Differential effects of interleukin-12 and interleukin-15 on expansion of NK cell receptor-expressing CD8$^+$ T cells.

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【running title】
Differential effects of IL-12 and IL-15 on T cells

Key words

CD94; NKG2A; NKG2D; cytolytic activity

Abstract

The cytolytic activity of cells expressing natural killer cell receptors(NKRs) depends on the balance between stimulatory and inhibitory signals. We investigated both inhibitory NK receptor (CD94/NKG2A) expression and stimulatory NKR (NKG2D) expression on T cells after stimulation with cytokines (IL-12 or IL-15).

Cytolytic NKR-expressing $CD8^+$ T cells were expanded from normal adult peripheral blood mononuclear cells using anti-CD3 monoclonal antibody and cytokines (IL-12 or IL-15). The proportion and absolute number of CD94/NKG2A-expressing T cells expanded by IL-12 were significantly larger than those of the cells expanded by IL-15. On the other hand, the proportion and absolute number of NKG2D-expressing T cells expanded by IL-15 were significantly larger than those of the cells expanded by IL-12. The proportions of NKG2D and intracellular granzyme A expression in CD94-expressing cells were much more increased in PBMCs cultured with IL-15 than those of cells cultured with IL-12. A real-time polymerase chain reaction assay showed that there was a 1.68-fold increase in NKG2D mRNA expression level and a 1.37-fold increase in DAP10 mRNA expression level in CD94-expressing cells expanded by IL-15 compared with those of the cells expanded by IL-12. The cytolytic activity levels of purified CD94-expressing cells from 8-day culture with IL-15 tested against ^{51}Cr-labeled K562 cells by standard 4-hour ^{51}Cr release assays without prior sensitization were much higher than those of cells from 8-day culture with IL-12.

IL-15 appears to be able to enhance the cytolytic activity of CD94/NKG2A-expressing cells through induction of NKG2D and intracellular granzyme expression much more efficiently than does IL-12.

Introduction

Natural killer cell receptors (NKRs) have been shown to be expressed on not only NK cells but also T cells [1-8]. These NKRs have a stimulatory or inhibitory function through their binding to major histocompatibility complex (MHC) class Ⅰ molecules or MHC class Ⅰ-like molecules. As one of the inhibitory receptors, CD94/NKG2A heterodimer recognizes an HLA-E that preferably binds to a peptide derived from the signal sequences of most HLA class I molecules [9-12]. On the other hand, the stimulatory NKG2D interacts with MHC class Ⅰ chain-related proteins A and B (MICA and MICB) [13,14]. Although NKG2D has no identifiable signaling motifs, it attains signaling capability through association with DNAX-activating protein of 10 kDa (DAP10) [7].

It has been reported that IL-12 [12,15], IL-15 [16-18], prostaglandin E2 [19] and transforming growth factor-β [20,21] can induce the expression of CD94/NKG2A on T cells. On the other hand, it has been reported that IL-2 [22-24], IL-12 [25] and IL-15 [26] can induce NKG2D expression on T cells. Cytolytic activity of NKR-expressing cells depends on the balance between stimulatory and inhibitory signals. However, only the expression of either CD94/NKG2A or NKG2D was examined in those studies. In the present study, we investigated cytolytic activity of both inhibitory NK receptor (CD94/NKG2A)-expressing and stimulatory NK receptor (NKG2D)-expressing T cells after stimulation with immobilized anti-CD3 monoclonal antibody (mAb) and IL-12 or IL-15 in peripheral blood mononuclear cells (PBMCs) for 8 days.

Materials and methods

Peripheral blood mononuclear cells

Normal adult peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors with informed consent from Hokkaido Red Cross Blood Center Sapporo.

Immunofluorescent staining for flow cytometric analysis and monoclonal antibodies

The phycoerythrin (PE) –conjugated monoclonal antibody (mAb) HP-3D9 (anti-CD94)

was obtained from Ancell (Bayport, MN, USA), and Z199 (anti-NKG2A) and ON72 (anti-NKG2D) were obtained from Immunotec (Marseilles, France). Fluorescein isothiocyanate (FITC) –conjugated anti-CD3 and anti-CD8 mAb were purchased from Pharmingen (SanDiego, CA, USA). FITC–conjugated anti-NKG2D was obtained from Serotec (Oxford, England). Anti-CD56 mAb, anti–granzyme A mAb and HI111 (anti-LFA-1) were obtained from Becton Dickinson (BD, San Jose, CA, USA). Anti-CD3 mAb OKT3 was obtained from Ortho Biotech(Raritan, NJ, USA). Anti-NKG2D mAb was obtained from R&D Systems (Minneapolis, MN). Intracellular granzyme A was stained using cytofix/cytoperm reagent according to the instructions of the manufacturer (BD). The fluorescence intensity of the cells was analyzed using a FACS Calibur (BD). Statistical analysis was performed using Student's t-test.

Expansion of NK receptor-expressing cells

For coating with anti-CD3 mAb, 24-well flat-bottom plates or tissue culture flasks were preincubated with OKT3 (1 μg/mL) in 100 mM Tris [tris(hydroxymethyl)aminomethane]-HCl buffer (pH 9.5) for 16 hours at 4°C. PBMCs (1 $\times$ 10^6/mL) were cultured on 24-well plates or tissue culture flasks in RPMI 1640 supplemented with 10% fetal calf serum with 2.5 ng/mL of reconminant human IL-12 or 5 ng/mL of recombinant human IL-15 (R&D Systems) at 37°C for 8 days as described previously [17,18]. Absolute numbers of $CD94^+/CD3^+$, $CD94^+/CD8^+$, $NKG2A^+/CD3^+$, $NKG2A^+/CD8^+$, $NKG2D^+/CD3^+$, $NKG2D^+/CD8^+$, $CD94^+/CD56^+$, and $CD94^+/NKG2D^+$ were calculated from multiplication of the total number of expanded cells and the proportion of these cells in expanded cells.

Immunomagnetic cell sorting

Purified $CD94^+$ cells (> 90% CD94 , as determined by flow cytometric analysis) were obtained by magnetic cell sorting (MACS) using magnetic microbeads according to the instructions of the manufacturer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Real-time polymerase chain reaction

Total RNA was prepared using a cQIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized from 1.2 mg total RNA using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 following the instructions of the manufacturer (Takara, Shiga, Japan). Assays-on-Demand Gene Expression products for NKG2D and DAP10 were obtained from Applied Biosystems(Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. Relative quantification assays for gene expression were performed using an Applied Biosystems 7300 real-time polymerase chain reaction system (Applied Biosystems).

Evaluation of cytolytic activity using 4-hour ^{51}Cr release assay

After 8 days of stimulation by immobilized anti-CD3 mAb with IL-12 or IL-15 in a T25 flask, CD94-expressing cells were purified by MACS. The cytolytic activities of purified CD94-expressing cells were tested against ^{51}Cr-labeled human erythroluekemic K562 cells (5×10^3) using a 4-hour ^{51}Cr release assay. K562 cells were obtained from Riken (Tsukuba, Japan).

Results

Expansion of NK receptor-expressing cells

There was a very small number of NK receptor-expressing $CD3^+/CD8^+$ T cells in PBMCs before culture. However, the proportion and absolute number of NK receptor-expressing $CD3^+/CD8^+$ T cells in PBMCs were dramatically increased after stimulation with immobilized anti-CD3 mAb and cytokines (Figure 1). The proportion and absolute number of CD94/NKG2A-expressing T cells expanded by IL-12 were significantly larger than those of the cells expanded by IL-15 ($CD94^+CD3^+/NKG2A^+CD3^+$: median 39.8% (range 26.4 - 54.7) / 36.4% (22.3 - 47.9) by Il-12 vs 19.1% (11.0 - 21.9) / 12.3% (5.5 - 15.1) by IL-15, n=9, $p < 0.01$). On the other hand, the proportion and absolute number of NKG2D-expressing T cells expanded by IL-15 were significantly larger than those of the cells expanded by IL-12 ($NKG2D^+CD3^+$: median 22.0% (range 9.8 - 46.1) by IL-12 vs

34.9% (22.6 - 65.0) by IL-15, n=9, $p < 0.05$). Also, the ratio of stimulatory NKR (NKG2D) to inhibitory NKRs (CD94 or NKG2A) on CD8 T cells expanded by IL-15 was significantly larger than those of the cells expanded by IL-12 ($NKG2D^+/CD94^+$: median 0.35 (range 0.19 - 0.81) by IL-12 vs 1.62 (0.82 - 4.04) by IL-15, $NKG2D^+/NKG2A^+$: 0.39 (0.23 - 1.22) by IL-12 vs 3.17 (0.95 - 6.44) by IL-15, n=9, $p < 0.01$) (Figure 2).

Immunomagnetic cell sorting

Purified $CD94^+$ cells (> 90% CD94 , as determined by flow cytometric analysis) were obtained by MACS. The proportions of NKG2D and intracellular granzyme A expression in CD94-expressing cells were much more increased in PBMCs cultured with IL-15 than those of cells cultured with IL-12 (NKG2D+ / Granzyme A: median 56.3% (range 18.6 - 68.7) / 24.3% (7.7 - 36.6) by IL-15 vs 34.6% (12.9 - 50.5) / 0.9% (0 -9.27) by IL-12, n=9, $p < 0.01$) (Figure 3). There was no difference in LFA-1 expression on CD94-expressing cells. Furthermore, a real-time polymerase chain reaction assay showed that there was a median 1.68-fold increase (range 1.03 - 2.39) in NKG2D mRNA expression level and a median 1.37-fold increase (range 1.10 - 2.36) in DAP10 mRNA expression level in CD94-expressing cells expanded by IL-15 compared with those of the cells expanded by IL-12 (Figure 4).

Cytolytic activity of CD94-expressing cells

The cytolytic activity levels of purified CD94-expressing cells from 8-day culture with IL-15 tested against ^{51}Cr-labeled K562 cells by standard 4-hour ^{51}Cr release assays without prior sensitization were much higher than those of cells from 8-day cultures with IL-12 (effector : target = 10 : 1, median 52.8% (range 41.2 - 70.5) by IL-15 vs 20.8% (18.1 - 30.7), n=15, $p <0.01$) (Figure 5). Furthermore, anti-NKG2D mAb (60 μ g/ml) significantly suppressed the cytolytic activity of CD94-expressing cells against K562 cells compared with the cytolytic activity of of cells without anti-NKG2D(effector : target = 10 : 1, median 19.0% (range 15.6 - 20.4) vs 52.8% (41.2 - 70.5) by IL-15, 9.5% (8.7 - 10.9) vs 20.8% (18.1 - 30.7) by Il-12, n=6, $p < 0.01$) (Figure 5).

Discussion

It has been reported that IL-12 [12,15,25] and IL-15 [16-18,26] can induce the expression of NK cell receptors on T cells. Derre et al. [12] showed that IL-12 induces the expression of CD94/NKG2A, and Mingari et al. [16] showed that IL-15 induces the expression of CD94/NKG2A. Ortaldo et al. [25] showed that IL-12 induces the expression of NKG2D, and Roberts et al. [26] showed that IL-15 induces the expression of NKG2D. However, the expression of both CD94/NKG2A and NKG2D was not assessed in those studies. We previously reported that CD94-expressing $CD8^+$ T cells with high cytolytic activity against patients' leukemic cells could be expanded by stimulation with immobilized anti-CD3 monoclonal antibody (mAb) and IL-15 [17,18]. In the present study, we investigated both CD94/NKG2A expression and NKG2D expression on T cells after stimulation with different cytokines (IL-12 and IL-15) in order to assess the role of the balance between inhibitory and stimulatory NKRs in the cytolytic activity.

We found that the proportion and absolute number of NKR-expressing $CD3^+/CD8^+$ T cells in PBMCs were dramatically increased after stimulation with immobilized anti-CD3 mAb and a cytokine (IL-12 or IL-15).

We investigated for the first time both inhibitory NK receptor (CD94/NKG2A) expression and stimulatory NK receptor (NKG2D) expression on T cells after stimulation. Furthermore, we showed differential effects of IL-12 and IL-15 on the expansion of NK cell receptor-expressing $CD8^+$ T cells. IL-12 induced significantly more CD94/NKG2A expression on T cells derived from PBMCs than did IL-15. On the other hand, IL-15 induced significantly more NKG2D expression on T cells derived from PBMCs than did IL-12. Also, IL-15 induced a significantly higher ratio of NKG2D to NKG2A. These expanded and purified CD94-expressing cells (>90% $CD94^+$) had CD8 and stimulatory receptor NKG2D expression but not CD56 expression on their surfaces and contained granzyme A in the cytoplasm. The proportions of NKG2D and intracellular granzyme A expression in CD94-expressing cells were much more increased in PBMCs cultured with IL-15 than those of cells cultured with IL-12. Also, a real-time polymerase chain reaction assay showed that there were higher

NKG2D and DAP10 mRNA expression levels in CD94- expressing cells expanded by IL-15 than those of the cells expanded by IL-12.

We also compared the cytolytic activitiy levels in purified CD94-expressing cells from 8-day culture with IL-12 or IL-15 against ^{51}Cr-labeled K562 cells by standard 4-hour ^{51}Cr release assays without prior sensitization. Purified CD94-expressing cells after IL-15 stimulation exhibited higher cytolytic activity than did cells after IL-12 stimulation. Furthermore, these cytolytic activities were significantly suppressed by anti-NKG2D mAb. Therefore, the cytolytic activity of CD94-expressing cells depends at least partially on NKG2D.

In this study, we have shown differential effects of IL-12 and IL-15 on the expansion of NK cell receptor-expressing $CD8^+$ T cells. IL-15 can induce a balance between expression of stimulatory NKRs and expression of inhibitory NKRs toward predominance of stimulatory signals. Therefore, IL-15 appears to be able to enhance cytolytic activity through induction of NKG2D and intracellular granzyme A expression much more efficiently than does IL-12.

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Titles and legends to figures

Figure 1.

Proportions of NKR-expressing cells before and after stimulation using different cytokines. Each datapoint shows the proportion of NKR-expressing cells before (Pre) and after anti-CD3 stimulation in the presence of IL-12 or IL-15 (n=9). Bars indicate the median of the datapoints.

* $p<0.01$ ** $p<0.05$

Figure 2.

Balance between stimulatory NKR and inhibitory NKRs on $CD8^+$T cells. Each datapoint shows the ratio of stimulatory NKR (NKG2D) to inhibitory NKRs (CD94 or NKG2A) on CD8-T cells (n=9). a) $NKG2D^+/CD94^+$, b) $NKG2D^+/NKG2A^+$. Bars indicate the median of the datapoints.

* $p<0.01$

Figure 3.

Proportions of NKG2D, LFA and intracellular granzyme A in $CD94^+$ cells purified by magnetic cell sorting (MACS). Each datapoint shows the proportion of NKG2D, LFA and intracellular granzyme A in CD94+ cells purified by MACS (n=9). a) $NKG2D^+$, b) LFA^+, c) granzyme A. Bars indicate the median of the datapoints.

* $p<0.01$

Figure 4.

Quantititative analysis of NKG2D mRNA and DAP10 mRNA expression in $CD94^+$ cells purified by magnetic cell sorting (MACS). Each datapoint shows the fold expression levels of NKG2D and DAP10 mRNA in $CD94^+$ cells expanded by IL-15 compared with expression levels in CD94 cells expanded by IL-12 (n = 9). a) NKG2D, b) DAP10. Bars indicate the median of the datapoints.

* $p<0.01$

Figure 5.

Cytolytic activities of CD94-expressing cells after stimulation with IL-12 or IL-15 against K562 cells with and without anti-NKG2D. Each datapoint shows the specific % ^{51}Cr release of CD94-expressing cells after stimulation with IL-12 or IL-15 against K562 with (n=6) and without (n=15) anti-NKG2D(α -ND). Bars indicate the median of the datapoints. (Effector-to-target ratio is 10:1.).

* $p<0.01$

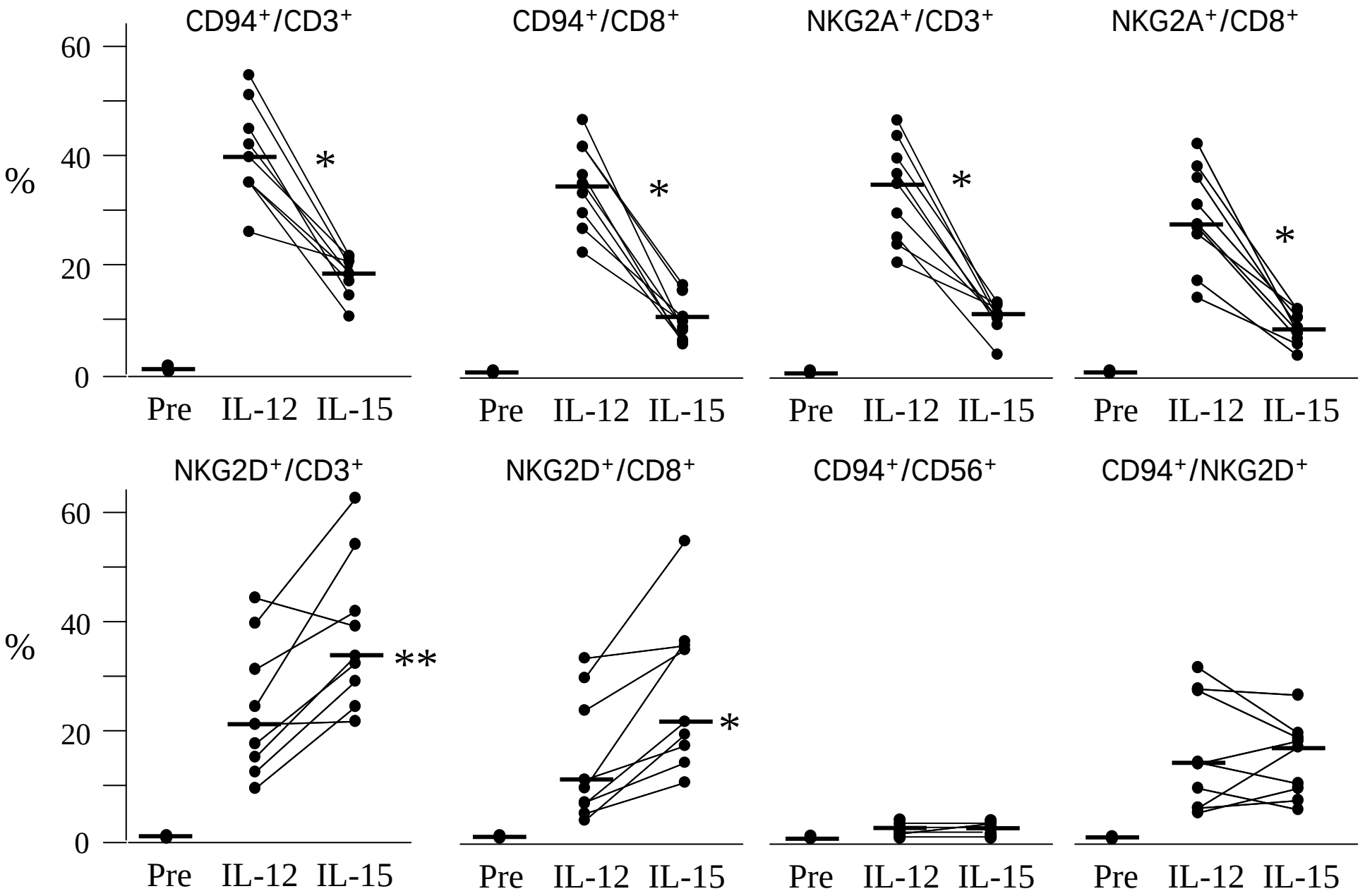
CD94+/CD3+
CD94+/CD8+
NKG2A+/CD3+
NKG2A+/CD8+
NKG2D+/CD3+
NKG2D+/CD8+
CD94+/CD56+
CD94+/NKG2D+
%
60
40
20
0
*
**
Pre
IL-12
IL-15

Figure 1.

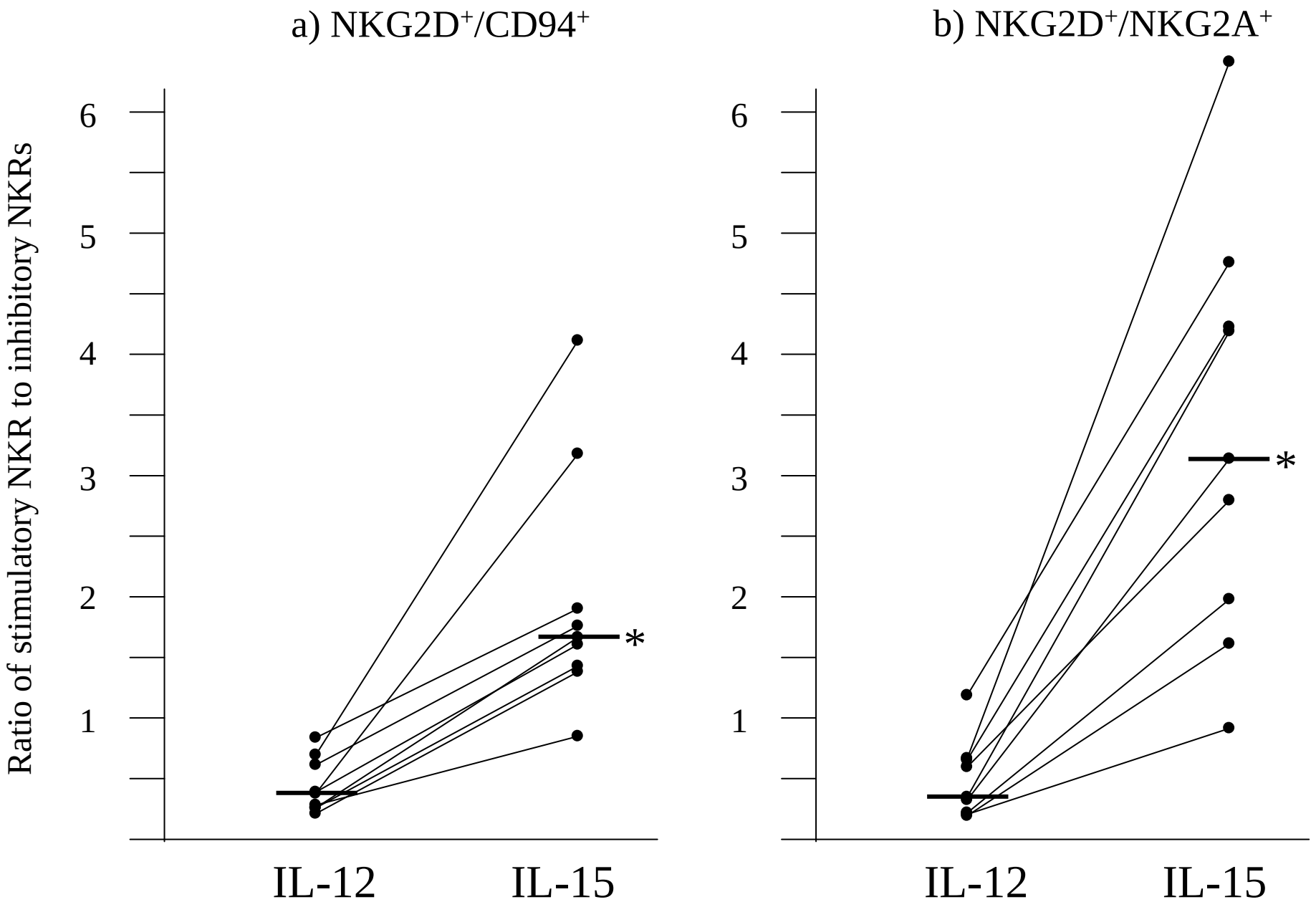
a) NKG2D+/CD94+
b) NKG2D+/NKG2A+
Ratio of stimulatory NKR to inhibitory NKRs
6
5
4
3
2
1
*
*
IL-12
IL-15
IL-12
IL-15

Figure 2.

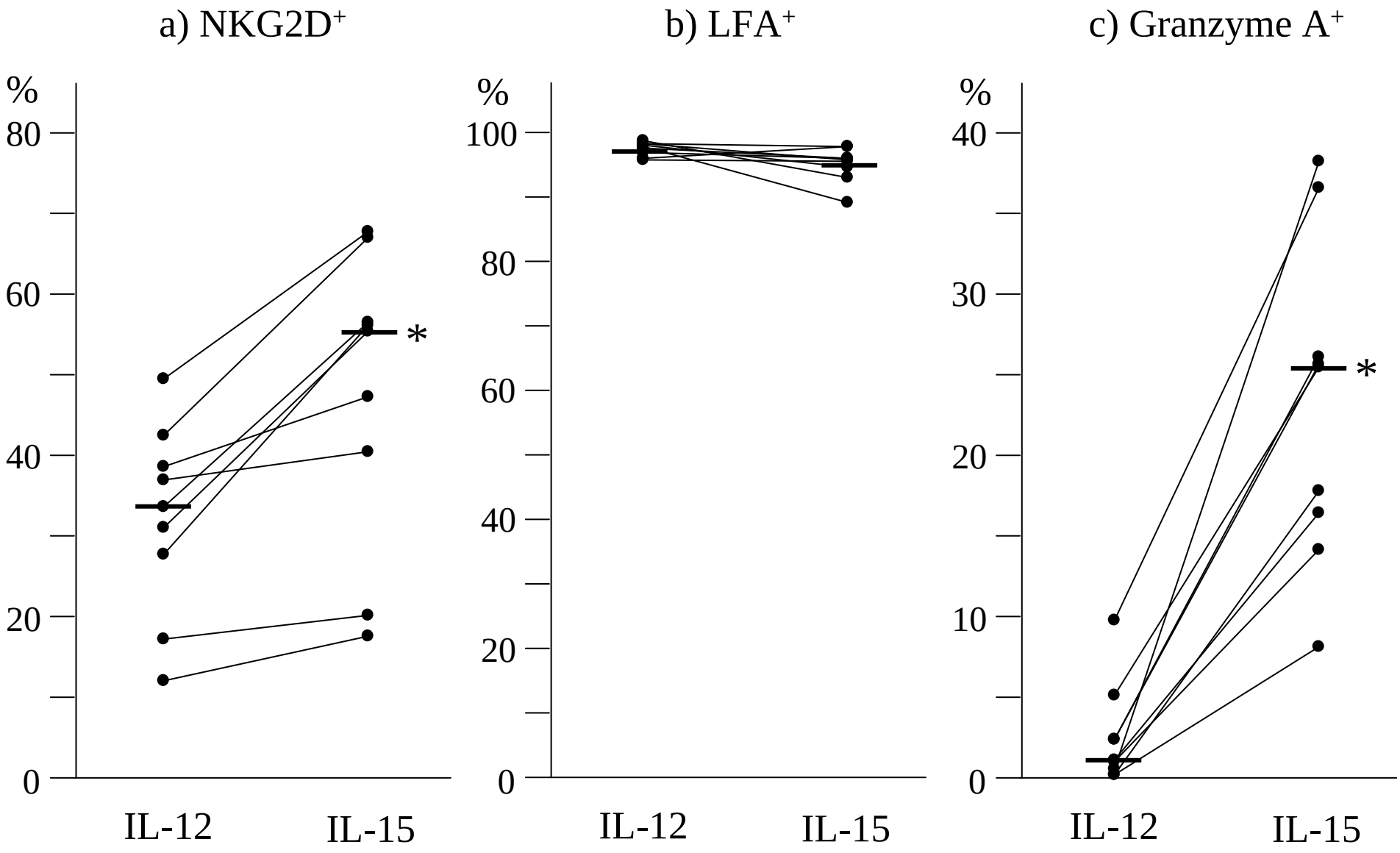

Figure 3.

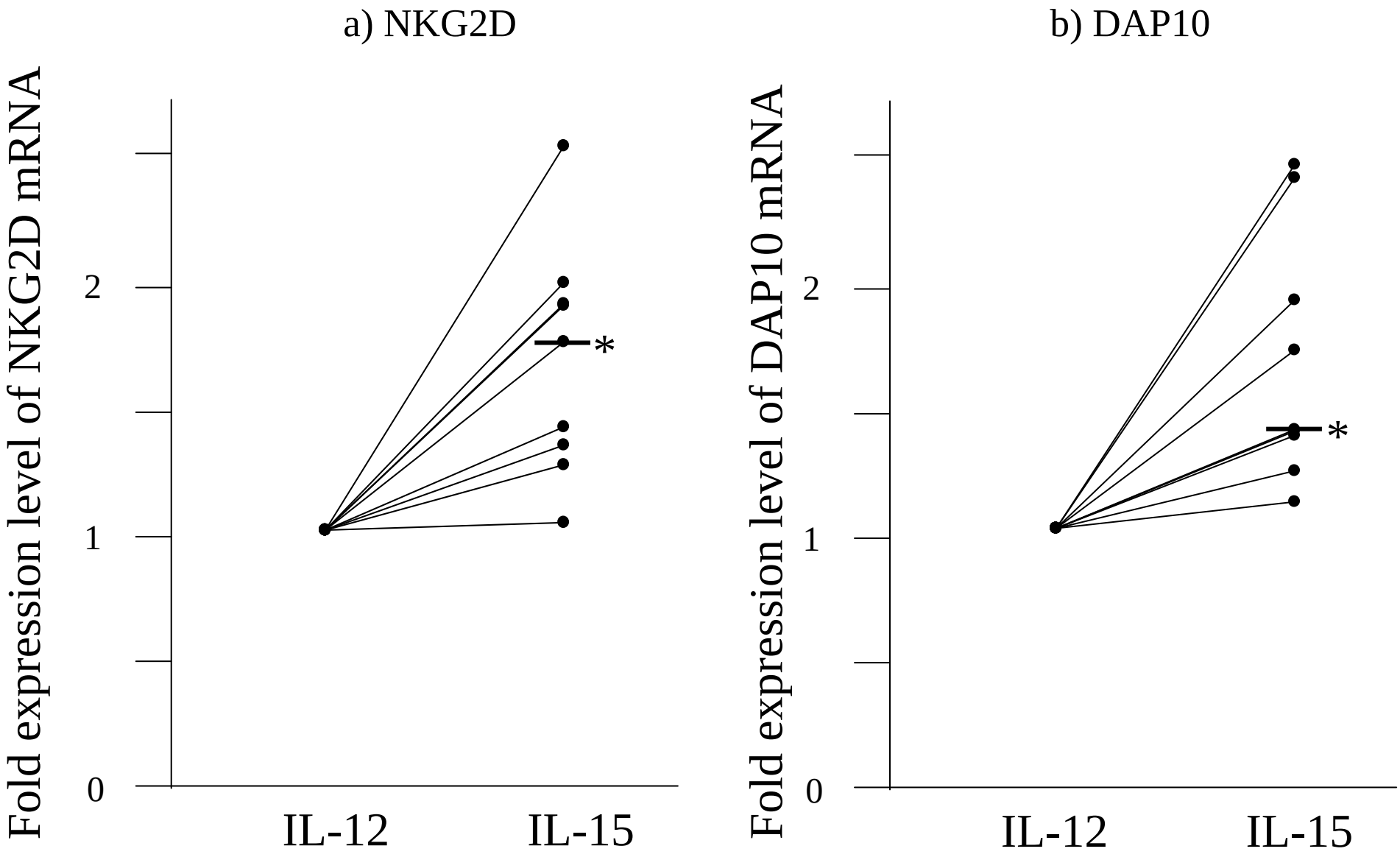
a) NKG2D
b) DAP10
Fold expression level of NKG2D mRNA
Fold expression level of DAP10 mRNA
0
1
2
IL-12
IL-15
*
*

Figure 4.

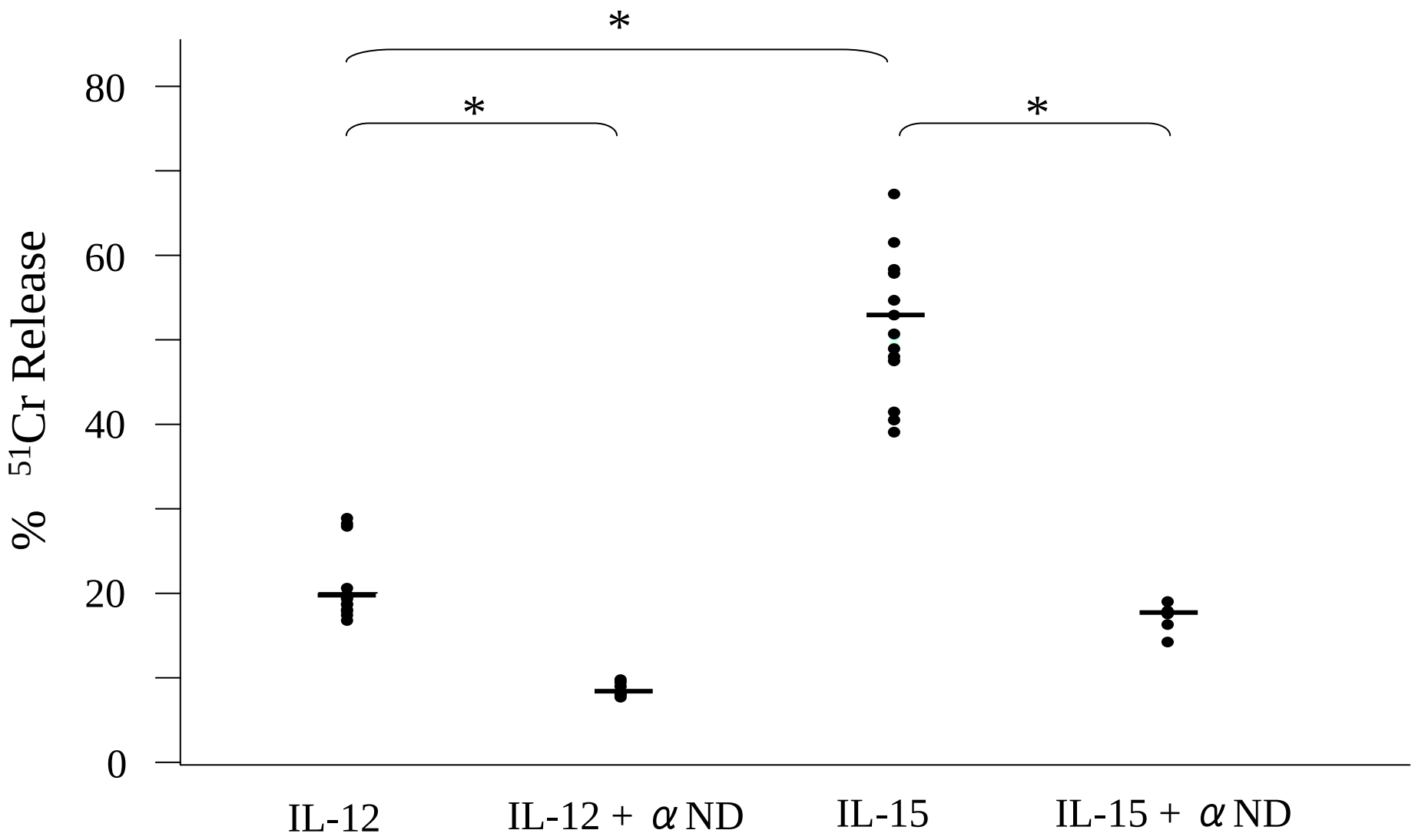

Figure 5.