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Killer-cell inhibitory receptors, CD158a/b, are upregulated by interleukin-2, but not interferon-γ or interleukin-4

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ALTHOUGH it is now accepted that killer-cell inhibitory receptors (KIRs), which were molecularly cloned in 1995, deliver negative signals to natural killer (NK) cells regarding the recognition of target cells, it is still unclear how the expression of these receptors on lymphocytes is regulated. Therefore, we investigated the regulation of expression of representative KIRs, CD158a and CD158b, by cytokines such as interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ). Neither IL-4 nor IFN-γ affected the expression of CD158a/b, but incubation for 48 h with IL-2, which enhances the killer activity of NK cells, upregulated the expression of the KIRs. This upregulation by IL-2 was also observed in CD16-positive cells sorted from total lymphocytes. In contrast, IL-4, which is a down-regulator of IL-2-induced killer responses, did not change the level of CD158a/b expression when added after the IL-2 treatment. These findings suggest that IL-2 plays an important role in the regulation of CD158a/b expression, and might be involved in controlling NK activity via regulating expression of these molecules.

Introduction

It is known that natural killer (NK) cells play an important role in the biodefense against virus-infected cells and neoplastic cells. Before it was determined what molecules NK cells recognize on target cells, it was observed that target cells that fail to express major histcompatibility complex (MHC) class I molecules are lysed by NK cells.[1] In 1995, several groups reported the molecular cloning of NK receptors[2–4] which deliver negative signals that inhibit killing.[5,6]

It is now widely accepted that NK cells recognize and lyse target cells through the interplay of two families of receptors.[7–9] One family consists of killer-cell activating receptors (KARs) and the other consists of killer-cell inhibitory receptors (KIRs). The activating signal can be overridden by a dominant negative signal from a KIR when the KIR interacts with its ligand on the target cell, although KARs, when occupied, trigger lysis of the target cell being recognized. Thus, it is crucial to analyze the regulation of the expression of KIRs in order to further our understanding of killer activity.

Classically, the NK activity assay (^{51}Cr-release assay) has been used to assess the cytotoxic function of NK cells. Natural cell-mediated cytotoxicity by NK cells has been defined as the ability to lyse neoplastic cells *in vitro* without definable prior activation. However, the augmentation of NK activity by several cytokines has been demonstrated. Interleukin-2 (IL-2) activates the cytotoxicity,[10] induces NK cell proliferation and stimulates increased production of granule contents.[11,12] In contrast, the augmentation of cytotoxicity by interferon-γ (IFN-γ) varies depending on the subject.[13] Interleukin-4 (IL-4) and transforming growth factor-β (TGF-β) are reported to be downregulatory cytokines of IL-2-induced killer responses.[14,15]

At present, the identification of the KIRs raises several interesting questions. How is the expression of KIRs affected by cytokines such as IL-2, which activates cytotoxicity, and IL-4, which inhibits IL-2-induced cytotoxicity? Answering these questions may yield insights into the process by which NK cells specifically recognize their target cells when NK cells are activated during the course of virus infection or neoplastic disease.

Materials and methods

Reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-human CD8, FITC-anti-human CD16, phycoerythrin (PE)-conjugated anti-human CD56, PE-anti-human

CD158a (EB6) and PE-anti-human CD158b (GL183)[16–19] were purchased from Immunotech (Marseille, France). Hapten-conjugated anti-human CD3, CD4, CD19 and CD33 antibodies and colloidal super-paramagnetic magnetic cell separation (MACS) microbeads conjugated to anti-hapten antibody were obtained from Miltenyl Biotec (Bergisch-Gladbach, Germany). Recombinant human IL-2, IL-4 and IFN-γ were obtained from Pharmabiotechnology (Hanover, Germany).

PBMC and NK cell preparation

Peripheral blood mononuclear cells (PBMC) obtained from 14 healthy donors were separated from heparinized blood by Lymphoprep (Nyegaard, Oslo, Norway) gradient cenfugation.[20] PBMC suspensions were washed twice in phosphate-buffered saline (PBS).

For further immunomagnetic separation of NK cells from the PBMC of six healthy subjects, the magnetic cell separation (MACS) system (Miltenyi, Biotec, Bergisch-Gladbach, Germany) was used.[21] This method is an indirect magnetic labeling system for depletion of human T cells, B cells and myeloid cells from PBMC to enrich untouched NK cells. In brief, hapten-conjugated monoclonal antibodies against CD3, CD4, CD19 and CD33 were added to 10^7 PBMC suspended in 80 µl of PBS, mixed well and incubated for 15 min at 10°C. Cells were washed carefully with 2 ml of PBS and resuspended in 80 µl of PBS. The colloidal super-paramagnetic MACS Microbeads conjugated to anti-hapten antibody were added to the cells, and the mixture was incubated for 15 min at 10°C. The cells were washed, collected and suspended in 500 µl of PBS/5 mM EDTA/0.5% bovine serum albumin (BSA). The cell suspension was applied to a prefilled depletion column, and the pass-through fraction containing unlabeled cells was collected and used as the enriched NK cells.

Cell phenotype

Surface phenotyping was carried out with a two-color immunofluorescence staining technique, using isotype-specific mouse anti-human antibody conjugated with either FITC or PE.[16] The stained cells were suspended in 0.5 ml of PBS per sample and analyzed by flow cytometry (Epics XL, Beckman Coulter, France). Lymphocyte subsets were identified by gating analysis and fluorescence profiles obtained for 10 000 cells of each sample. Negative controls for each experiment were performed with FITC- and PE-labeled mouse immunoglobulin-G (IgG).

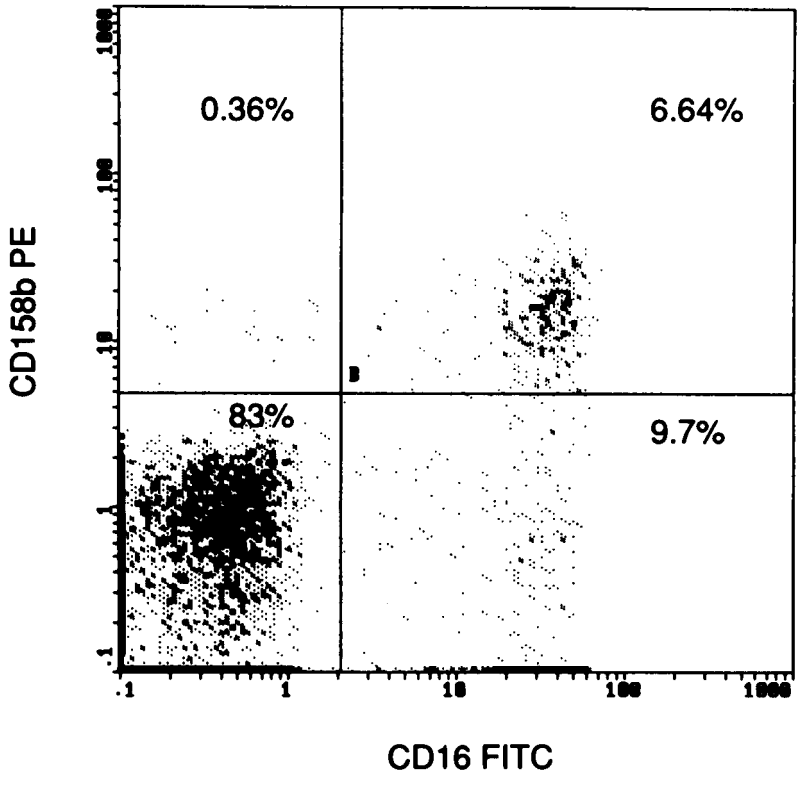

FIG. 1. Two-dimensional flow cytometric histogram of a representative sample. CD16-FITC is plotted versus CD158b-PE. Sixteen and three-tenths per cent of the lymphocytes were CD16-positive NK cells, of which 40.6% (6.64% of all lymphocytes) expressed CD158b.

Table 1. The expression on PBMC of KIRs in healthy subjects (n=14)

Cell population	Total lymphocytes		CD16-positive cells		CD8-positive cells	
KIRs*	CD158a	CD158b	CD158a	CD158b	CD158a	CD158b
% of Total lymphocytes	2.11±1.46**	6.34±5.10	1.68±1.43	5.52±4.58	1.03±0.58	2.51±1.66

*KIRs: killer inhibitory receptors; **mean±SD.

Cell culture

One million cells were cultured in RPMI1640 medium containing 10% fetal calf serum (Biological Industries, Israel) in a tissue culture dish (Becton Dickinson, Flanklin, NJ, USA). The cultures were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 48 h. In the experiment of IL-4 addition after treatment with IL-2, the cells were first cultured in medium with IL-2 for 24 h, then collected and washed twice with PBS. The cells were resuspended in medium containing IL-4 and incubated at 37°C for 24 h. Each experiment was carried out in duplicate.

Statistical analysis

All data were collected in a computer database and analyzed using the StatView-J 4.02 program (Abacus Concept, Berkeley, CA, USA). The Mann–Whitney u-test was performed for each set of laboratory variables. For all statistical tests, significance was defined as $P<0.05$.

Results

The expression of CD158a and CD158b

Figure 1 shows the expression of CD158 versus CD16 as a marker for NK cells as a scatter plot in a representative sample. In all samples, CD16-positive cells showed a bimodal distribution of the surface receptors CD158a/b, as can be seen in this subject. CD8-positive cells also generally showed a bimodal distribution of CD158a/b. However, four samples showed a trimodal distribution of CD158a. We judged whether CD158a was positive or not compared with

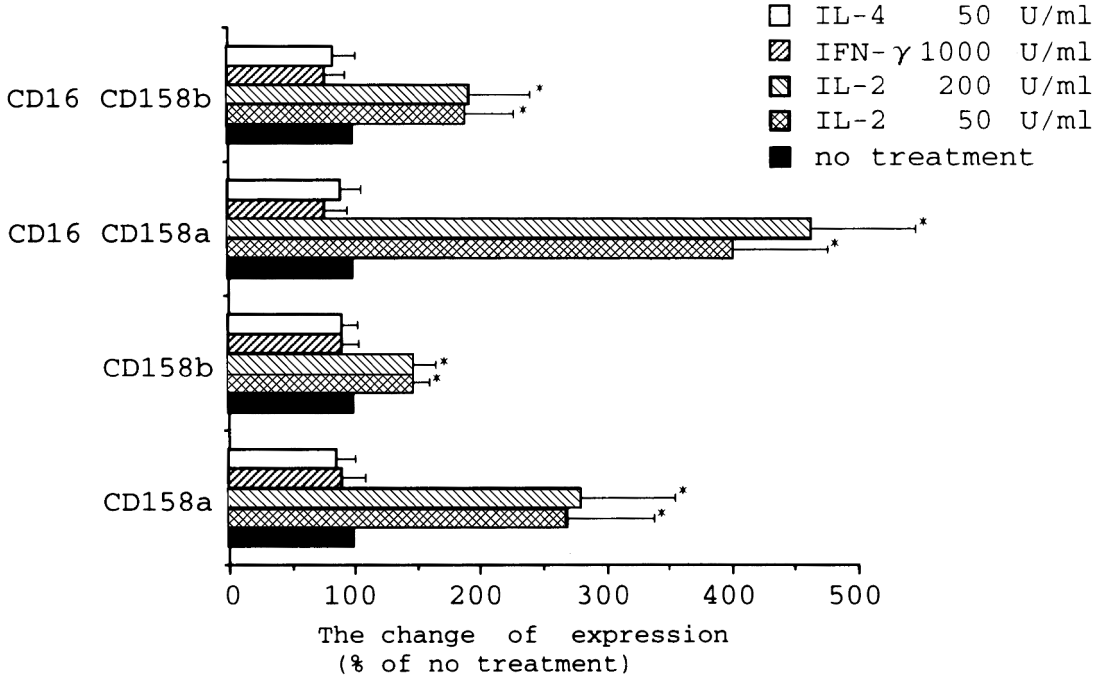

FIG. 2. Regulation of KIRs on PBMC by several cytokines. The cells were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 48 h without cytokine, or with IL-2 (50 or 200 U/ml), IL-4 (50 U/ml), or IFN-γ (1000 U/ml). Neither IL-4 nor IFN-γ affected the expression of CD158a/b, but IL-2 upregulated their expression on lymphocytes. Asterisks indicate a significant increase compared to no treatment in each group with a P value of <0.05. Data are shown as the mean (thick bar) ±SD (thin bar).

Table 2. The effect of IL-4 on the expression of KIRs on PBMC after treatment with IL-2

	24 h		48 h		
	No treatment	IL-2 50 U/ml	No treatment	IL-2 50 U/ml	IL-2 50 U/ml (first 24 h)* IL-4 50 U/ml (last 24 h)
CD158a (% of lymphocytes)	1.7±0.4	2.4±0.3	1.9±0.2	2.8±1.0	2.4±1.1
CD158b	3.7±1.5	6.6±2.8	4.1±1.4	7.8±2.5	6.5±2.7

*The PBMC were cultured in medium containing IL-2 for 24 h, and in medium containing IL-4 for the next 24 h (See Materials and methods). The expression of KIRs on PBMC treated with IL-2 followed by IL-4 did not differ from that on PBMC treated with IL-2 alone, suggesting that IL-4 does not influence the CD158a/b expression after IL-2 treatment.

control/mouse-IgG, although cells expressing CD158a weakly seemed to be functionally similar to CD158a-negative cells. This discrete distribution pattern enabled reliable quantification of receptor-negative or positive subpopulations. The expression of CD158a/b on various cell types is summarized in Table 1. CD158a/b were expressed on CD8 positive cells to a lesser degree than on CD16-positive cells. In most samples, CD158a was expressed on fewer CD16-positive or -negative cells than CD158b. These observations are in accordance with those made in previous studies.[22,23]

IL-2 upregulates the expression of CD158a and CD158b

To investigate the regulation of CD158a/b receptors, we assessed their response to stimulation by cytokines. First, we investigated the effects of IL-2, IL-4 and IFN-γ on the expression of CD158a/b on the PBMC. The results are shown in Fig. 2. Neither IL-4 nor IFN-γ affected the expression of CD158a/b, but IL-2 affected their expression on CD16-positive cells. The upregulation by IL-2 was observed to lesser degree in CD16-negative cells, probably mostly on CD8-positive cells. These effects of IL-2 were dose-dependent (data not shown).

Although IL-4 did not change the expression of these molecules in our study, a previous study showed that IL-4 is a downregulatory cytokine of IL-2-induced killer responses. Therefore, we further investigated the effects of IL-4 on PBMC treated with IL-2. The level of expression of CD158a/b after IL-2 stimulation was not changed by treatment with IL-4, further indicating that IL-4 does not downregulate the expression of CD158a/b (Table 2).

Table 3. The change of the expression of KIRs when enriched NK cells are stimulated by IL-2

	No treatment	IL-2 50 U/ml
CD158a	9.0±5.8*	13.9±6.9**
CD158b	23.8±8.1	27.2±9.3**

*Mean±SD (%), n=6; **P<0.05 versus no treatment.

IL-2 acts directly on NK cells in the upregulation of CD158a/b

IL-2 contributes to T cell growth[24] in addition to augmenting the killer activity of NK cells. It is thus possible that the upregulation of CD158a/b by IL-2 is mediated through other cells, especially T cells. To test this, we treated enriched NK cells with IL-2 and observed that CD158a/b molecules were also upregulated by IL-2 in these NK cells, just as in PBMC (Table 3).

Discussion

Although NK cells had been defined as TCR/CD3-negative cells that lyse neoplastic or virus-infected cells in an HLA-unrestricted manner, in contrast to the HLA-restricted lysis by T cells, doubts concerning MHC-unrestricted recognition arose from the phenomenon of hybrid resistance in mice and from the observations that HLA-expressing cell lines are often more resistant to NK cells than non-HLA expressing lines.[1,25] Subsequently, the NK receptors, which recognize MHC class I molecules and deliver negative signals to NK cells, were identified in several laboratories.[26] Based on these findings, it is now considered that NK activity may involve a balance between activating signals from non-self-recognition and inhibitory ones from self-recognition. However, it is still unclear how this delicate balance is regulated. Therefore, we investigated the regulation by cytokines of representative KIRs, CD158a and CD158b, which react with certain HLA molecules related to Cw4, 5, 6 and Cw1, 3, 7, 8, respectively.[17–19]

Our study demonstrated that expression of CD158a/b molecules was upregulated by IL-2, but not by IFN-γ or IL-4. IL-2 is released from T cells, and in turn activates T cells. These activated T cells release other lymphokines, such as IL-3, IL-5 and IL-

6, in addition to IL-2. There was thus a possibility that when PBMC are treated with IL-2, the resulting upregulation of CD158a/b may be dependent on cytokines other than IL-2. To test this, we prepared NK cells from PBMC using the MACS system. The upregulation of CD158a/b molecules was also observed in these enriched NK cells prepared from PBMC, suggesting that IL-2 is an important, direct regulator of the expression of these molecules.

IL-2 upregulated the KIRs in this study although it is well known that IL-2 augments cytolytic activity.[13] These facts seem to be contradictory. It is now thought that NK activity is dependent on the following: (i) the proliferation and sustainment of NK cells, (ii) the augmentation and accumulation of intracellular granules, (iii) the expression of activating receptor or fas ligand on the cell surface, (iv) the blockage of killer potency via inhibitory signals. IL-2 contributes to (i), (ii) and partly to (iii) in activated NK cells, which have high potency to lyse target cells. In our study, the upregulation of the expression of CD16, activating receptor,[27] was also observed by IL-2 treatment. When activated NK cells lyse virus-infected cells or neoplastic cells, they must specifically distinguish non-MHC class I cells from normal cells under the delicate balance required for specific killing. To this end, the expression of KIRs may be upregulated on the activated NK cells. IFN-γ, which is also released by activated NK cells and augments the cytolytic activity of NK cells,[13] did not affect the expression of these molecules in our experiments. A previous study showed that IFN-γ upregulated MHC class I molecules, which are the ligands of KIRs, on the target cells.[28] These observations lead us to speculate that the augmentation of the expression of KIRs on NK cells along with the enhancement of expression of MHC class I molecules on target cells by IFN-γ enables activated NK cells to lyse non-MHC class I cells and to specifically conserve MHC class I-expressing cells. When this balance is disturbed, an attack on the self by NK cells might occur. From the clinical viewpoint, there might be a failure to express KIRs in patients with autoimmune diseases in whom NK cells are upregulated. To confirm this speculation, further studies are required.

On the other hand, it is known that IL-4 inhibits IL-2-induced cytotoxity.[14,15] We found that IL-4 did not change the expression of KIRs on NK cells and also did not affect their expression after treatment with IL-2. These findings suggest that the suppression of cytotoxity by IL-4 is independent of the regulation of KIR expression. In a recent study, it was demonstrated that the inhibition of natural cytotoxicity by IL-4 was due to the inhibition of the induction of granzyme B expression by IL-2.[29]

In conclusion, IL-2, but not IFN-γ or IL-4, upregulates the expression of CD158a/b molecules on lymphocytes, suggesting that IL-2 might enhance the ability of NK cells to distinguish target cells according to MHC class I expression.

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