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Expression of CD94/NKG2-A on Human T Lymphocytes Is Induced by IL-12: Implications for Adoptive Immunotherapy[1]

Laurent Derre,* Murielle Corvaisier,* Marie-Christine Pandolfino,† Elisabeth Diez,* Francine Jotereau,* and Nadine Gervois[2]*

NK cell receptors (NKRs) are expressed on a subset of human T cells, predominantly CD8$^+$, within which they can modulate TCR-mediated functions. In an attempt to identify the mechanisms leading to NKR expression, we analyzed the capacity of IL-12 to modulate the expression by T cells of the components of the CD94/NKG2-A inhibitory receptor, a member of the C-type lectin-like family of NKR. We show that IL-12 induces the expression of NKG2-A and/or CD94 by CD8$^+$ T cells in culture, and that this induction was mediated neither by IFN-γ nor by IL-15. We also show, using the redirected killing assay, that IL-12-induced expression of both CD94 and NKG2-A led to the acquisition by T cells of a functional inhibitory receptor. Expression of the CD94/NKG2-A inhibitory receptor was also induced by IL-12 during T cell Ag stimulation so that in the presence of this cytokine a high proportion of melanoma-reactive CTL induced from PBL by melanoma peptide stimulation expressed this receptor. This study emphasizes the implication of IL-12 in the modulation of immune responses through NKR induction. ***The Journal of Immunology*****, 2002, 168: 4864–4870.**

Natural killer cell receptors (NKRs),[3] initially discovered in NK cells, have been shown to be expressed in a subset of T cells (for reviews, see Refs. 1–3). These NKRs exhibit inhibitory or activatory function, and most of them specifically bind to MHC class I or MHC class I-like molecules. Inhibitory receptors have cytoplasmic immunoreceptor tyrosine-based inhibitory motifs that operate through recruitment of tyrosine phosphatases (4). Activating receptors associate with adaptor proteins, DAP12 (or DAP10), containing immunoreceptor tyrosine-based activatory motifs, which recruit and activate protein tyrosine kinases (5). This new category of lymphocyte membrane molecules may play a role in the regulation of T cell activation and of immune responses in vivo.

In humans, NKRs belong to two distinct molecular families. Members of the Ig superfamily, including killer cell Ig-like receptors and leukocyte Ig-like receptors, also called Ig-like receptors, belong to the first one. Type II transmembrane proteins containing a C-type lectin domain, including CD94, NKG2, and NKRP1A, belong to the other one. It has been reported that CD94 can be expressed as a homodimer or as a heterodimer associated with various NKG2 molecules that determine the nature of the transduced signal: NKG2-A and NKG2-B mediating an inhibitory signal (6, 7), NKG2-C an activatory one (8). The class Ib molecule HLA-E is specifically recognized by CD94/NKG2-A, CD94/NKG2-B, and CD94/NKG2-C complex (9–11). HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors (12). In contrast, the activating NKG2-D molecule does not associate with CD94, but forms homodimers that interact with inducible ligands MICA and MICB (13).

A potential role of NKRs in virus- and tumor-specific immune responses in vivo has been suggested by recent data obtained on patients suffering from HIV infections (14), melanoma (15–21), or T cell lymphoma (22). Therefore, it is important to define the mechanisms that lead to NKR expression in T lymphocytes. In this context, we observed that >60% of human melanoma-specific CD8$^+$ T cell clones, generated in vitro by stimulating PBL with peptide-pulsed presenting cells and cytokines (IL-6, IL-12, IL-2, and IL-7), expressed the CD94/NKG2-A receptor. In view of the already known implications of two cytokines, IL-15 (2) or TGF-β (23, 24), in the induction of the CD94/NKG2-A receptor on T cells, we checked whether one of the four cytokines used in our assays to generate these clones could achieve a similar effect. We demonstrate that IL-12, a cytokine known to play a central role in polarization of Th1 response, induces de novo expression of CD94/NKG2-A by human CD8$^+$ T cells.

Materials and Methods

Isolation of lymphocyte populations

PBL were isolated from blood of healthy donors by a Ficoll (Eurobio, Les Ulis, France) density gradient. In some experiments, PBL were first depleted of CD94$^+$ cells by negative selection using anti-CD94 mAb, and magnetic beads coated with goat anti-mouse Ig (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, CD8$^+$ cells were sorted from the CD94$^-$ cell population by positive selection using magnetic beads coated with anti-CD8 mAb (Miltenyi Biotec). In one experiment, NKG2-A-positive and NKG2-A-negative populations were also sorted using anti-NKG2-A mAb and magnetic beads coated with anti-mouse Ig (Dynabeads; Dynal Biotech, Oslo, Norway).

Abs and cytokines

mAb specific for CD94 (HP-3B1), NKG2-A (Z199), and mouse IgG2a isotypic control were obtained from Immunotech (Marseille, France). PE-conjugated mAbs specific for CD3, CD4, CD8, and CD94 were also obtained from Immunotech. FITC-conjugated rabbit F(ab′)$_2$ and mouse IgG were obtained from BioAtlantic (Nantes, France).

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[3] Abbreviations used in this paper: NKR, NK cell receptor; MART-1, melanoma Ag recognized by T cells-1; TIL, tumor-infiltrating lymphocyte.

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Anti-CD3 Ab used for T cell stimulation was a generous gift of L. Toujas (Centre Régional de Lutte contre le Cancer, Rennes, France).

Anti-human IFN-γ mAb was purchased from R&D Systems (Wiesbade Nordenstadt, Germany). Anti-human IL-15 mAb was a generous gift of Y. Jacques (Institut National de la Santé de la Recherche Médicale, Nantes, France).

IL-2 was purchased from Chiron (Suresnes, France), and the three other cytokines (IL-6, IL-7, and IL-12) were purchased from Sigma-Aldrich (St. Louis, MO).

Stimulation by cytokines combined or not with anti-CD3 mAb

PBL or purified $CD8^+$ T cells were plated in 24-well plates (10^6 cells/well) with RPMI 1640 medium containing 8% human serum (local production). At the onset of the culture, the following cytokines were added: IL-2, IL-6, IL-7, and IL-12 (Sigma-Aldrich), either separately or in combination. Concentrations used were 5 ng/ml for IL-2, IL-6, and IL-7, and ranging from 0.001 to 100 ng/ml for IL-12.

Purified CD8 T cells were cultured with a range of IL-12 concentrations using culture plates coated with anti-CD3 mAb (10 μg/ml).

In some experiments, anti-human IFN-γ or anti-IL-15 mAb was added to the culture at a final concentration of 5 μg/ml.

Generation of melanoma peptide-specific T cells

PBL from HLA-A*0201 healthy donors or melanoma patients were stimulated by irradiated M17 melanoma cells loaded with the Melan-A/melanoma Ag recognized by T cells-1 (MART-1) $peptide_{26–35}$ analog ELAGIGILTV. Loading was done by incubating M17 cells with the peptide (1 μM) at 37°C in serum-free medium for 2 h. Stimulator cells were washed twice to eliminate unbound peptide. Stimulations were performed in 24-well culture plates by mixing 5×10^5 peptide-pulsed stimulator cells and 1×10^6 responder PBL in RPMI medium containing 8% human serum and cytokines. Irradiated stimulator cells were added again twice, at 7-day intervals. Cytokines added to these cultures were as follows: either IL-6 and IL-12 (5 ng/ml each) for the first week and IL-2 (10 U/ml) and IL-7 (5 ng/ml) thereafter, or IL-6 only for the first week and IL-2 only (50 U/ml) thereafter.

Tetramers

HLA-A0201/peptide α3-mutated monomers were a gift of F. Lang (Institut National de la Santé et de la Recherche Médicale, Nantes, France) and were generated as previously described (25). Recombinant proteins were produced as inclusion bodies in *Escherichia coli* XA90F′Lac[Q1], dissolved in 8 M urea, and refolded with 15 μg/ml Melan-A peptide analog (ELAGIGILTV) or NA17-A peptide (VLPDVFIRC). Tetramerization was performed as previously described (26). Briefly, HLA monomers were biotinylated for 4 h at 30°C with 6 μg/ml BirA (Immunotech), purified on monoQ column (Pharmacia, St. Quentin en Yvelines, France), and tetramerized with PE-labeled streptavidin (Sigma-Aldrich) at a molar ratio of 4:0.8.

Flow cytometry

For simple fluorescence labeling, cells were stained with the corresponding mAb (anti-CD94 or anti-NKG2-A), followed by an FITC-conjugated Ab as second-step reagent.

For double fluorescence labeling, cells were incubated with 1) primary (anti-CD94 or anti-NKG2-A) Ab and washed; 2) goat anti-mouse FITC-labeled Ab and washed; 3) mouse IgG; and 4) PE-labeled Abs (anti-CD3, anti-CD4, anti-CD8, or anti-CD94).

For tetramer staining, cells (0.5×10^6) were 1) stained with tetramers and the primary NK receptor-specific Ab for 30 min at 4°C and washed, and 2) stained with goat anti-mouse FITC-labeled Ab for 20 min at 4°C and washed.

After the end of the staining steps, the cells were washed and analyzed on a flow cytometer (FACScan; BD Biosciences, Mountain View, CA).

Assay for cytolytic activity

Cytolytic activity of T cell was tested against the ^{51}Cr-labeled murine mastocytoma $Fc\gamma R^+$ P815 cell line by a CD3 mAb-redirected killing assay. Briefly, 10^3 ^{51}Cr-labeled P815 were incubated with T cells (E:T ratio, 10:1), in the presence of purified anti-CD3 mAb (0.05 μg/ml). CD3-redirected lysis of labeled P815 cells was modulated by the presence of indicated mAbs (0.1–10 μg/ml) added at the initiation of the assay. After 4 h, 25 μl supernatant was mixed with 100 μl scintillation liquid (Optiphase Supermix; Wallac, Turku, Finland) for measurement of radioactive content. Data are expressed as the percentage of specific lysis.

Results

A high proportion of melanoma-specific CD8 T cell clones derived from in vitro stimulation of PBL by tumor peptides expresses the CD94/NKG2-A receptor

In keeping with previous studies suggesting that the CD94/NKG2-A receptor might affect the response of melanoma-specific CTL, we analyzed the expression of this receptor by a panel of 88 melanoma-reactive CTL clones. These clones have two distinct origins: 49 were derived from tumor-infiltrating lymphocytes (TIL) after a short 10-day culture of tumor fragments with IL-2, followed by a limiting dilution culture of the TIL also in the presence of IL-2, or the other 39 clones were derived from healthy donor PBL stimulated three times at 1-wk intervals with melanoma-associated Ag peptide-pulsed allogeneic melanoma cells or autologous mature DC, in the presence of IL-6 and IL-12 for 1 wk and of IL-7 and IL-2 for 2 wk. As shown in Fig. 1, despite a similar array of peptide specificity, very different fraction of these two categories of clones expressed the CD94/NKG2-A receptor: 60 and 5% of the PBL and TIL clones, respectively.

Involvement of IL-12 in the induction of CD94 expression by $CD8^+$ T cells

To check whether one or several of the four cytokines used to generate the PBL-derived clones were involved in the frequent expression of CD94 and NKG2-A by these clones, we tested whether various combinations of these cytokines, and each one separately, did influence CD94 and NKG2-A expression by cultured PBL or TIL, following or not a T cell stimulation. After 2 wk of culture with these cytokines, unstimulated total PBL were analyzed by flow cytometry for the coexpression of CD94 and CD3. Results from one representative experiment are shown in Fig. 2*a*. The percentage of $CD94^+$ T cells among the $CD3^+$ T cells was higher in all IL-12-supplemented cultures.

The same experiment was performed using IL-12 concentrations for 0.01–10 ng/ml, and culture was analyzed for the coexpression of CD4 or CD8 and CD94 after 14 days. As shown in Fig. 2*b*, CD94 expression was induced in a dose-dependent fashion by IL-12 on $CD8^+$ PBL. By contrast, a very low fraction of $CD4^+$ PBL cells was $CD94^+$, irrespective of the amount of IL-12 added in the culture.

Similar experiments were also performed using lymphocytes from two melanoma-invaded lymph nodes (M47 and M48). As shown in Table I, the fraction of $CD94^+$ TIL was significantly higher in the presence than in the absence of IL-12 among one TIL population. For the other, although the fraction of CD94/

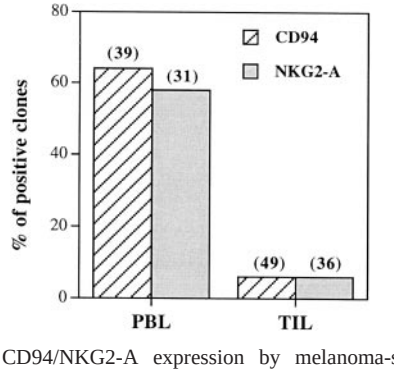

FIGURE 1. CD94/NKG2-A expression by melanoma-specific CTL clones derived from TIL or from in vitro peptide-stimulated PBL. The expression of CD94 and NKG2-A was determined by flow cytometry. Results are expressed as percentage of NKR-positive clones. Number of tested clones is indicated *above* each bar.

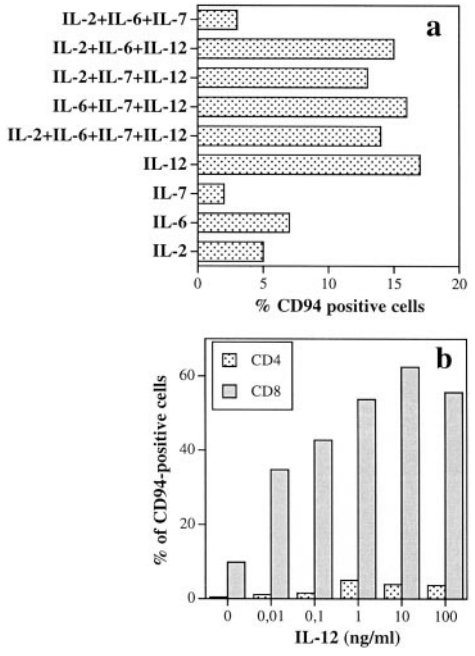

FIGURE 2. IL-12-induced expression of CD94 in $CD8^+$ PBL. $CD8^+$ PBL were cultured for 2 wk in the presence of different cytokines (*a*) or in the presence of increasing doses of IL-12 (*b*). CD94 expression was analyzed by flow cytometry among $CD3^+$ cells (*a*) or among $CD4^+$ or $CD8^+$ cells (*b*).

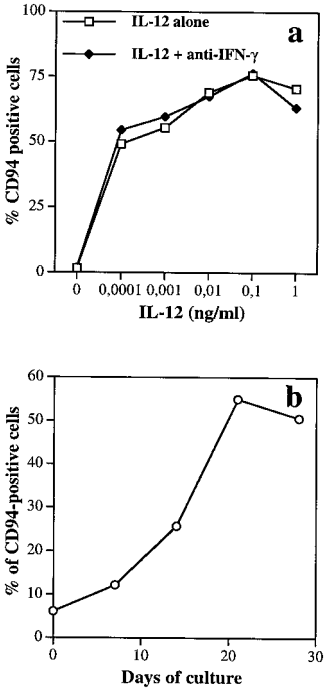

FIGURE 3. *a*, Expression of CD94 by $CD8^+CD94^-$ PBL cultured with IL-12 is not affected by the presence of anti-IFN-γ mAb. $CD8^+CD94^-$ PBL were stimulated with coated anti-CD3 mAb (10 μg/ml) in the presence of increasing IL-12 concentrations, and in the presence or not of anti-IFN-γ Ab (5 μg/ml). After 2 wk, cells were analyzed by flow cytometry for the expression of CD94. *b*, Time course of CD94 expression was evaluated on $CD8^+CD94^-$ PBL cultured in IL-12 (5 ng/ml).

$NKG2\text{-}A^+$ TIL was not increased by IL-12, the level of expression of this receptor was increased.

We then checked whether IL-12 could induce de novo expression of CD94 by $CD8^+$ T cell in the context of a polyclonal stimulation by an anti-CD3 Ab. $CD94^-CD8^+$ PBL were sorted, stimulated with anti-CD3 Ab, and cultured in the presence of various concentrations of IL-12. Data represented in Fig. 3*a* show that high fractions of CD3-stimulated $CD8^+$ T cells expressed CD94 when cultured with IL-12 concentrations as low as 1 pg/ml, and that this fraction increased with the concentration of IL-12 until 0.1 ng/ml. As shown in kinetic data in Fig. 3*b*, CD94 expression was already detectable by a fraction of cultured T cell after 7 days of culture, but such fraction increased until day 21 of the culture.

To control whether increased CD94/NKG2-A expression induced by IL-12 could result in great part from the selective growth of a low fraction of preexisting $CD94^+NKG2\text{-}A^+$ T cells, we analyzed the polyclonality of these cells using a panel of Vβ Abs. A normal Vβ diversity (data not shown) was observed, which allows us to exclude the existence of a pauciclonal expansion in these cultures.

We finally tested whether IL-12 could induce CD94/NKG2-A expression by melanoma-specific preestablished CTL clones. As shown in Table II, T cell clones that completely lacked CD94 and NKG2-A expression in the absence of IL-12 were not induced to express these molecules by an 8-day culture with IL-12. However, CTL clones that already expressed CD94 in the absence of IL-12 showed an increase both of the fraction of cells expressing this receptor and of the level of CD94 expressed per cell after 8-day culture with IL-12. This was also the case for NKG2-A expression.

Table I. *TIL from two melanoma patients cultured with or without IL-12*

TIL	IL-12	% of $CD94^+$ Cells[a]	% of $NKG2\text{-}A^+$ Cells[a]
M47	−	14 (99)	12 (105)
	+	24 (277)	21 (374)
M48	−	27 (137)	25 (135)
	+	29 (287)	31 (90)

[a] The percentages of cells positive for CD94 and NKG2-A were evaluated at day 14 by flow cytometry. The mean fluorescence is indicated in parentheses.

Neither IFN-γ nor IL-15 was relaying IL-12-induced expression of CD94

Since IFN-γ is a strong mediator of IL-12 biological effects (27), and since IL-15 is known to be an inducer of the CD94/NKG2-A

Table II. *T cell clones cultured with or without IL-12*

T Cell Clones	IL-12	% of $CD94^+$ Cells[a]	% of $NKG2\text{-}A^+$ Cells[a]
Mel 1–37	−	20 (28)	19 (29)
	+	46 (43)	47 (44)
Mel F-5	−	6 (27)	ND
	+	78 (55)	ND
ELA-3	−	24 (30)	ND
	+	92 (79)	ND
M199.6.1	−	3 (36)	ND
	+	38 (25)	ND
M28.10B	−	26 (25)	24 (23)
	+	68 (68)	50 (60)
M77-84	−	0	0
	+	0	0
M199.7.5	−	0	ND
	+	0	ND

[a] The percentages of cells positive for CD94 and NKG2-A were evaluated after 8-day culture with or without IL-12 by flow cytometry. The mean fluorescence is indicated in parentheses.

receptor, we investigated whether the effect of IL-12 on CD94 expression by $CD8^+$ T lymphocytes could be mediated by one of these two cytokines. To this end, $CD94^-CD8^+$ lymphocytes were stimulated with anti-CD3 Ab and cultured in the presence of different amounts of IL-12 (from 0.0001 to 1 ng/ml), with or without saturating concentrations of anti-human IFN-γ or of anti-IL-15 mAb. As shown in Fig. 3*a*, after 14 days of culture, IL-12 concentrations as low as 1 pg/ml induced CD94 expression by ~50% cultured $CD8^+$ cells. Furthermore, a similar fraction of PBL cells was induced to express CD94 by IL-12 in the presence of anti-IFN-γ mAb (Fig. 3*a*), or in the presence of anti-IL-15 mAb (data not shown). Consequently, the effect of IL-12 on expression of CD94 by $CD8^+$ T cells was not dependent on IFN-γ or IL-15 production.

Lack of coordinated induction of CD94 and NKG2-A expression by IL-12

CD94 is usually expressed at the cell surface in association with a NKG2 family member. We analyzed in this study whether IL-12 induced in a coordinated fashion the expression of CD94 and NKG2-A. To this end, we stained $CD8^+$ PBL with CD94- and NKG2-A-specific mAbs, after anti-CD3 mAb stimulation in the presence of IL-12. As shown in Fig. 4 and Table III, a significant fraction of cultured PBL was $CD94^+$ but $NKG2\text{-}A^-$. This suggested that some T cells were induced by IL-12 to express CD94 either as a homodimer or as a heterodimer containing another NKG2 molecule.

To distinguish between these two possibilities, we tried to establish whether $CD94^+NKG2\text{-}A^-$ T cells could express a known functional CD94/NKG2 receptor. In the absence of available Abs reactive against all NKG2 isoforms, we performed a PCR analysis of NKG2 mRNA expression on $NKG2\text{-}A^-$ and $NKG2\text{-}A^+$ T cells, whose staining profiles are provided in Fig. 5*a*. Both subpopulations expressed the CD94, NKG2-C, and NKG2-E mRNAs, while NKG2-B and, as expected, NKG2-A mRNAs were poorly expressed by the $NKG2\text{-}A^-$ subset (data not shown). Therefore, the $CD94^+NKG2\text{-}A^-$ subpopulation induced by IL-12 lacked expression of the CD94/NKG2-B receptor, but could have expressed one of the activatory receptors, CD94/NKG2-C or NKG2-E.

As far as mRNA expression does not always correlate with protein expression, we used a functional assay to determine whether the $CD94^+NKG2\text{-}A^-$ T cells expressed a functional CD94-containing receptor. To this end, we analyzed the ability of an anti-CD94 mAb to modulate TCR-dependent lysis in a redirected lysis assay against P815 target cells. As shown in Fig. 5*b*, anti-CD3 mAb induced a strong cytolytic activity by both the $CD94^+NKG2\text{-}A^-$ and $CD94^+NKG2\text{-}A^+$ T cell subpopulations. However, addition of anti-CD94 mAb did not affect the lysis of the $CD94^+NKG2\text{-}A^-$ subpopulation, while it inhibited in a dose-dependent manner that of the $CD94^+NKG2\text{-}A^+$ subpopulation, as described previously. Consequently, this suggests that $CD94^+NKG2\text{-}A^-$ T cells do not express a CD94/NKG2-C or CD94/NKG2-E activatory receptor.

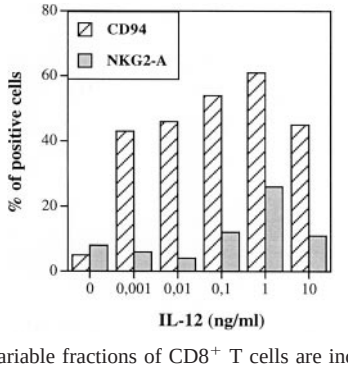

FIGURE 4. Variable fractions of $CD8^+$ T cells are induced to express CD94 and NKG2-A after a culture with IL-12. $CD8^+CD94^-$ cells were stimulated with coated anti-CD3 mAb (10 μg/ml) and further expanded for 2 wk with increasing IL-12 concentrations (from 0.001 to 10 ng/ml). Cells were analyzed by flow cytometry for the expression of CD94 and NKG2-A.

Table III. *$CD8^+$ PBL from different healthy donors cultured in the presence of IL-12 express CD94 and NKG2-A on variable cell fractions*[a]

Healthy Donors	$CD94^+$ Cells (%)[a]	$NKG2\text{-}A^+$ Cells (%)[a]
29	72	45
46	88	40
50	85	52
52	79	10
54	63	50

[a] The percentages of cells positive for CD94 and NKG2-A were evaluated at day 14 by flow cytometry after 2 wk of culture supplemented with IL-12 at 10 ng/ml.

IL-12 addition to peptide stimulation cultures, commonly used to derive peptide-specific T cells, favors the generation of CD94/NKG2-A-expressing CTL

We described recently (28) a relatively simple and efficient procedure to obtain high avidity Melan-A/MART-1-specific T cells from PBL, for use in immunotherapy. During the first step of the establishment of this procedure, four cytokines (IL-6 and IL-12 for the first week of culture, and IL-2 and IL-7 afterward) had been used, according to culture methods developed by others (29). In a second step, to make this culture protocol simpler for immunotherapy applications, we tested the contribution of these four cytokines for an optimal production of Melan-A-specific CTL. We showed that IL-6 and IL-2 were sufficient for optimal induction of high avidity T cells specific for the Melan-A/MART-1 26–35 A27L analog (ELAGIGILTV) (28). In this study, we compared the fraction of PBL-derived Melan-A/MART-1 peptide-specific CTL induced by in vitro peptide stimulation, in the presence or in the absence of IL-12 and IL-7. Tetramers were used to label Melan-A/MART-1 peptide-specific CTL. As shown in Fig. 6 and Table IV, a significant fraction of Melan-A-specific CTL induced in the presence of IL-12 expressed the CD94/NKG2-A (23 and 47%), while a very low fraction of these CTL derived in the absence of IL-12 expressed this receptor (0 and 5%).

Discussion

We show in this study that when $CD8^+$ PBL or melanoma TIL were cultured in the presence of IL-12, a significant fraction of these expressed NKG2-A and/or CD94. We could establish that this effect was due to IL-12 induction of NKG2-A and/or CD94 expression rather than to a selective expansion by IL-12 of T cells previously expressing these molecules. Indeed, this effect was still observed on sorted $CD94^-CD8^+$ T cells, and Vβ diversity of $CD94^+$ T cells derived from this population was normal. This effect was already detectable in most cultures after 7 days of culture with IL-12 and in all of them after 14 days. Therefore, IL-12 induces de novo expression of NKG2-A and/or CD94 by $CD8^+$ T cells. Furthermore, neither IFN-γ, a key downstream factor induced by IL-12, nor IL-15, a known inducer of the CD94/NKG2-A receptor, was implicated such in IL-12-mediated induction of CD94 on T cells as far as anti-IFN-γ and anti-IL-15 mAbs failed to abolish this effect. A short culture with IL-12 also increased the

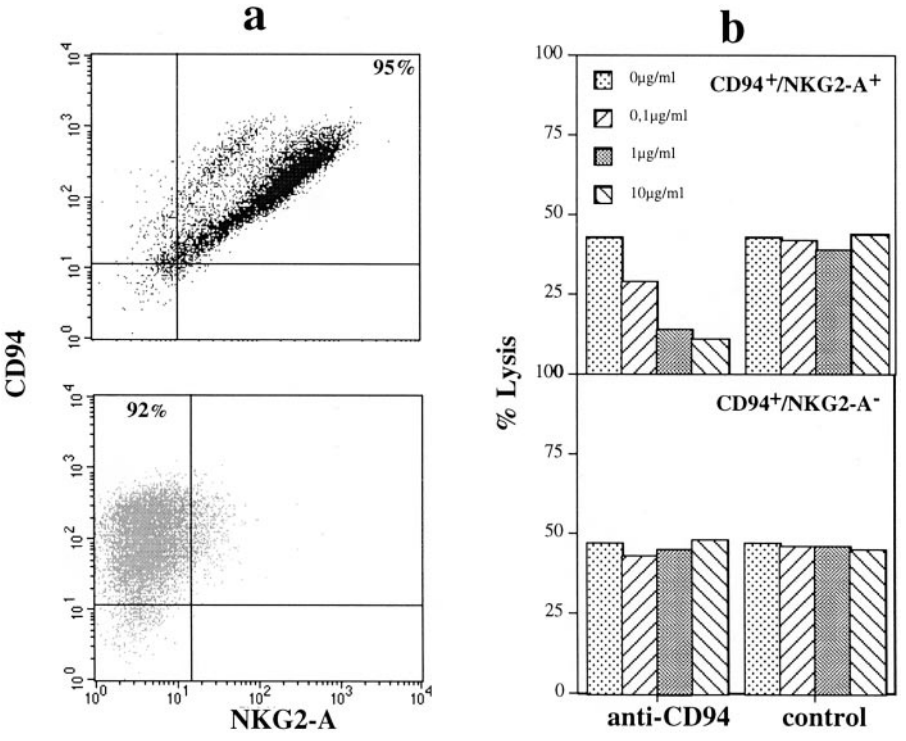

FIGURE 5. Cross-linking of $CD94^+$ $NKG2\text{-}A^+$ cells (but not $CD94^+NKG2\text{-}A^-$ cells) inhibits the CD3-induced cytolytic activity against $Fc\gamma R^+$ target cells. $CD8^+$ $CD94^-$ PBL were stimulated with anti-CD3 mAb. After 2 wk of culture in the presence of IL-12 (5 ng/ml), $NKG2\text{-}A^+$ T cells were positively sorted using immunomagnetic beads. *a*, The two sorted populations, $NKG2\text{-}A^+$ (*top panel*) and $NKG2\text{-}A^-$ (*bottom panel*), were expanded by a polyclonal PHA stimulation and then analyzed for the surface expression of CD94 and NKG2-A. *b*, $CD94^+NKG2\text{-}A^+$ and $CD94^+NKG2\text{-}A^-$ populations were analyzed for cytolytic activity in a redirected killing assay in the presence (or in the absence) of anti-CD94 mAb or mouse IgG2a isotypic control (from 0.1 to 10 μg/ml). The E:T ratio was 10:1. Data are expressed as percentages of specific ^{51}Cr release.

expression of CD94 and NKG2-A by previously established CTL clones. Both the fraction of cells and the level of expression of these two molecules were significantly higher than in the absence of IL-12. However, de novo expression of CD94 and NKG2-A failed to be induced by IL-12 on preestablished CTL clones that lacked these molecules.

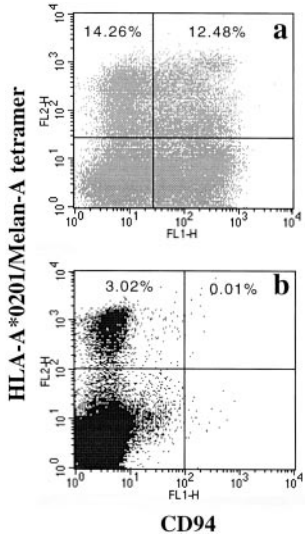

FIGURE 6. IL-12 induces the expression of CD94 and NKG2-A by a significant fraction of Melan-A/MART-1-specific CTL derived from in vitro peptide-stimulated PBL. PBL were stimulated with Melan-A_{26-35}A27L peptide-loaded M17 melanoma cells, for 3 wk, in the presence of IL-6 and IL-12 for the first week and IL-2 and IL-7 thereafter (*a* corresponds to patient D in Table IV), or in the presence of IL-6, but not IL-12 for the first week and of IL-2 only thereafter (*b* corresponds to patient B in Table IV). Stimulated PBL were stained with soluble PE-HLA-A*0201/Melan-A_{26-35}A27L tetramer and FITC-Ab specific for CD94. Numbers in the dot plots indicate the percentages of tetramer-positive/CD94-negative or CD94-positive cells.

IL-12-dependent induction of CD94 expression by $CD8^+$ PBL was observed as well in the absence of any TCR stimulus than following a CD3- or a peptide-specific stimulation. Nonetheless, much higher fractions of $CD8^+$ T cells were induced to express CD94 following a TCR stimulation (60–90 vs 15%). Therefore, TCR stimulation strongly potentiates IL-12-dependent induction of CD94 and NKG2-A expression by $CD8^+$ T cells. This may be due to the expression of IL-12R induced by TCR stimulation. This might explain conflicting data that IL-12 failed to induce the expression of CD94/NKG2-A receptor by superantigen-activated T cells (2). This could be due to a lack of IL-12R induction by the toxic shock syndrome toxin-1-stimulated T cells, as already reported for the $V\delta1^+$ $\gamma\delta$ T cell subset (30).

Although CD3 stimulation in the presence of IL-12 might induce PBL to express CD94 only, peptide stimulation in similar conditions systematically induced T cells to coexpress CD94 and NKG2-A. The reason for this difference is unclear. It might be related to the nature or strength of the T cell stimulation: TCR cross-linking by an anti-CD3 mAb vs MHC-peptide presentation by a melanoma cell line or mature DC. It may be that these two modes of TCR triggering activate distinct signaling pathways or the same one at different levels, therefore resulting in the activation

Table IV. *HLA-A*0201/Melan-A tetramer-positive cells and CD94-positive T cells in PBL stimulated by peptide-pulsed melanoma cells in the presence or absence of exogenous IL-12*

Donors	IL-12	Melan-A-Specific Cells (%)[a]	$CD94^+$ Cells (%)[b]	$NKG2\text{-}A^+$ Cells (%)[c]
A	−	0.24	5	5.5
B	−	3.09	0.3	0
C	+	4.60	23	22
D	+	27.77	47	45

[a] Percentages of tetramer-positive cells.
[b] Percentages of CD94-positive cells among Melan-A-specific cells.
[c] Percentages of NKG2-A-positive cells among Melan-A-specific cells.

of both or only one gene. Since a stimulation by peptide-pulsed melanoma cells in the presence of IL-12 induced coexpression of CD94 and NKG2-A as efficiently as peptide-pulsed mature DC in similar conditions, it seems unlikely that costimulation was critical for IL-12-mediated NKG2-A induction as far as melanoma cells do not express known costimulation molecules. Expression of CD94, but not NKG2-A, has already been reported in T cells, especially in $CD4^+$ T cells (2, 23).

Induction of CD94 expression in the absence of NKG2-A suggested that CD94 could have been expressed in association with another NKG2 member. However, although NKG2-C and NKG2-E mRNAs were expressed by $CD94^+NKG2\text{-}A^-$ T cells, cross-linking of CD94 by a specific mAb failed to modulate the redirected lysis, suggesting that these cells did not express a functional CD94 containing inhibitory or activatory receptor. Therefore, it is likely that CD3-stimulated PBL had been induced to express a CD94 nonfunctional homodimer by IL-12.

Previous studies had shown that IL-12 is a strong inducer of NKRP1-A molecule (CD161) by human NK cells (31, 32), $\alpha\beta$ T cells (31), and $V\delta2^+$ $\gamma\delta$ T cells (30). Our data show that IL-12 also is an inducer of CD94 and NKG2-A, two genes located in the same gene locus as NKRP1-A, the NK gene complex (33). Our data show that the transcription of at least three genes from this locus coding for these C-type lectin receptors is regulated by the same cytokine.

The present and previous studies (2, 23, 24) therefore show that several cytokines, TGF-β, IL-15, and IL-12, produced by APCs and B cells, can induce the expression of the CD94/NKG2-A inhibitory receptor by $CD8^+$ T cells. Interestingly, IL-10 and TGF-β modulate the expression of the IL-12R (34) and could therefore affect IL-12-induced expression of CD94. Whether this induction contributes to the normal T cell homeostasis remains to be established. It has been reported that oligoclonal T cells that express killer cell Ig-like receptors in normal individuals could be autoreactive T cells, and that expression of these inhibitory receptors would contribute to tolerance by these cells (35). Therefore, IL-12 and IL-10 produced by dendritic cells could contribute to the expression of the CD94/NKG2-A receptor during T cell cross-priming.

While in vivo treatment by IL-12 has been essentially stopped, IL-12 is still used in vitro for the production of Ag-specific T cells for research or clinical purposes. Our results suggest that using this cytokine might compromise the function of Ag-specific T cells obtained as far as a majority of them will express the inhibitory CD94/NKG2-A receptor. It might thus be better to avoid using IL-12, inasmuch as other cytokines can support the optimal induction of Ag-specific T cells in vitro (28).

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