cells.** (**A**) The NC of CD16-KHYG-1 cells expressing PD-1-GFP (NK-PD-1-GFP) towards K562 cells that expressed either mCherry (K562-mCherry, squares, as the control group) or PD-L1-mCherry (K562-PDL1-mCherry, triangles) was measured with varying effector/target ratios. (**B**) The ADCC of NK-PD-1-GFP towards Daudi cells that expressed mCherry (Daudi-mCherry, squares, as the control group) or PD-L1-mCherry (Daudi-PD-L1-mCherry, triangles) was measured in the presence of rituximab, which was pre-incubated with Daudi cells for 30 min and present throughout the 4-hour incubation period. Results are representatives of three independent experiments. Error bars represent SDs (****, $p < 0.0001$, *by Student's t test*).

**Figure 2. PD-1 and PD-L1 molecules accumulate at the IS in NK cells.** Live-cell imaging of NK-GFP or NK-PD-1-GFP effector cells (green) conjugated with target cells (red) that expressed mCherry or PD-L1-mCherry. (**A**) K562 cells were used as CD16-KHYG-1 susceptible target cells for evaluating NC. (**B**) Daudi cells in the presence of rituximab were used as CD16-KHYG-1 susceptible target cells for evaluating ADCC. Fluorescence intensity profiles of green and red signals as indicated were measured across the white line in the right panel. Shown are representatives of live cell images from at least 15 repeat samplings for each condition taken by confocal microscopy at 15-60 min after mixing the cells. Scale bars: 5 μm.

**Figure 3. PD-1 signaling does not affect the conjugation between NK and target cells.** NK-GFP or NK-PD-1-GFP cells were mixed with the their target cells, as indicated, at a 1:2

ratio for 30 min. Cells were fixed, and then analyzed by flow cytometry. The ratios of GFP and mCherry double-positive events to total GFP-positive NK cells were calculated to determine the percentage of conjugation. K562 cells (**A**) were used for NC and Daudi cells (**B**) were used for ADCC, where rituximab was included throughout the experiment. Error bars represent SDs. Data are from three independent experiments. NS, Not significant.

**Figure 4. PD-1 engagement blocks lytic granule polarization.** Two representative images (1 and 2) for projections of 3D-reconstructions of paired cells between NK cells and their target cells at 30 mins after conjugation are shown on the left for measuring NC (**A**) and ADCC (**B**). Quantitative data are shown on the right for measuring the distance between lytic granules and center of synapse. Cells were stained with anti-perforin (*green,* for lytic granule staining) and phalloidin (*red,* for F-actin staining). NK cells were identified by perforin staining and marked by dash lines. IS was identified by accumulated F-actin combining with DIC images. In ADCC assays (B), NK cells were pretreated with Rituximab to trigger CD16 signaling. Scale bars: 5 μm. The mean distance of the perforin cluster to IS in each NK-target conjugate (represented by individual data points) was calculated and summarized in the right panel. The average values and SDs are shown by the horizontal lines (****, $p < 0.0001$, *by Student's t test*).

**Figure 5. PD-1 signaling blocks the accumulation of ILK to the IS in NK cells.** Conjugates were made between NK and K562 cells, as indicated. Cells were fixed and stained with anti-perforin (*green*), anti-ILK (*red*), and DAPI (*blue*, for nuclei). (**A**) Representative images for projections of 3D-reconstructions of paired NK-K562 cells without (*upper panels*) or with (*lower panels*) PD-1/PD-L1 engagement at 30 min after conjugation. Scale bars: 5 μm. (**B**) Quantification of the percentage of NK-target conjugates

583 showing accumulation of ILK at the IS at different time points after conjugation. n indicates
584 the total number of conjugates counted.

585

586 **Figure 6. A new model for PD-1 regulation of NK cell functions.** In the absence of
587 PD-1/PD-L1 engagement (*left*), the activation of activating receptors in the NK cell induces
588 the accumulation of perforin-containing granules and ILK to the IS when it encounters a
589 target cell. This leads to a consequent degranulation and cytotoxic killing of the target cell.
590 However, in the presence of PD-1/PD-L1 engagement (*right*), which represents inhibitory
591 signaling, the polarization of perforin and ILK is blocked, rendering the inhibition of NK
592 cytotoxicity and survival of the target cell.

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## REFERENCES


1. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J 1992; 11:3887-95.

2. Ma W, Gilligan BM, Yuan J, Li T. Current status and perspectives in translational biomarker research for PD-1/PD-L1 immune checkpoint blockade therapy. J Hematol Oncol 2016; 9:47.

3. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. Sci Transl Med 2016; 8:328rv4.

4. Kuchroo VK, Anderson AC, Petrovas C. Coinhibitory receptors and CD8 T cell exhaustion in chronic infections. Curr Opin HIV AIDS 2014; 9:439-45.

5. Pedoeem A, Azoulay-Alfaguter I, Strazza M, Silverman GJ, Mor A. Programmed death-1 pathway in cancer and autoimmunity. Clin Immunol 2014; 153:145-52.

6. Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. Mucosal Immunol 2011; 4:288-93.

7. Zhang YH, Tian M, Tang MX, Liu ZZ, Liao AH. Recent Insight into the Role of the PD-1/PD-L1 Pathway in Feto-Maternal Tolerance and Pregnancy. Am J Reprod Immunol 2015; 74:201-8.

8. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008; 26:677-704.

9. Patsoukis N, Sari D, Boussiotis VA. PD-1 inhibits T cell proliferation by upregulating p27 and p15 and suppressing Cdc25A. Cell Cycle 2012; 11:4305-9.

10. Riley JL. PD-1 signaling in primary T cells. Immunol Rev 2009; 229:114-25.

11. Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, et al. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat Immunol 2009; 10:1185-92.

12. Wei F, Zhong S, Ma Z, Kong H, Medvec A, Ahmed R, et al. Strength of PD-1 signaling differentially affects T-cell effector functions. Proc Natl Acad Sci U S A 2013; 110:E2480-9.

13. Santarpia M, Karachaliou N. Tumor immune microenvironment characterization and response to anti-PD-1 therapy. Cancer Biol Med 2015; 12:74-8.

14. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 2009; 206:3015-29.

15. Alvarez IB, Pasquinelli V, Jurado JO, Abbate E, Musella RM, de la Barrera SS, et al. Role played by the programmed death-1-programmed death ligand pathway during innate immunity against Mycobacterium tuberculosis. J Infect Dis 2010; 202:524-32.

16. Benson DM, Jr., Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. Blood 2010; 116:2286-94.

17. Beldi-Ferchiou A, Lambert M, Dogniaux S, Vely F, Vivier E, Olive D, et al. PD-1 mediates functional exhaustion of activated NK cells in patients with Kaposi sarcoma. Oncotarget 2016; 7:72961-77.

18. Pesce S, Greppi M, Tabellini G, Rampinelli F, Parolini S, Olive D, et al. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. J Allergy Clin Immunol 2017; 139:335-46 e3.

19. Bryceson YT, Long EO. Line of attack: NK cell specificity and integration of signals. Curr Opin Immunol 2008; 20:344-52.

20. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. Annu Rev Immunol 2013; 31:227-58.

21. Long EO. Regulation of immune responses through inhibitory receptors. Annu Rev Immunol 1999; 17:875-904.

22. March ME, Long EO. beta2 integrin induces TCRzeta-Syk-phospholipase C-gamma phosphorylation and paxillin-dependent granule polarization in human NK cells. J Immunol 2011; 186:2998-3005.

23. Barber DF, Faure M, Long EO. LFA-1 contributes an early signal for NK cell cytotoxicity. J Immunol 2004; 173:3653-9.

24. Anikeeva N, Somersalo K, Sims TN, Thomas VK, Dustin ML, Sykulev Y. Distinct role of lymphocyte function-associated antigen-1 in mediating effective cytolytic activity by cytotoxic T lymphocytes. Proc Natl Acad Sci U S A 2005; 102:6437-42.

25. Bryceson YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. J Exp Med 2005; 202:1001-12.

26. Zhang M, March ME, Lane WS, Long EO. A signaling network stimulated by beta2 integrin promotes the polarization of lytic granules in cytotoxic cells. Sci Signal 2014; 7:ra96.

27. Alpert MD, Harvey JD, Lauer WA, Reeves RK, Piatak M, Jr., Carville A, et al. ADCC develops over time during persistent infection with live-attenuated SIV and is associated with complete protection against SIV(mac)251 challenge. PLoS Pathog 2012; 8:e1002890.

28. Jang JH, Huang Y, Zheng P, Jo MC, Bertolet G, Zhu MX, et al. Imaging of Cell-Cell Communication in a Vertical Orientation Reveals High-Resolution Structure of Immunological Synapse and Novel PD-1 Dynamics. J Immunol 2015; 195:1320-30.

29. Grier JT, Forbes LR, Monaco-Shawver L, Oshinsky J, Atkinson TP, Moody C, et al. Human immunodeficiency-causing mutation defines CD16 in spontaneous NK cell cytotoxicity. J Clin Invest 2012; 122:3769-80.

30. Song H, Kim J, Cosman D, Choi I. Soluble ULBP suppresses natural killer cell activity via down-regulating NKG2D expression. Cell Immunol 2006; 239:22-30.

31. Alpert MD, Heyer LN, Williams DE, Harvey JD, Greenough T, Allhorn M, et al. A novel assay for antibody-dependent cell-mediated cytotoxicity against HIV-1- or SIV-infected cells reveals incomplete overlap with antibodies measured by neutralization and binding assays. J Virol 2012; 86:12039-52.

32. Pentcheva-Hoang T, Chen L, Pardoll DM, Allison JP. Programmed death-1 concentration at the immunological synapse is determined by ligand affinity and availability. Proc Natl Acad Sci U S A 2007; 104:17765-70.

33. Orange JS. Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol 2008; 8:713-25.

34. Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, et al. The immunological synapse. Annu Rev Immunol 2001; 19:375-96.

686 35. Liu D, Bryceson YT, Meckel T, Vasiliver-Shamis G, Dustin ML, Long EO.
687 Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic
688 immune synapses. Immunity 2009; 31:99-109.

689 36. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M,
690 Saito T. Programmed cell death 1 forms negative costimulatory microclusters that
691 directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J Exp Med
692 2012; 209:1201-17.

693 37. Liu D, Peterson ME, Long EO. The adaptor protein crk controls activation and
694 inhibition of natural killer cells. Immunity 2012; 36:600-11.

695 38. Mace EM, Dongre P, Hsu HT, Sinha P, James AM, Mann SS, et al. Cell biological
696 steps and checkpoints in accessing NK cell cytotoxicity. Immunol Cell Biol 2014;
697 92:245-55.

698 39. Kamphorst AO, Ahmed R. Manipulating the PD-1 pathway to improve immunity.
699 Curr Opin Immunol 2013; 25:381-8.

700 40. Fuller MJ, Callendret B, Zhu B, Freeman GJ, Hasselschwert DL, Satterfield W, et al.
701 Immunotherapy of chronic hepatitis C virus infection with antibodies against
702 programmed cell death-1 (PD-1). Proc Natl Acad Sci U S A 2013; 110:15001-6.

703 41. Abeyweera TP, Kaissar M, Huse M. Inhibitory receptor signaling destabilizes
704 immunological synapse formation in primary NK cells. Front Immunol 2013; 4:410.

705 42. Eissmann P, Davis DM. Inhibitory and regulatory immune synapses. Curr Top
706 Microbiol Immunol 2010; 340:63-79.

707 43. Zinselmeyer BH, Heydari S, Sacristan C, Nayak D, Cammer M, Herz J, et al. PD-1
708 promotes immune exhaustion by inducing antiviral T cell motility paralysis. J Exp
709 Med 2013; 210:757-74.

710 44. Schleinitz N, March ME, Long EO. Recruitment of activation receptors at inhibitory
711 NK cell immune synapses. PLoS One 2008; 3:e3278.

712 45. Dieckmann NM, Frazer GL, Asano Y, Stinchcombe JC, Griffiths GM. The cytotoxic
713 T lymphocyte immune synapse at a glance. J Cell Sci 2016; 129:2881-6.

714 46. Dustin ML, Groves JT. Receptor signaling clusters in the immune synapse. Annu Rev
715 Biophys 2012; 41:543-56.

716 47. Kinashi T. Overview of integrin signaling in the immune system. Methods Mol Biol
717 2012; 757:261-78.

718 48. Springer TA, Dustin ML. Integrin inside-out signaling and the immunological
719 synapse. Curr Opin Cell Biol 2012; 24:107-15.

720 49. Azoulay-Alfaguter I, Strazza M, Pedoeem A, Mor A. The coreceptor programmed
721 death 1 inhibits T-cell adhesion by regulating Rap1. J Allergy Clin Immunol 2015;
722 135:564-7.

723 50. Saunders PA, Hendrycks VR, Lidinsky WA, Woods ML. PD-L2:PD-1 involvement
724 in T cell proliferation, cytokine production, and integrin-mediated adhesion. Eur J
725 Immunol 2005; 35:3561-9.

726 51. Das A, Long EO. Lytic granule polarization, rather than degranulation, is the
727 preferred target of inhibitory receptors in NK cells. J Immunol 2010; 185:4698-704.

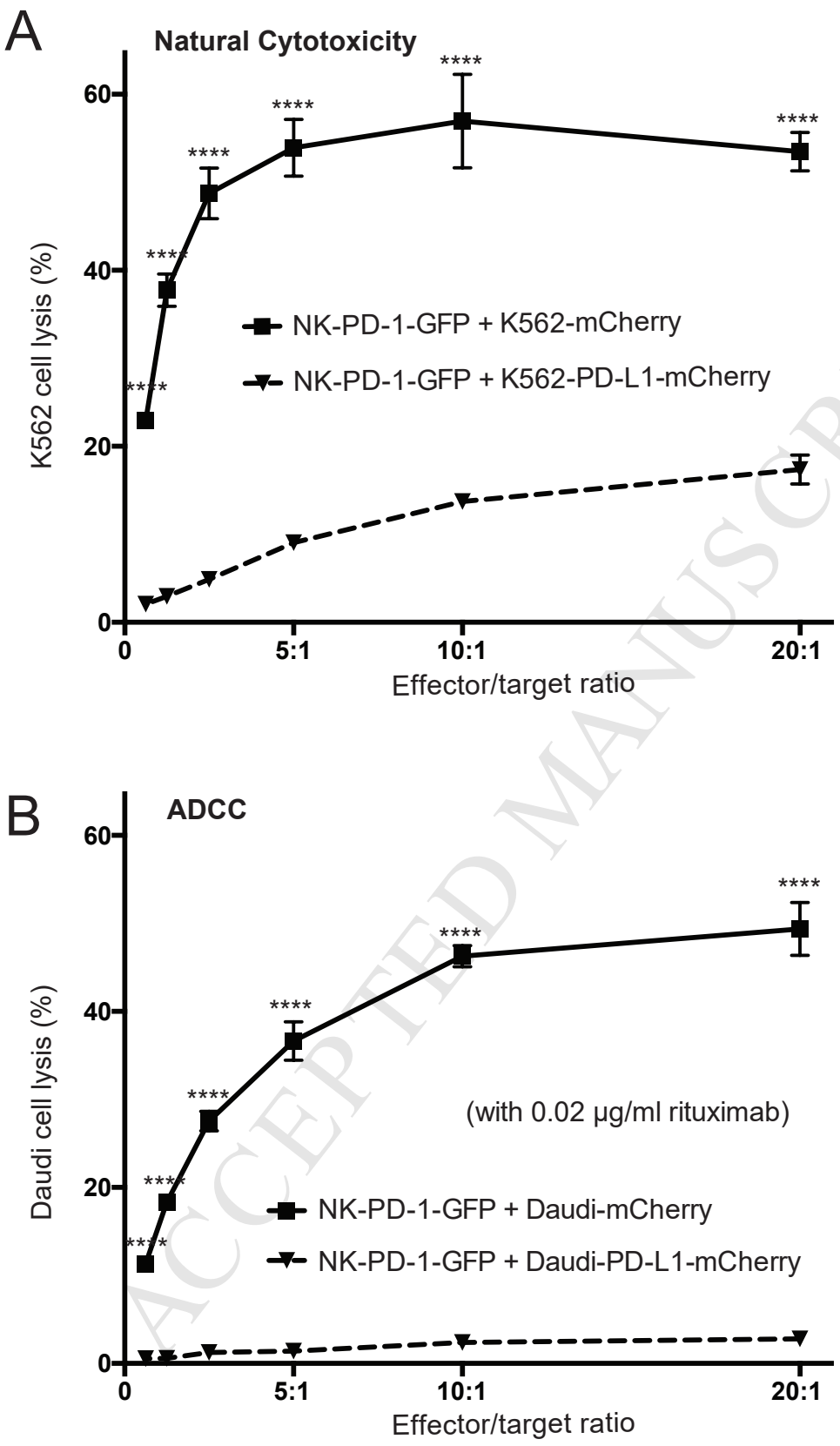
728 52. Wiesmayr S, Webber SA, Macedo C, Popescu I, Smith L, Luce J, et al. Decreased
729 NKp46 and NKG2D and elevated PD-1 are associated with altered NK-cell function
730 in pediatric transplant patients with PTLD. Eur J Immunol 2012; 42:541-50.

731 53. Norris S, Coleman A, Kuri-Cervantes L, Bower M, Nelson M, Goodier MR. PD-1
732 expression on natural killer cells and CD8(+) T cells during chronic HIV-1 infection.
733 Viral Immunol 2012; 25:329-32.
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Figure 1

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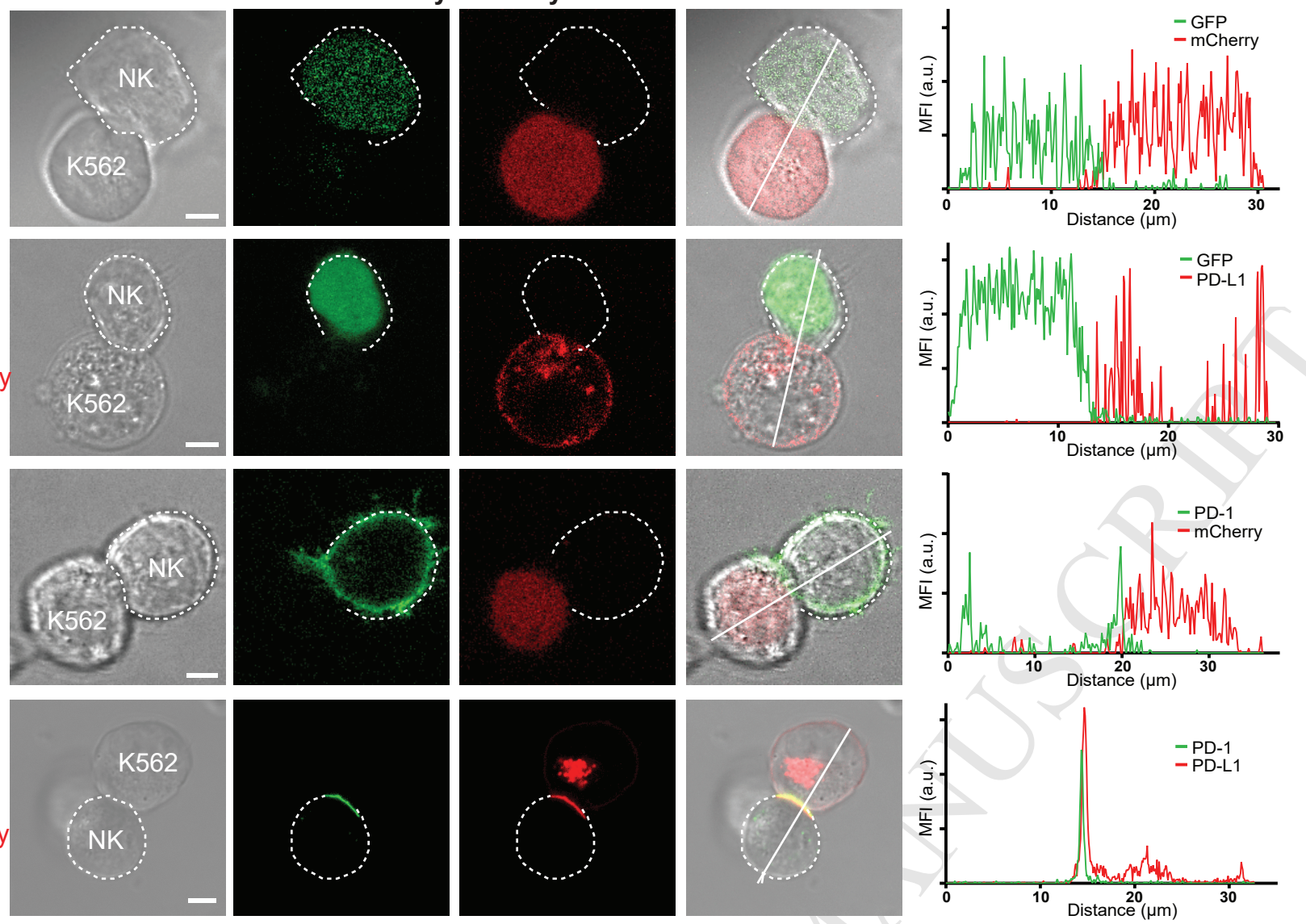



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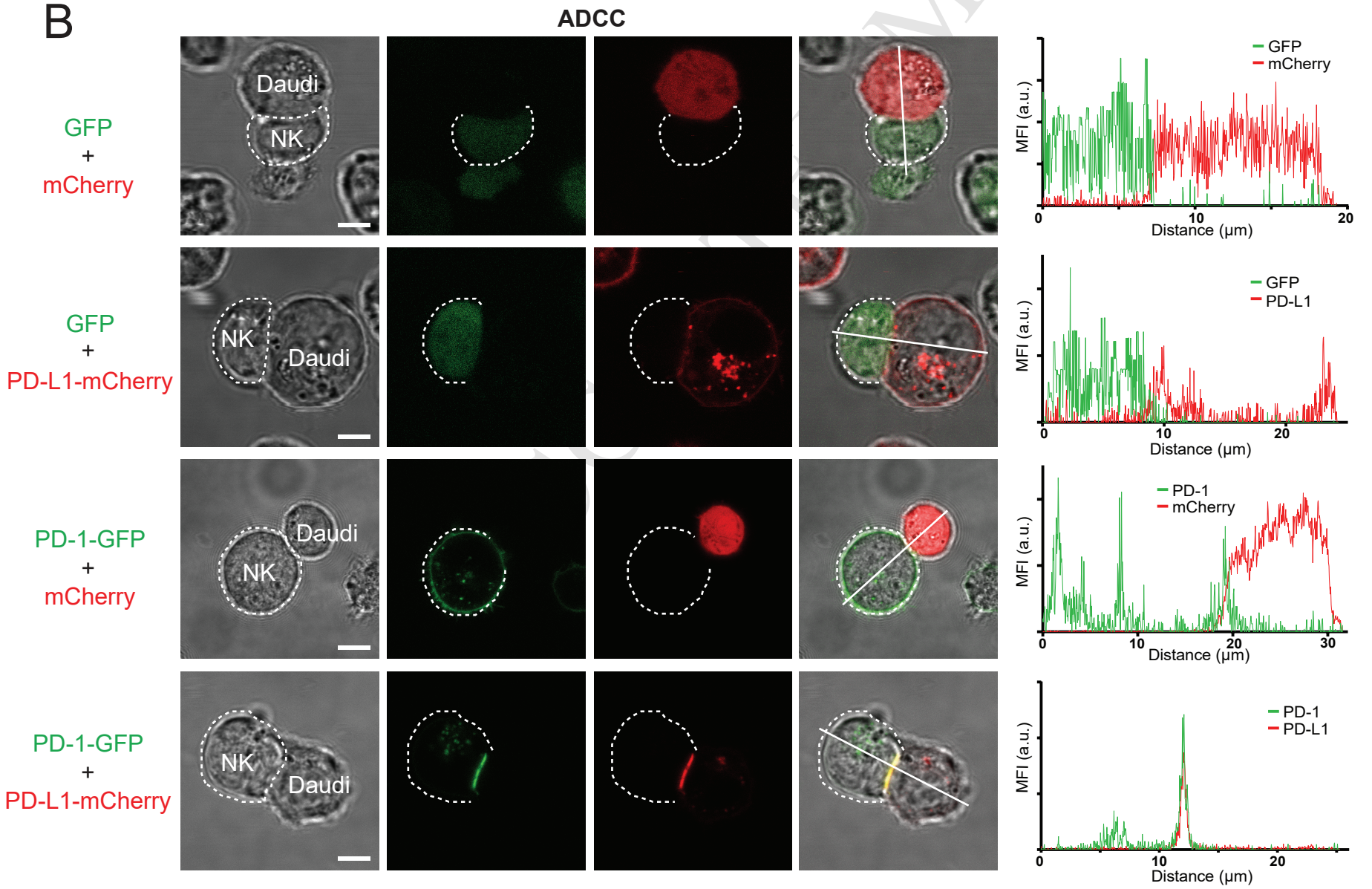
Figure 2

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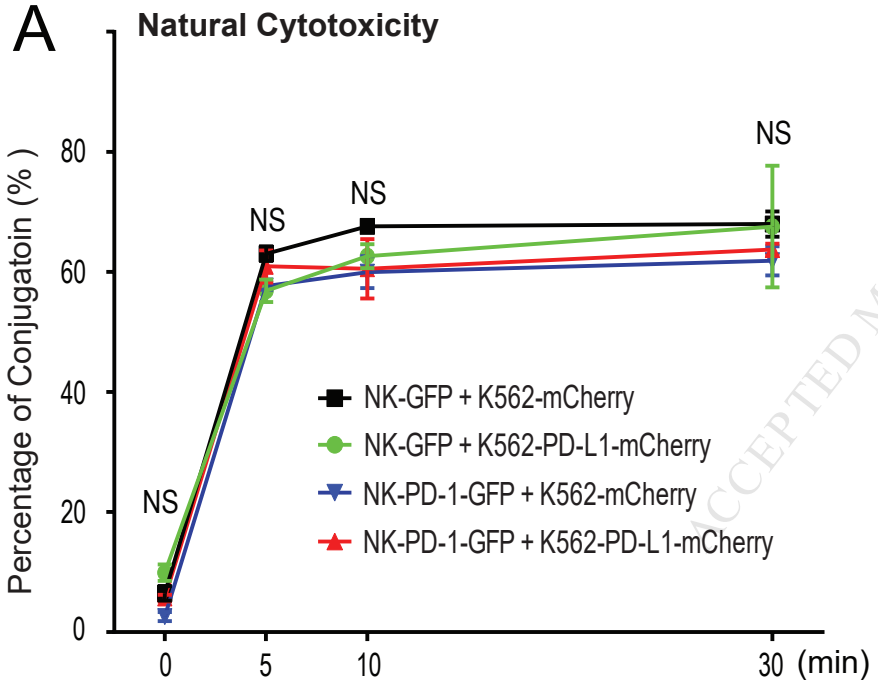


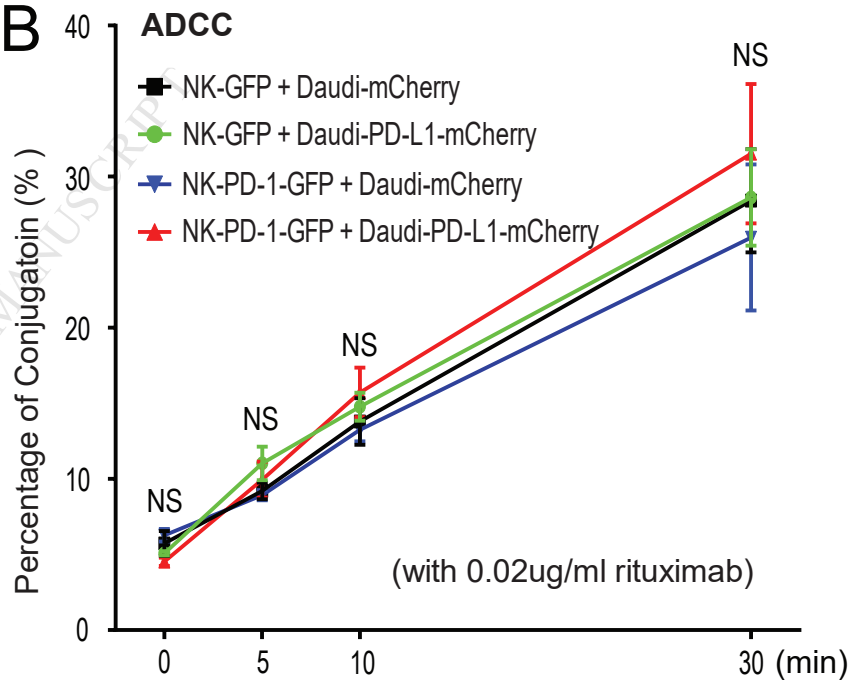

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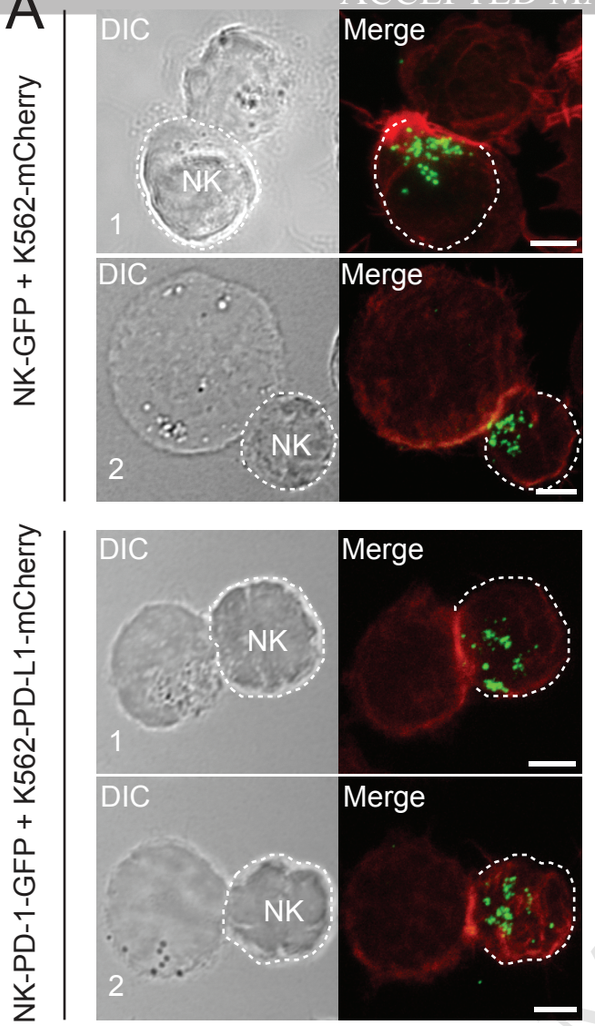
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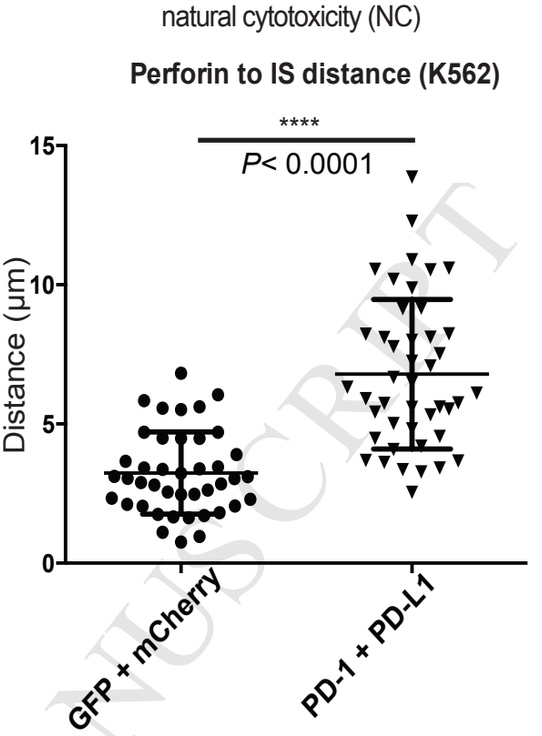


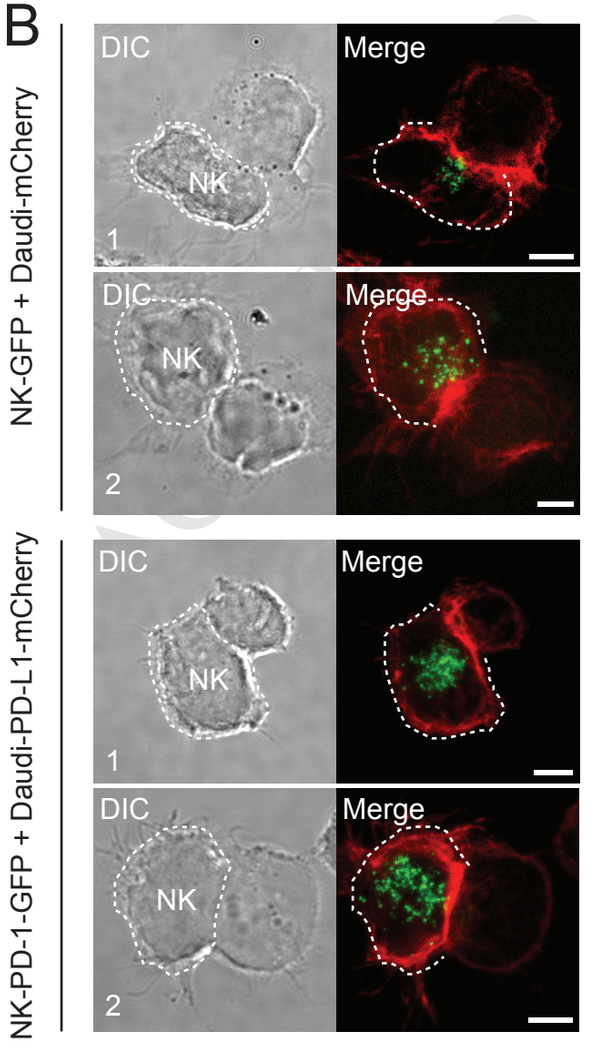




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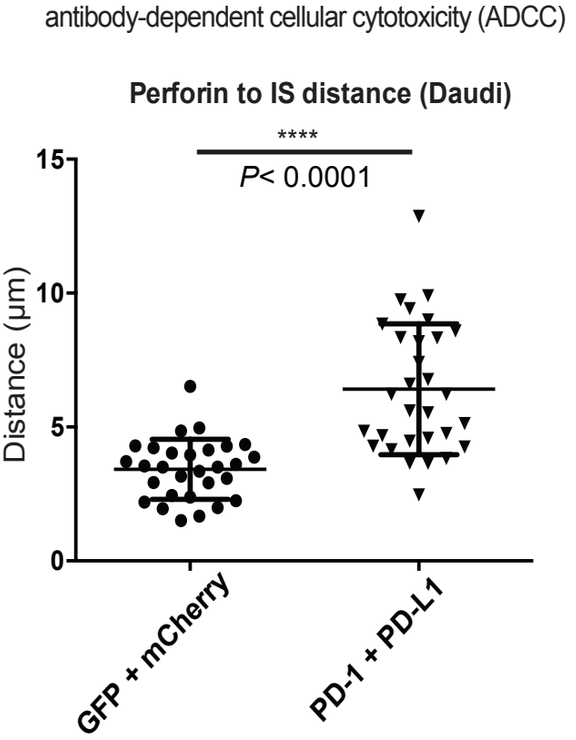
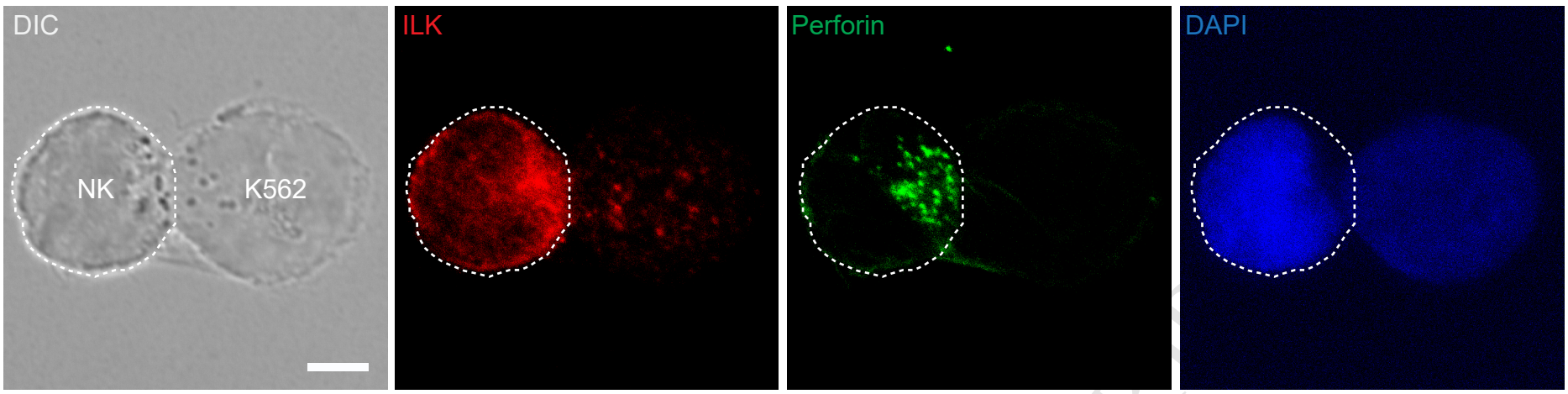



Figure 5

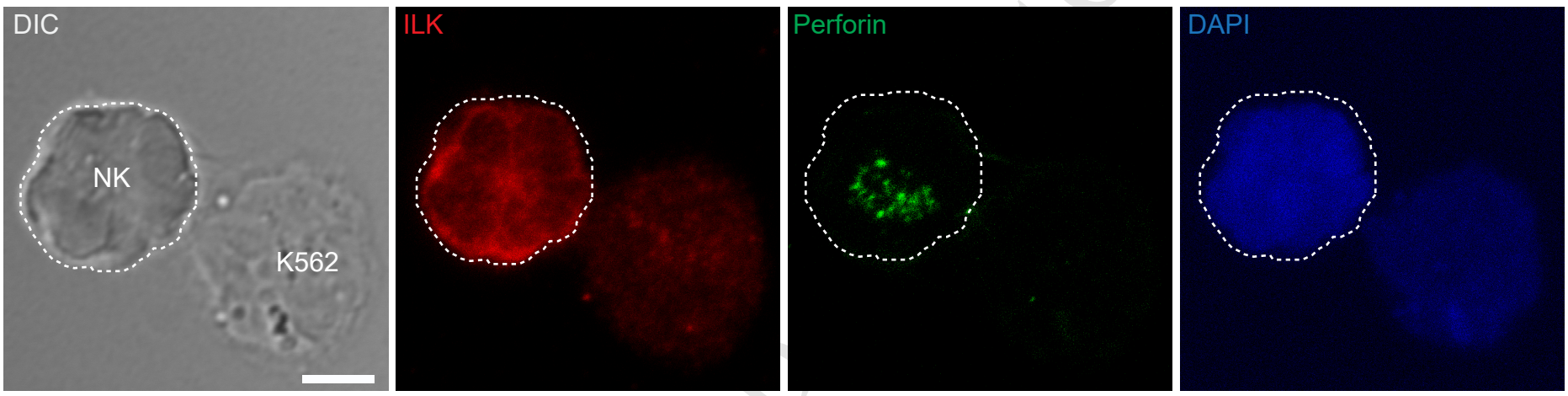
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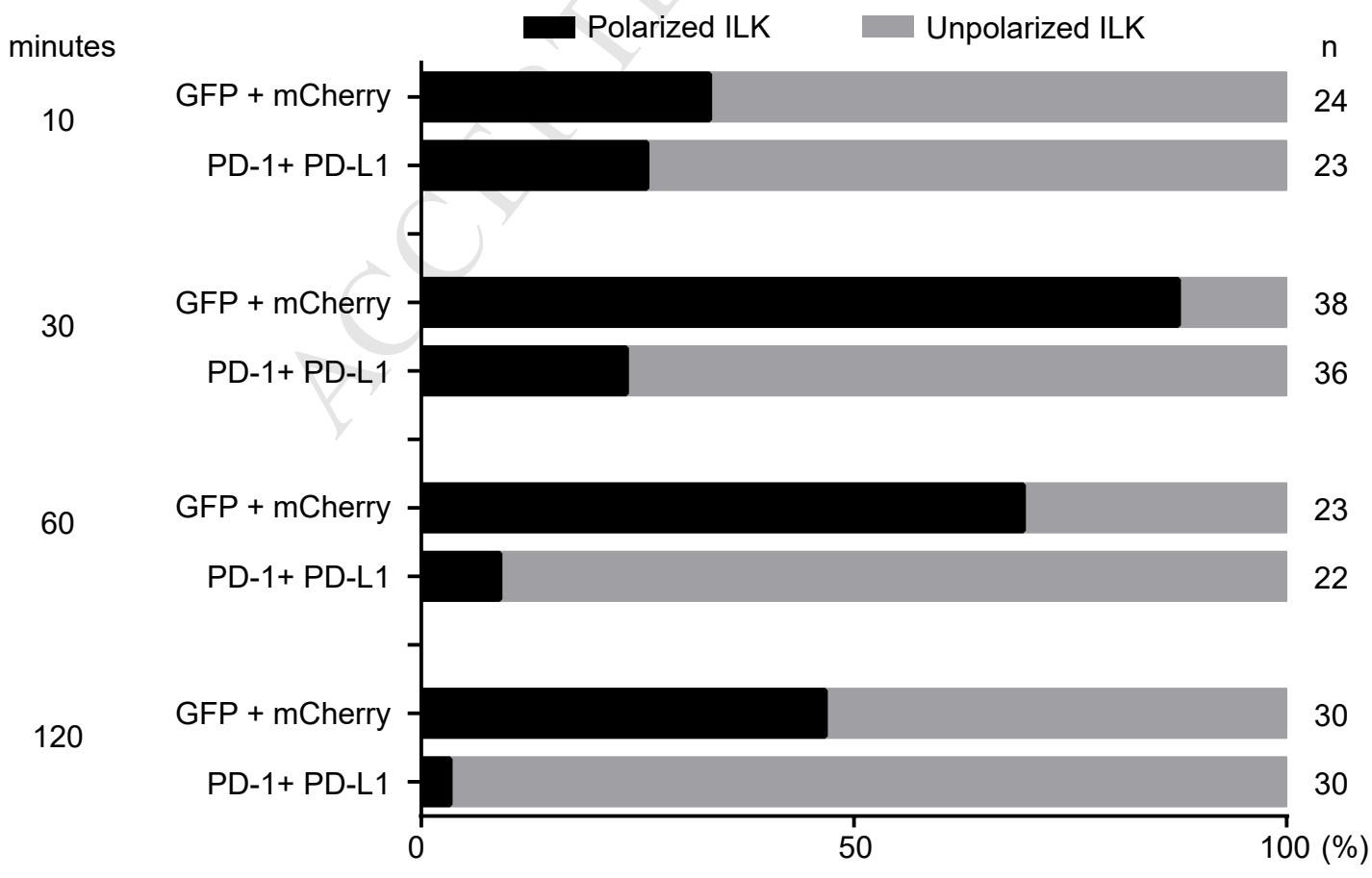
NK-GFP + K562-mCherry




NK-PD-1-GFP + K562-PD-L1-mCherry

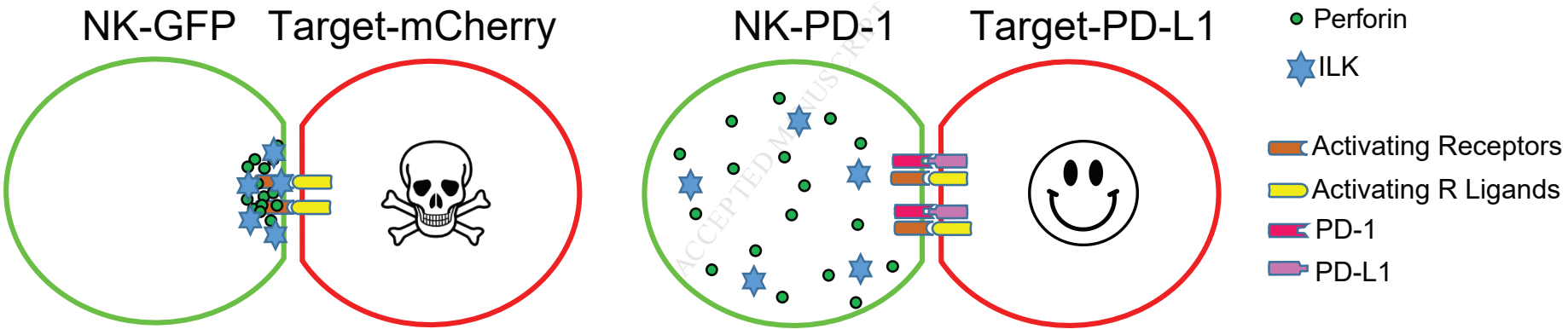



B



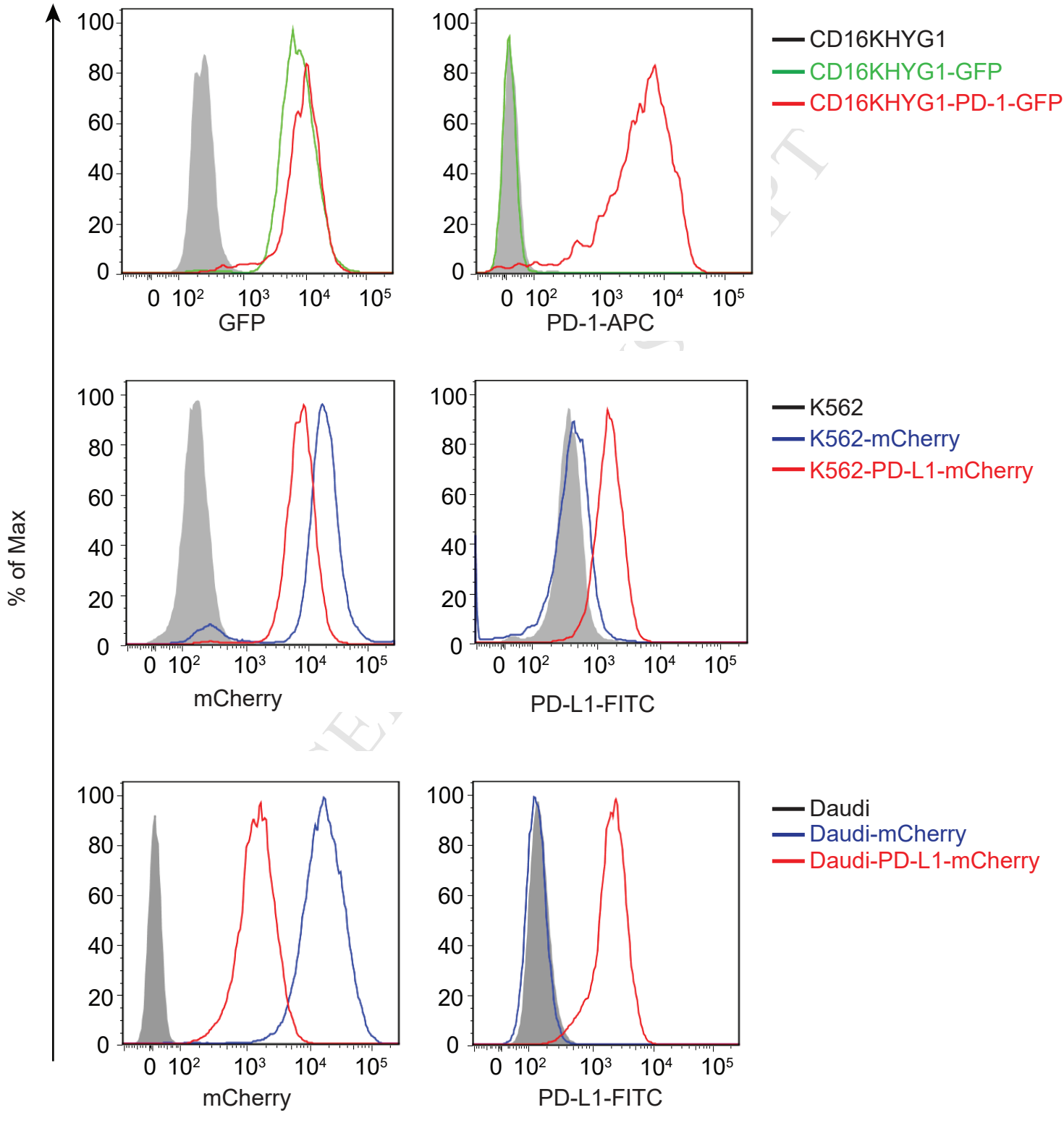
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# Figure 6



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# Figure E1



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# Figure E2

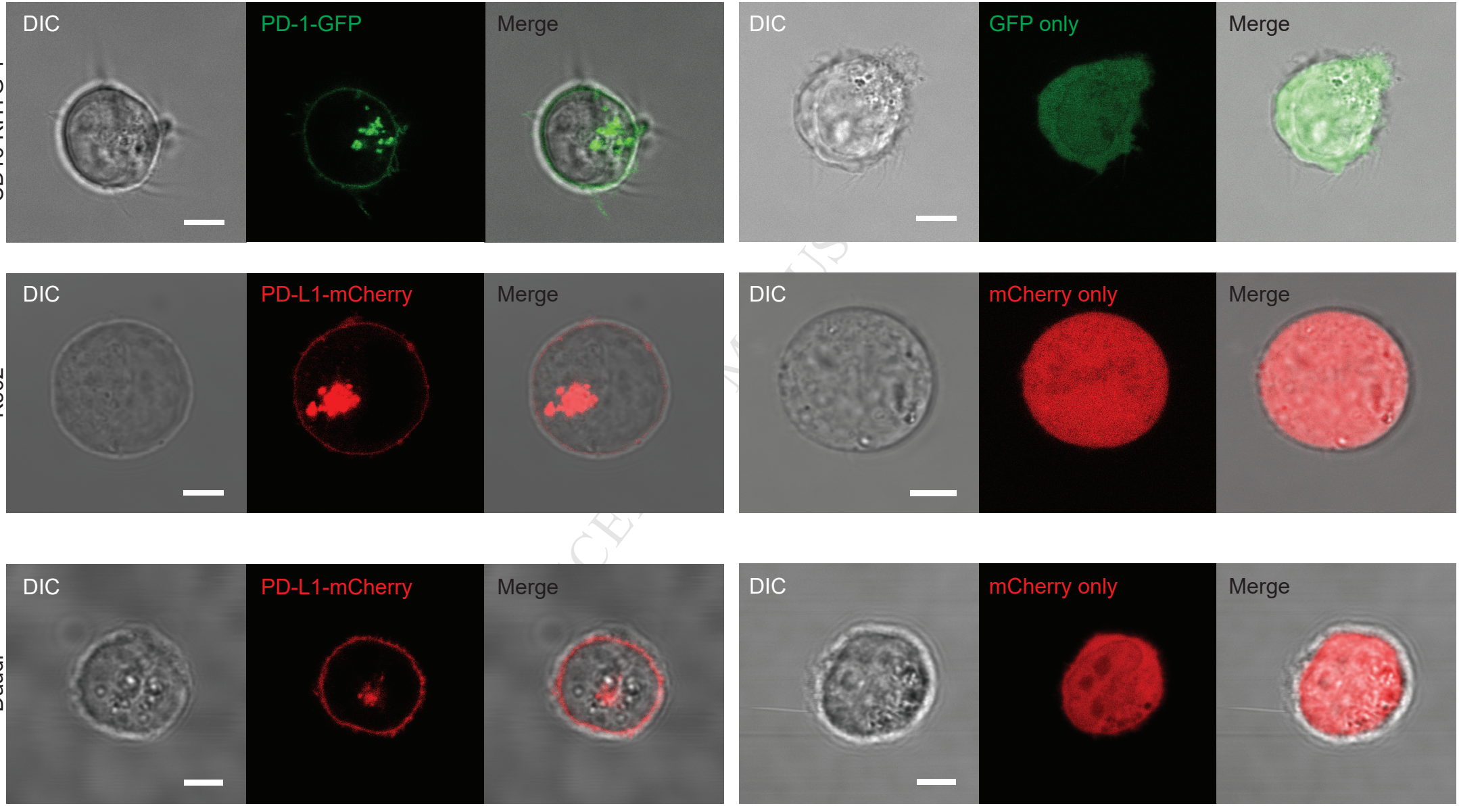
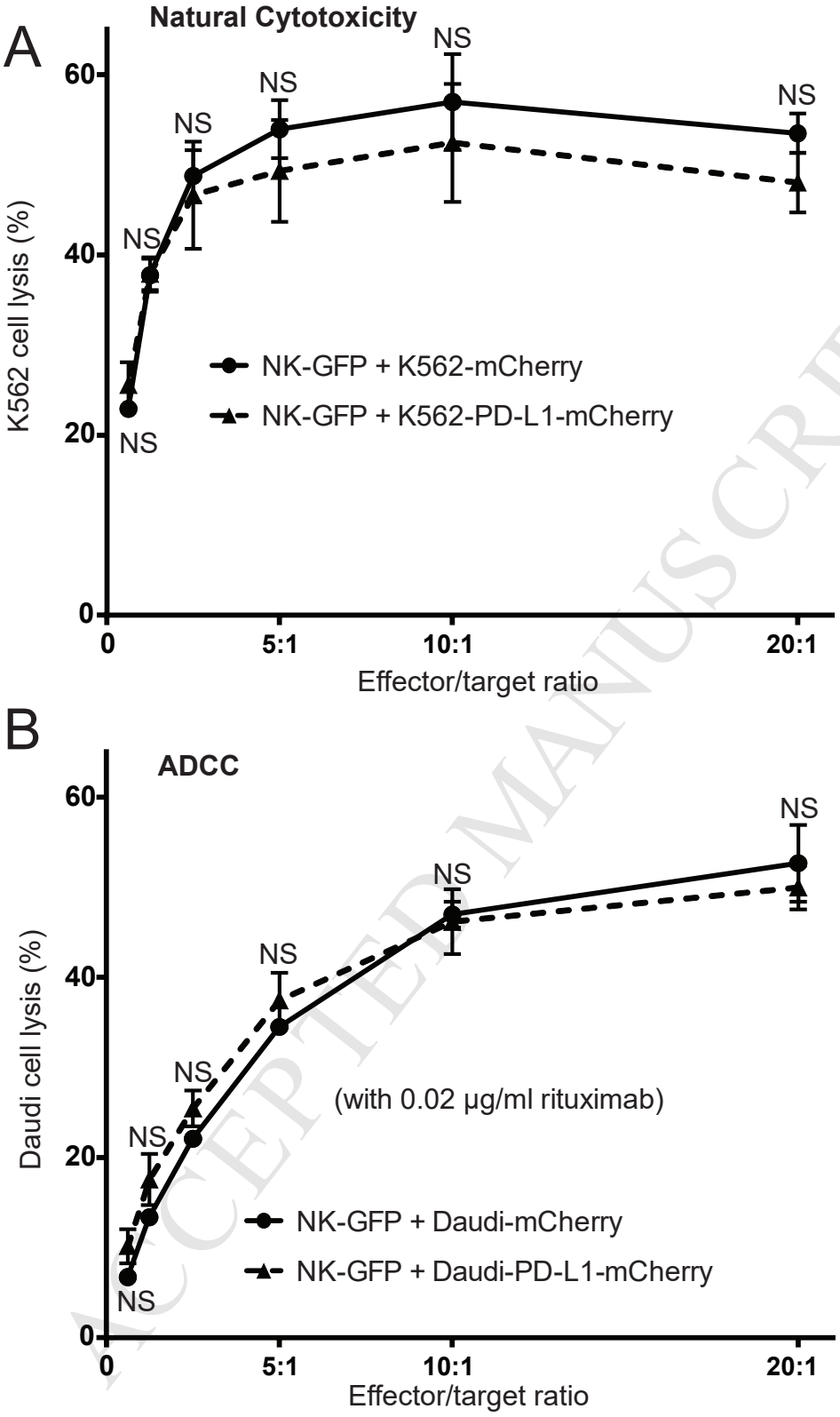


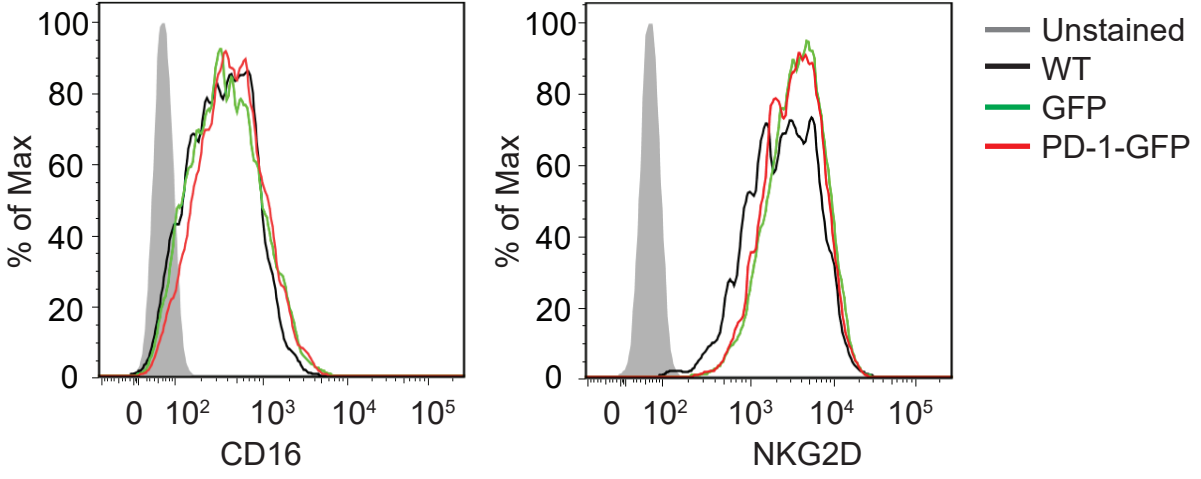
Figure E3




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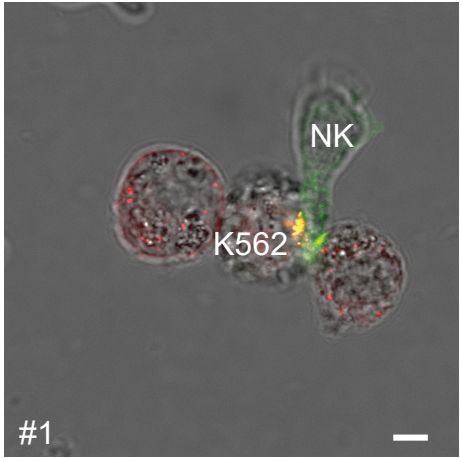
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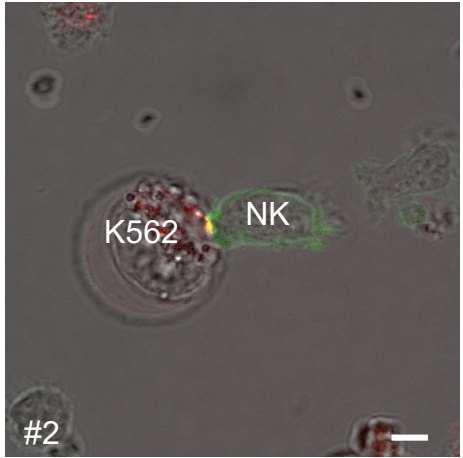
# Figure E4



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# Figure E5



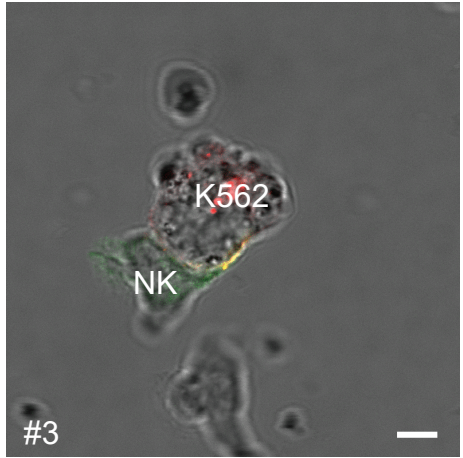
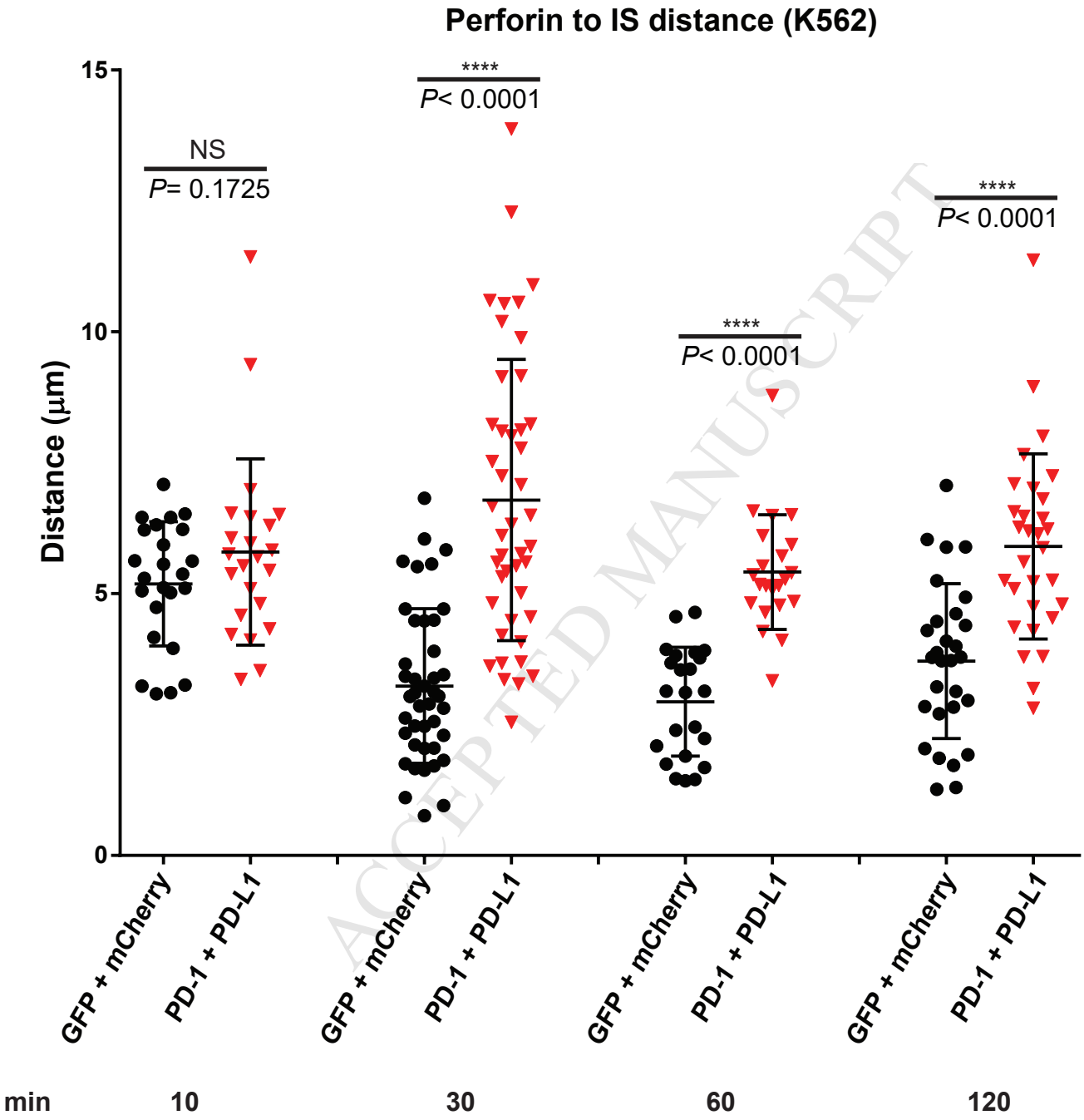



Figure E6

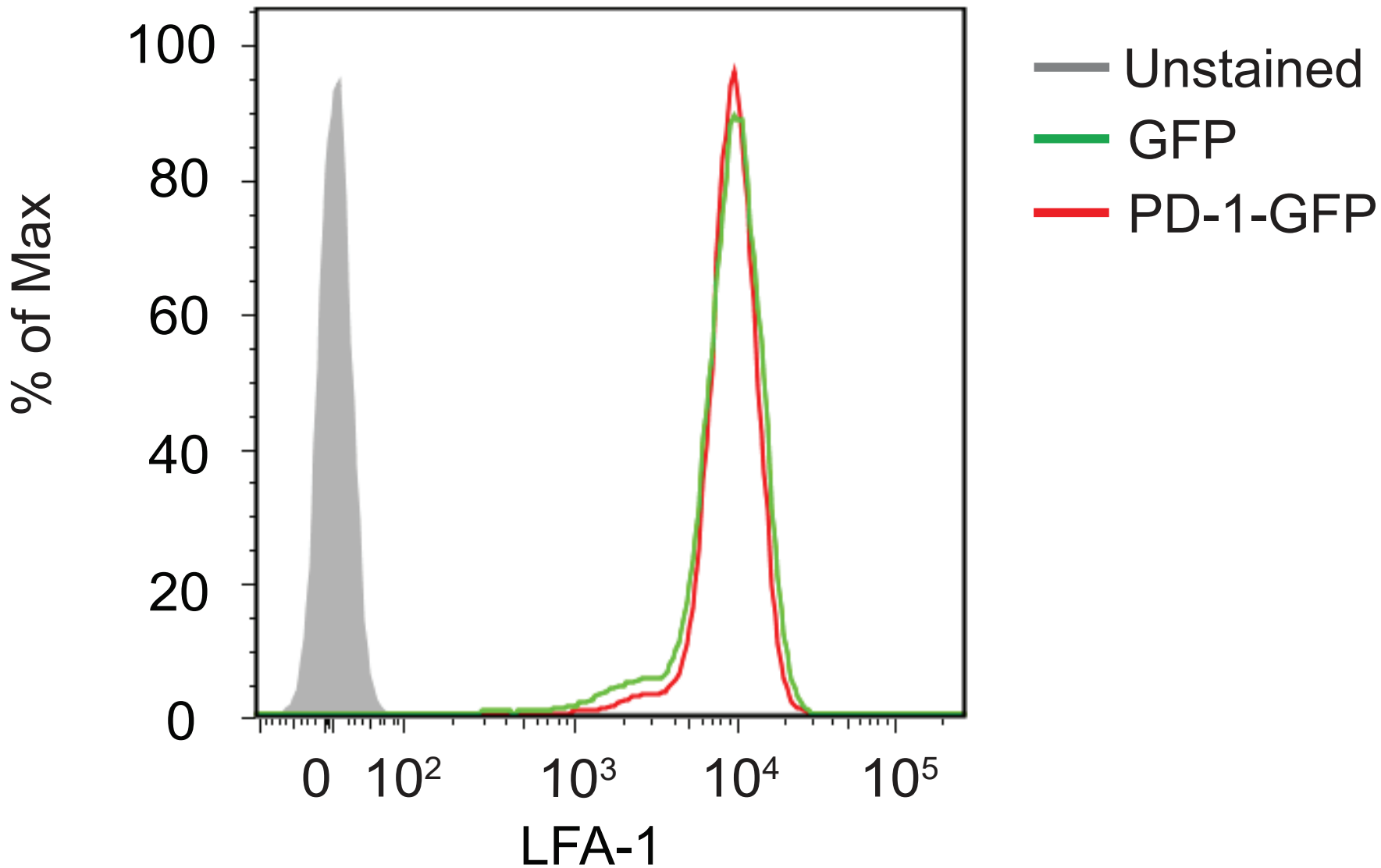
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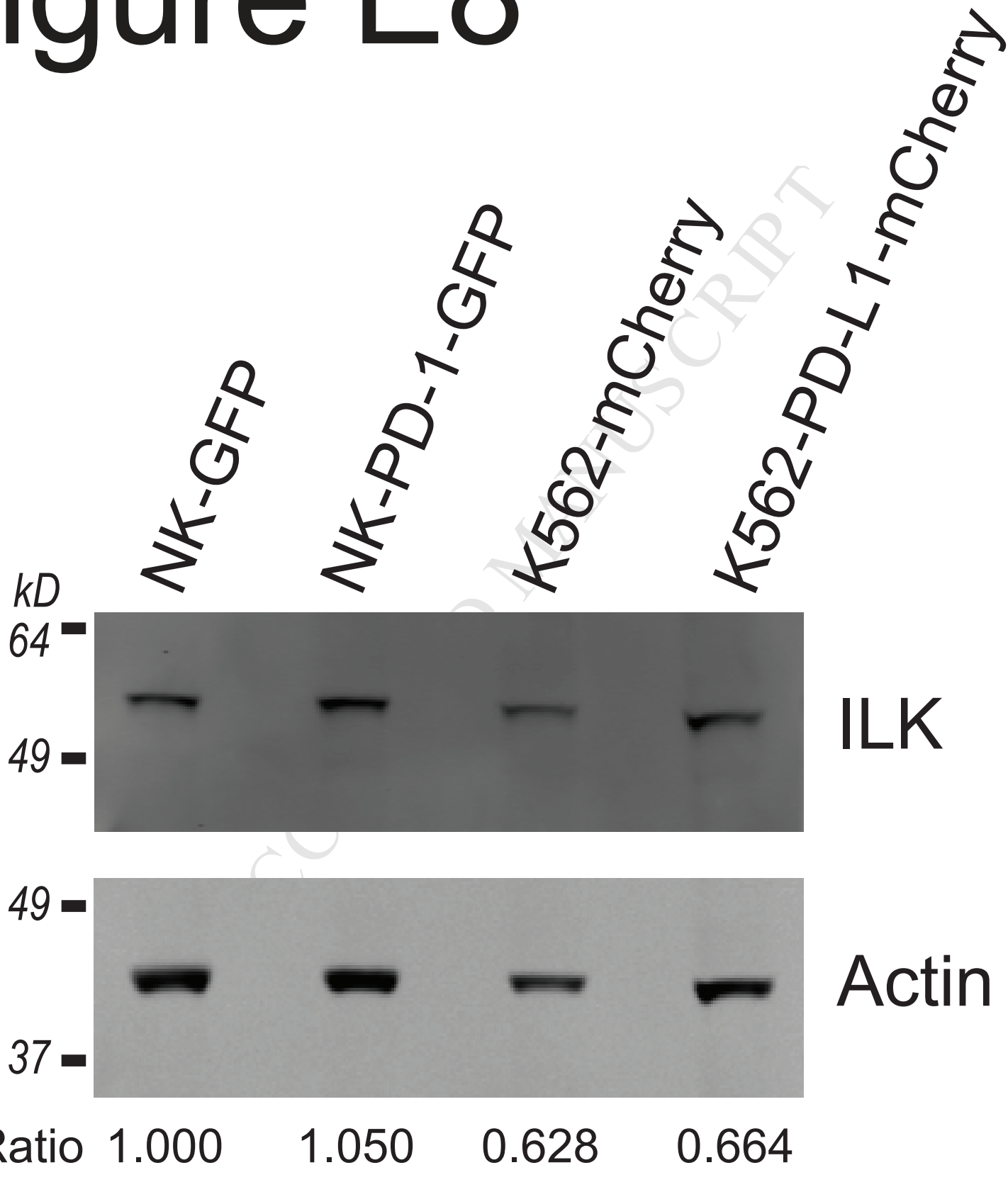
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# Figure E7



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# Figure E8



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**Supplementary Figure Legends:**

**Figure E1. Verification of PD-1 and PD-L1 molecule expression in NK and target cells, respectively, by flow cytometry.** First row, histograms of wild-type (*gray*), GFP- (*green*), or PD-1-GFP- (*red*) transduced CD16-KHYG-1 cells tested by flow cytometry for GFP fluorescence (*left*) and APC-tagged PD-1 antibody staining (*right*). Second and third rows, histograms of wild type (*gray*), mCherry- (*blue*), or PD-L1-mCherry- (*red*) transduced K562 (*second row*) or Daudi (*third row*) cells measured by flow cytometry for mCherry florescence (*left*) and FITC-tagged PD-L1 antibody staining (*right*).

**Figure E2. Fluorescence protein-tagged PD-1 and PD-L1 are expressed on the plasma membrane of NK and target cells, respectively.** Representative confocal differential interference contrast and fluorescence images of GFP and mCherry for a single cell that expressed GFP or PD-1-GFP (*green*) in CD16-KHYG-1 and mCherry or PD-L1-mCherry (*red*) in K562 or Daudi cells. Scale bars: 5 μm.

**Figure E3. The cytotoxicity of NK cells in the absence of PD-1 is not affected by the presence of PD-L1 in target cells.** (**A**) The NC of NK-GFP cells towards K562-mCherry (*circles*) or K562-PDL1-mCherry (*triangles*) cells was measured by the $^{51}$Cr-release assay with varying NK/K562 (effector/target) ratios. (**B**) The ADCC of NK-GFP towards Daudi-mCherry (*circles*) or Daudi-PDL1-mCherry (*triangles*) cells was measured by the $^{51}$Cr-release assay in the presence of rituximab as in Fig. 1. Results are representatives of three independent experiments. Error bars represent SDs. NS, Not significant.

**Figure E4. PD-1-GFP expression does not affect the surface expression of activating receptors CD16 and NKG2D in NK cells.** Histograms of flow cytometry analyses of cell surface staining by CD16 (*left*) and NKG2D (*right*) antibodies in wild-type (*black*), GFP- (*green*), or PD-1-GFP- (red) transduced CD16-KHYG-1 cells.

**Figure E5. PD-1 and PD-L1 molecules accumulate at IS in primary NK cells.** Representative live-cell imaging of PD-1-GFP infected primary cells (green) conjugated with K562-PD-L1-mCherry target cells (red). Images were taken by confocal microscopy at 15-60 min after conjugation. Scale bars: 5 μm.

**Figure E6. PD-1 engagement blocks lytic granule polarization during two-hour conjutations.** The mean distance of the perforin cluster to the IS in each NK-target conjugate (represented by individual data points) was calculated and summarized for each indicated time point after conjugation. 'GFP' and 'mCherry' represent NK-GFP and K562-mCherry, respectively. 'PD-1' and 'PD-L1' represents NK-PD-1-GFP and K562-PD-L1-mCherry, respectively. The average values and SDs are shown by the horizontal lines (NS, Not significant; ****, *$p \leq 0.0001$, by Student's t test*).

**Figure E7. PD-1-GFP expression does not affect the overall expression of LFA-1 in NK cells**. Histogram of flow cytometry analysis of the staining of LFA-1 by monoclonal antibody against LFA-1 in GFP- (*green*) or PD-1-GFP- (red) transduced CD16-KHYG-1 cells.

**Figure E8. No difference of ILK expression within the NK cells or the K562 cells.** ILK and loading control (actin) proteins were probed with the antibodies as indicated in the labeled NK and K562 cells. The ratio of ILK to actin is calculated and normalized in the bottom line.

**Supplementary Movie Legends:**

**Movie S1. PD-1 and PD-L1 molecules accumulate at NK IS.** Time−lapse images of NK-PD-1-GFP cells *(green)* mixed with K562-PD-L1-mCherry cells *(red)*. The time interval between two frames is 3 seconds. Scale bar represents 15 μm.

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