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IL-15 induces CD8+ T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion

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ABSTRACT

During the last years several authors have described a small population of CD8+ T cells expressing NK receptors (NKRs). Although their origin remains largely unknown, we have recently demonstrated that IL-15 is capable of inducing NKR expression in purified human CD8+CD56− T cells. In this study we show that IL-15-driven NKR induction in CD8+ T cells was linked with CD56 *de novo* acquisition, consistent with an effector-memory phenotype, increased anti-apoptotic levels, high granzyme B/perforin expression and with the ability of displaying *in vitro* NK-like cytotoxicity. Interestingly, dissection of NKR functional outcome in IL-15-cultured CD8+ T cells revealed: (i) that NKG2D cross-linking was able *per se* to upregulate degranulation levels and (ii) that KIR and NKG2A cross-linking upregulated secretion of cytokines such as IFN-γ, TNF-α, IL-1β and IL-10. These results suggest that IL-15 is capable of differentiating CD8+ T cells into NK-like T cells displaying a regulatory phenotype.

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Introduction

In the last years, it has been shown that NK receptors could be expressed, not only by NK cells, but also by T cells. In early studies, killer immunoglobulin-like receptors (KIR) were found to be expressed in a small fraction of T cells from normal donors expressing either TCRαβ or TCRγδ, mostly CD8+ T cells (Mingari et al. 1996, 1995). Together with KIR, CD94/NKG2A was also found to be expressed in CD8+ T cells (Mingari et al. 1995). These receptors were shown to provide T cells with the ability to recognize HLA class I molecules on target cells, leading to a decrease in CTL cytotoxicity (Mingari et al. 1996, 1995; Speiser et al. 1999). It is now known that besides inhibitory receptors, CD8+ T cells also express activating receptors, (Bauer et al. 1999; Groh et al. 2001) and the notion that NK receptor expression in CD8+ T cells could modulate T cell function, by increasing or decreasing TCR threshold accordingly to the triggering of activating or inhibitory NKRs, is now emerging. Despite this knowledge, the environmental signals that trigger NK receptor acquisition by CD8+ T cells remain largely unknown. In a previous study, we have found that the γ-common cytokine IL-15, known to be crucial to NK and invariant NKT cell development and homeostasis, as well as for the activation, survival and differentiation of both memory and naïve CD8+ T cells (Alves et al. 2003; Fehniger and Caligiuri 2001; Huntington et al. 2009; Mortier et al. 2009; Pek et al. 2010), was capable of inducing the expression of NKRs in purified CD8+CD56− T cells after a prolonged exposure (Correia et al. 2009). Between those NKRs there were both inhibitory and activation and from different structural families: KIR (KIR2DL2/3, KIR2DL4), C-type lectin-like receptors (NKG2A, NKG2D), the most common NK marker, CD56, and the recently considered the truly bona-fide marker of NK cells, the natural cytotoxicity receptor (NCR) NKp46 (Correia et al. 2009).

Although the term "NKT cell" is mainly used to describe CD1d-restricted NKT cells expressing an invariant TCR chain, it also includes CD1d-unrestricted NKT cells with an oligoclonal TCR, known also as NK-like T cells, which are less studied. NK-like T cells have been described to be mostly CD8+CD56+ T cells and, contrarily to mice, constitute the NKT cell population mostly enriched in human livers (Kenna et al. 2003; Norris et al. 1999). In this context, we have previously hypothesized that those NK-like T cells do not necessarily consist in a different lineage, but could in part rather be CD8+ T cells that under certain conditions as under pro-

Abbreviations: KIR, killer immunoglobulin-like receptor; NCR, natural cytotoxicity receptor; NKG2, natural killer cell lectin-like receptor gene 2; NKR, NK receptor.

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longed exposure to IL-15 in a favorable environment such as the liver, differentiate and acquire NK receptors (Correia et al. 2009). In the present study we have extended these results by characterizing the functional phenotype of IL-15-induced CD8+NKR+ T cells and dissecting the physiological outcome of triggering NKRs on CD8+ NKR+ T cells. To our knowledge, this is the first evidence showing that KIR and NKG2A mAb cross-linking can induce the secretion of a varied pattern of cytokines by CD8+ T cells. The results add further knowledge to the biology of NKR expression by CD8+ T cells and strongly suggest that IL-15 is one environmental factor involved in the generation of regulatory NK-like CD8+ T cells.

Material and methods

Reagents and monoclonal antibodies

RPMI-1640 Glutamax®, fetal bovine serum (FBS) and amphotericin B/penicillin/streptomycin (APS) were from Gibco BRL (Paisley, Scotland). 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and the live/dead® Viability/Cytotoxicity Kit (for mammalian cells) were purchased from Molecular Probes (Amsterdam, The Netherlands). Human serum was obtained from Cambrex (New Jersey, USA). Recombinant human IL-15 was obtained from R&D Systems (Minneapolis, USA). Permeabilization buffer (Foxp3 Staining Buffer Set) is from eBioscience (San Diego, CA, USA). The monoclonal antibodies (mAb) used are listed in Table 1.

Cell lines

K562 (human immortalized myeloid leukemia), Jurkat E6.1 (human immortalized T cell leukemia) and P815 (murine mastocytoma) cell lines were obtained from European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cell lines were cultured in 75 cm^2 flasks (Nunc, Roskilde, Denmark) in RPMI-1640 Glutamax® supplemented with 10% inactivated FBS and 1% APS solution at 37 °C, 5% CO_2 and 95% humidity. The medium was regularly changed and the cells were always washed twice before use.

Isolation of peripheral blood lymphocytes and T cell subsets

Peripheral blood lymphocytes and T cell subsets were freshly isolated in every experiment and obtained as described previously (Correia et al. 2009). Briefly, peripheral blood mononuclear cells (PBMC) were obtained from buffy coats after centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Contaminating red blood cells were lysed in lysis solution (10 mM Tris, 150 mM NH_4Cl, pH 7.4), 10 min at 37 °C. Peripheral blood lymphocytes (PBL) were obtained by overnight culture in Petri dishes. Purified CD8+ CD56− T cells were obtained after two-step isolation: First, CD8+ T cells were isolated from PBL using a CD8 negative isolation kit (Miltenyi Biotec). Then, the negatively selected CD8+ T cells were subjected to positive isolation with CD8 beads (Miltenyi Biotec), obtaining a population >99.5% CD8+CD3+CD56−. This investigation was approved by the Institutional Ethics Committee and informed consent was obtained from all the blood donors.

Culture conditions and CFSE proliferating assay

Purified CD8+CD56− T cells were cultured in RPMI-1640 Glutamax® supplemented with 10% human serum and 1% APS solution, at 37 °C, 5% CO_2, 95% humidity, during 12 days with 10 ng of IL-15 (R&D Systems). For proliferation studies, 10^7 cells/ml cells were labeled with CFSE at a final concentration of 10 μM for 10 min at 37 °C with occasional mixing, and then washed twice with PBS/20% FBS prior to cell culture, as described previously (Correia et al. 2009). Rounds of cell division were determined by sequential halving of CFSE-fluorescence intensity after the period of culture.

Cell-mediated cytotoxicity and redirected killing assays

The cytotoxicity assays were performed using the live/dead® Viability/Cytotoxicity Kit (Molecular Probes). Briefly, target cells were previously labeled with 3,3′-dioctadecyloxacarbocyanine ($DiOC_{18}(3)$) and washed twice with PBS. For redirected killing assays, the FcR positive P815 target cells were pre-incubated with 10 μg of anti-CD3 Ab (OKT3), or the respective mouse isotype con-

Table 1
List of antibodies used in the study.

Antibody	Conjugate	Company	Isotype	Clone
CD3	APC	Immunotools	mIgG2a	MEM-57
CD8	PE-Dy647	Immunotools	mIgG2a	MEM-31
CD56	PE	Immunotools	mIgG2a	MEM-188
CD56	APC	Immunotools	mIgG2a	MEM-188
KIR2DL2/S2/L3 (NKAT2)	PE	BD	mIgG2a	DX27
KIR2DL4 (CD158d)	PE	R&D Systems	mIgG2a	181703
NKG2A (CD159a)	PE	Coulter	mIgG2b	Z199
NKp46 (CD335)	PE	Coulter	mIgG1	BAB281
NKG2D (CD314)	PE	Coulter	mIgG1	ON72
Granzyme B	PE	Immunotools	mIgG1	HC4
Perforin	PE	Immunotools	mIgG2a	Delta G9
CCR7	APC	eBioscience	mIgG2a	3D12
CD45RA	PE-Dy647	Immunotools	mIgG2b	MEM-56
Bcl-2	PE	Caltag Laboratories	mIgG1	100
CD107a (LAMP-1)	PE	BD		H4A3
Mouse IgG	PE	Immunotools	mIgG1	PPV-06
Mouse IgG	PE	Immunotools	mIgG2a	713
Mouse IgG	PE	Immunotools	mIgG2b	GC198
Rabbit anti-mouse (RAM)	None	Dako Cytomation	-	-
Mouse IgG	None	eBioscience	mIgG1	P3
CD3	None	eBioscience	mIgG2a	OKT3
CD56	None	Biolegend	mIgG1	HCD56
NKp46 (CD335)	None	Biolegend	mIgG1	9E2
KIR2DL2/3/S2 (NKAT2)	None	Biolegend	mIgG2a	DX27
KIR2DL4 (CD158d)	None	R&D Systems	mIgG2a	181703
NKG2A (CD159a)	None	R&D Systems	mIgG2a	131411
NKG2D (CD314)	None	Biolegend	mIgG1	1D11

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trol (mIgG), for 20 min at 4 °C and again washed twice. Then, target cells were platted together with the effector cells at a 5:1 E:T ratio in 96-well plates and propidium iodide (PI) was added for counterstaining accordingly to manufacturer instructions. The cells were then incubated for 3 h at 37 °C and immediately acquired in a flow cytometer. The percentage of dead target cells was determined accordingly to $DiOC_{18}(3)$+PI+ cells. The specific cytotoxicity was calculated as follows: % specific cytotoxicity = (% of dead target cells in the presence of effector cells) − (% of dead target cells alone).

CD107a degranulation assay after NKR crosslinking

CD107a degranulation studies were performed on CD8+ T cells obtained after 12-day culture with IL-15. The supernatant was collected and re-used as culture media in the assays. In order to do NKR cross-linking, the CD8+ T cells were pre-incubated separately with 10 μg of the different NKR functional-grade antibodies (see Table 1), anti-CD3 antibody (OKT3) or isotype control antibodies for 30 min at 4 °C and washed twice. Then, mAb-labeled cells were ressuspended in the originally collected culture supernatant and added to 96-well culture plates previously coated with rabbit anti-mouse (RAM) antibodies. Then, 10 μl of CD107a-PE antibody or mIgG-PE isotype control were added and cells were incubated during 4 h at 37 °C. Afterwards, cells were washed twice with PBS and acquired in the flow cytometer.

Cytokine quantification by fluorescent bead immunoassay (FlowCytomix)

A fluorescent bead immunoassay was used for quantitative detection by flow cytometry of IFNγ, IL-1β, IL-6, IL-10, MIP-1β, and TNF-α (FlowCytomix Assay, Bender MedSystems GmbH, Austria) in culture supernatants, accordingly to the manufacturer instructions. This assay allows the identification of different cytokines based on two different populations (A and B) in the flow cytometer due to the use of two sets of beads with different sizes, and discrimination of the different cytokines within two populations according to variations in the intensities of an internally fluorescent dye detected in the FL-3 channel; while allowing the quantification of each specific cytokine in the FL-2 channel. The supernatants were obtained after performing NKR crosslinking of NKRs on IL-15-cultured CD8+ T cells, as described above, and culturing cells for 48 h at 37 °C. Supernatants were collected, centrifuged at 13,000 × *g* for 15 min and stored at −20 °C until used. After processing samples according to manufacturer's instructions, they were acquired in a FACSAria flow cytometer (BD, Mountain View, CA) and the amount of cytokine (pg/ml) determined by using a FlowCytomix 2.3 Software (Bender MedSystems).

Flow cytometry determinations

Extracellular cell stainings were performed at 4 °C for 30 min in PBS in 96-well round-bottom plates. For intracellular stainings, cells were previously fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience), accordingly to the manufacturer instructions. Irrelevant mouse mAbs were always used as negative controls to define background staining. After staining, cells were washed and acquired in a FACSCalibur (BD, Mountain View, CA) and analyzed using CellQuest or FlowJo softwares.

Statistical analysis

Statistical analyses were performed using Excel or GraphPad Prism 5 software. Student's *t*-test was used to test the significance of the differences between group means. Statistical significance was defined as $p < 0.05$.

Results

IL-15 induces de novo formation of CD8+CD56+ T cells co-expressing other NKRs

As we have previously shown (Correia et al. 2009), after 12-days of culture with IL-15, purified CD8+CD56− T cells were capable of up-regulating and/or *de novo* expressing several NK receptors, including KIR (KIR2DL2/3, KIR2DL4), C-lectin-like receptors (NKG2A, NKG2D), natural cytotoxic receptors, NCR (NKp46), and the most NK common marker, CD56 (Fig. 1). In Fig. 1(A), it is possible to see that acquisition of the different NKRs by CD8+ T cells after 12 days in culture with IL-15 occurs mainly in cells that underwent several cycles of division, as determined by CFSE loss (see CD56 and NKp46). Indeed, the initial purified CD8+ T cell population was negative for CD56 and NKp46, while the other NKRs were only slightly expressed (Fig. 1(B), day 0). After culture with IL-15 there is a clear CD56 (about 20%) and NKp46 (about 5%) de novo expression in the CD8+ T cell population, as well as the increased expression of the other NKRs (ranging between 6 and 12%) (Fig. 1(B), day 12), demonstrating that the initial CD8+CD56− T cell population was capable of *de novo* expressing and/or up-regulating NKRs (Fig. 1(A) and (B)). Noteworthy, after the period of culture, the cells remain CD3+CD8+ (Fig. 1(C)). Although NKG2D was already expressed by almost all CD8+CD56− T cells, culture with IL-15 induced an up-regulation in the mean fluorescence intensity (MFI), indicating that this cytokine is able to up-regulate further NKG2D expression levels (Fig. 1(E)). Importantly, simultaneous analysis of CD56 and the other NKRs revealed that the majority of CD8+ T cells expressing NKRs also co-expressed the CD56 marker (Fig. 1(D)), indicating that NKR expression by CD8+ T cells is closely linked to *de novo* CD56 acquisition.

IL-15-induced CD8+NKR+ T cells display an effector memory phenotype and express high levels of Bcl-2

Next, we wanted to determine markers of differentiation and survival in the IL-15-induced CD8+NKR+ T cells. In order to use a representative marker for CD8+NKR+ T cells we used CD56 since we have found it to be the most accurate marker of *de novo* NKR expression and shown to be co-expressed with the other NKRs (see Fig. 1). Based on the expression of CD45RA and CCR7 markers, four differentiation phenotypes can be defined: CD45RA+CCR7+ (naïve, T_N), CD45RA−CCR7+ (central memory, T_{CM}), CD45RA−CCR7− (effector memory, T_{EM}) and the CD45RA+CCR7− (CD45RA+ effector memory, T_{EMRA}). As seen in Fig. 2(A), IL-15-induced CD8+ CD56+ T cells were shown to be mainly CD45RA+ and CCR7−, corresponding to a CD45RA+ effector memory phenotype (T_{EMRA}). In some experiments, a part of CD8+CD56+ T cells were CD45RA−CCR7− effector memory cells (T_{EM}), showing that IL-15 induced CD8+CD56+ T cell generation is consistent with a differentiation towards an T_{EM}/T_{EMRA} effector memory phenotype.

On the other hand, Bcl-2 has been shown to play a central role in preventing CD8+ T cell apoptosis upon TCR triggering (Geginat et al. 2003). Analysis of intracellular Bcl-2 by flow cytometry revealed that the population obtained after 12 day-culture with IL-15 has higher expression of Bcl-2, as determined by MFI levels, than the initial purified CD8+CD56− T cell population (Fig. 2(B), day 0 vs. day 12). Noteworthy, simultaneous analysis of Bcl-2 and CD56 revealed that nearly all CD8+CD56+ T cells were Bcl-2-positive (Fig. 2(C), upper dot-plot) and expressed significantly higher levels of Bcl-2 (Fig. 2(C), lower graph) when compared to CD8+CD56− T cells,

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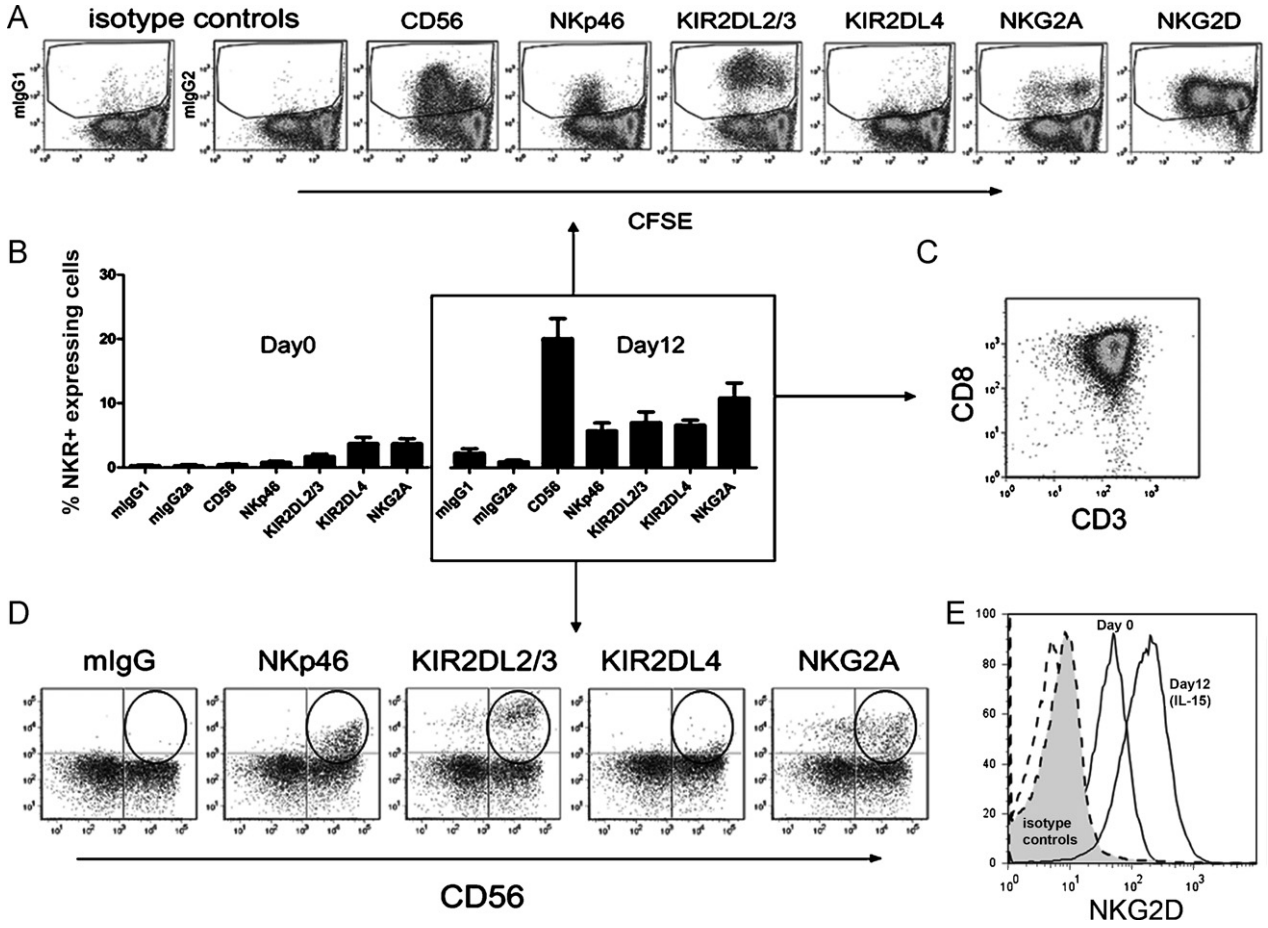

Fig. 1. IL-15-driven induction of NK receptors in purified CD8+CD56− T cells is paralleled by CD56 acquisition. Purified CD8+CD56− T cells were obtained as indicated in Material and Methods, labeled with CFSE and cultured with IL-15 (10 ng/ml) for 12 days. (A) Dot-plots showing NKR expression in CD8+ T cells after 12 days with IL-15 vs. CFSE halving. Isotype controls (mIgG1 and mIgG2) are included. (B) Graph showing the percentage of NKR+CD8+ T cells at day 0 (without IL-15) and after 12 days of culture with IL-15 for all NKRs, (mean ± SEM, $n = 8$ independent experiments), $p < 0.05$ in all conditions compared with mIgG. (C) Dot-plot showing that after 12-day culture with IL-15 all cells remain CD8+CD3+. (D) Dot-plots illustrating CD56 vs. NKR expression in IL-15 cultured CD8+ T cells. (E) Histogram showing upregulation of NKG2D expression in CD8+ T cells after 12 day-culture with IL-15 compared with day 0. Correspondent isotype controls for each condition are shown. Data is representative from at least 7 independent experiments.

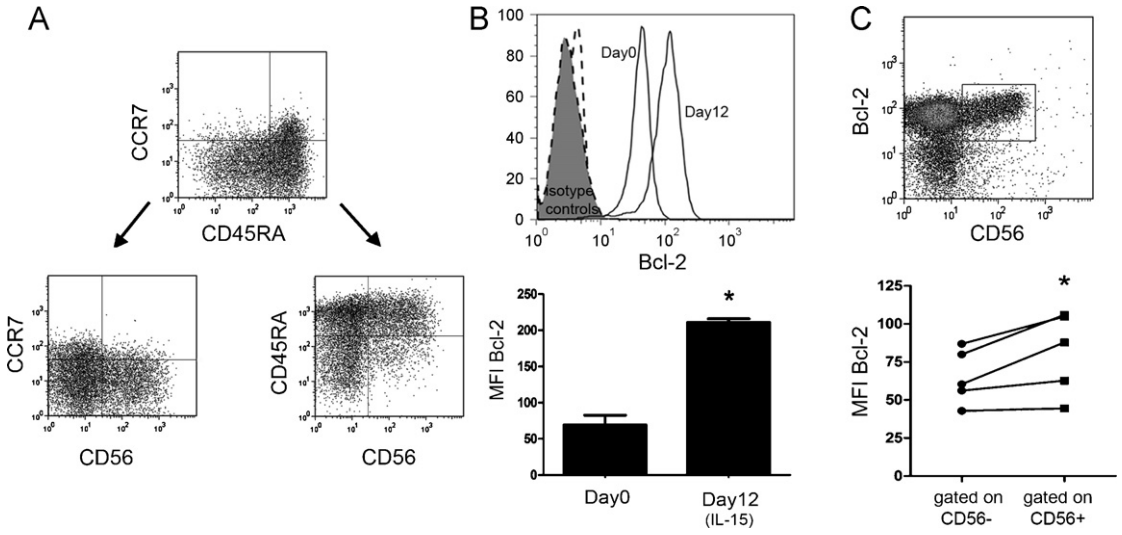

Fig. 2. IL-15-induced CD8+NKR+ T cells display an effector memory phenotype and express high levels of Bcl-2. Purified CD8+CD56− T cells were obtained as indicated in Material and Methods and cultured with IL-15 (10 ng/ml) for 12 days. (A) Dot-plots showing CD45RA and CCR7 expression (upper dot-plot) and CD45RA and CCR7 vs. CD56 co-expression (lower dot-plots). One representative experiment out of 6 different experiments performed is shown. (B) Histogram illustrating intracellular Bcl-2 expression in purified CD8+CD56− (day 0) and after culture with IL-15 (day 12). Correspondent isotype controls for each condition are shown (upper histogram). Graph showing intracellular MFI of Bcl-2 protein determined by flow cytometry at day 0 and after 12 days in culture with IL-15 (lower graph). (C) Representative dot-plot showing Bcl-2 vs. CD56 expression after 12 days in culture with IL-15 (upper dot-plot). The MFI Bcl-2 values (mean ± SEM) on gated CD56− and CD56+ populations are indicated (lower graph) for 5 independent experiments. $^*p < 0.05$.

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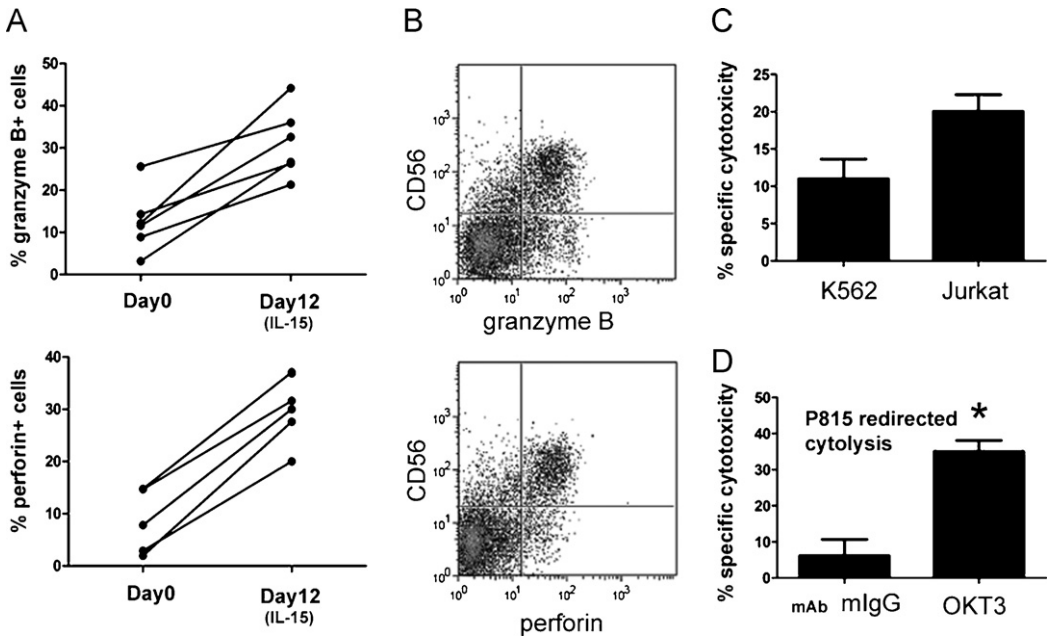

Fig. 3. IL-15-generated CD8+CD56+ T cells are granzyme B+/perforin+ and display cytotoxicity towards different targets. Purified CD8+CD56− T cells were obtained as indicated in Material and Methods, and cultured with IL-15 (10 ng/ml) for 12 days. (A) Graphs shows a statistically significant increase ($p < 0.05$) in the percentage of granzyme B (upper graph) and perforin (lower graph) positive CD8+ T cells from day 0 to day 12 after culture with IL-15 ($n = 6$ independent experiments). (B) Dot-plots showing representative experiments of CD56 vs. granzyme B (upper dot-plot) or perforin (lower dot-plot) expression after 12 days in culture with IL-15. (C-D) IL-15-cultured CD8+ T cells were examined for cytotoxicity using the live/dead® Viability/Cytotoxicity Kit (Molecular probes) as described in Material and Methods: (C) Graph showing the specific cytotoxicity (mean ± SEM, $n = 3$ independent experiments) of IL-15 cultured CD8+ T cells towards K562 and Jurkat cells. (D) Graph showing the specific cytotoxicity (mean ± SEM, $n = 3$ independent experiments) of IL-15 cultured CD8+ T cells towards P815 cells in redirected assays using OKT3 or an isotype control (mIgG). $^*p < 0.05$.

suggesting that the IL-15 generated CD8+CD56+ T cells show an increased anti-apoptotic potential.

IL-15-cultured CD8+ T cells express high levels of granzyme B and perforin and can display MHC-unrestricted cytotoxicity

In order to assess if the IL-15-differentiated CD8+NKR+ T cells have acquired a cytotoxic phenotype, we went to examine the intracellular expression of granzyme B and perforin. As observed in Fig. 3(A), purified CD8+CD56− T cells already expressed some levels of granzyme B and perforin (day 0). However, after 12 days of culture with IL-15 there was a clear increase in the percentage of granzyme B/perforin positive CD8+ T cells (day 12), which was consistently observed in all experiments (Fig. 3(A)). Importantly, simultaneous determination of granzyme B/perforin and CD56 showed that the large majority of the generated CD8+CD56+ T cells were granzyme B and perforin positive (Fig. 3(B)), indicating that *de novo* expression of CD56 in IL-15-differentiated CD8+ T cells is paralleled with granzyme B/perforin acquisition.

In the light of these results, we wanted to ascertain if our IL-15-cultured CD8+ T cells could display cytolytic activity. To that purpose, K562 cells (that lack MHC class I molecules) and Jurkat cells were used as targets. Interestingly, the cytotoxicity of freshly purified CD8+CD56− T cells prior to culture with IL-15 against either cell line was completely absent (no specific cytotoxicity shown). However, after a 12-day period culture with IL-15, the generated CD8+ T cells were capable of displaying measurable levels of cytotoxicity towards both K562 and Jurkat cells (Fig. 3(C)). In parallel, as illustrated in Fig. 3(D), redirected cytotoxic assays using P815 cells showed that CD3 cross-linking (OKT3) led to increased cytolysis of P815 cells compared with the control (mIgG). Overall, these results show that a fraction of CD8+ T cells obtained after 12 day-culture with IL-15 were capable of acquiring killer function, displaying both TCR-mediated and MHC-unrestricted (NK-like) cytotoxicity.

IL-15-cultured CD8+ T cells express functional NKRs: cytotoxic granule and cytokine release

An important issue arising from these results was to ascertain whether the NKRs expressed *de novo*/up-regulated on CD8+ CD56− T cells upon culture with IL-15 were functional. Thus, we performed analysis of cytotoxic granules release and analysis of cytokine secretion after NK receptor triggering using the bulk IL-15-cultured CD8+ T cells. Controls included mouse IgG (negative) and OKT3 (positive) antibodies. As shown in Fig. 4, control (mIgG-treated) IL-15-cultured CD8+ T cells already expressed low but detectable levels of CD107a, which was not observed in freshly

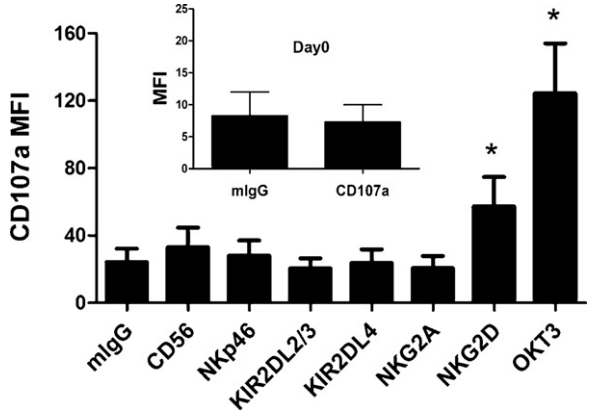

Fig. 4. NK receptors induced by IL-15 are functional and capable of modulating CD107a degranulation. Purified CD8+CD56− T cells were obtained, cultured with IL-15 (10 ng/ml) for 12 days and analyzed for CD107a expression after mAb crosslinking, as described in Material and Methods. The graph represents CD107a levels (MFI) after crosslinking of the different NKRs and OKT3. Mouse IgG antibodies were used as crosslinking control. Data is presented as mean ± SEM, $n = 7$ independent experiments, $^*p < 0.05$. The graph inset shows that freshly isolated CD8+CD56− T cells do not express measurable CD107a levels at the cell surface (day 0).

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isolated CD8+ CD56− T cells (Fig. 4, inset), confirming that after culture with IL-15 CD8+ T cells acquire a cytotoxic potential compared with resting CD8+ T cells. As expected, OKT3 cross-linking induced a marked increase in CD107a levels (Fig. 4) in almost all cells, indicating that IL-15-cultured CD8+ T cells are highly prone to respond to TCR/CD3 stimulation. Interestingly, regarding degranulation after NK receptor specific cross-linking, contrarily to the other NKRs studied, NKG2D was *per se* capable of inducing a statistically significant increase in the percentage of CD107a expressing cells when compared to the control (mIgG) (Fig. 4).

Cytokine secretion was assessed after 48 h of NK receptor cross-linking by using fluorescent bead immunoassay for IFN-γ, TNF-α, IL-10, IL-1β and MIP-1β, as described in Material and Methods. Fig. 5(A) shows representative dot-plots illustrating cytokine discrimination (according to FSC/SSC parameters and FL-3 fluorescence intensity) and quantification (according to FL-2 fluorescence intensity). As shown in Fig. 5(B), CD8+ T cells cultured 12 days with IL-15 (mIgG-treated) produced measurable amounts MIP-1β (4381 ± 2739 pg/ml), TNF-α (1043 ± 212.7 pg/ml) and IFN-γ (1825 ± 1228 pg/ml), and also slight amounts of IL-10 (183 ± 60.21 pg/ml) and IL-1β (202.7 ± 96.67 pg/ml). IL-6 was not detected in the supernatants. After CD3 cross-linking, there was a marked increase in IFN-γ, IL-10, IL-1β and TNF-α secretion, with IFN-γ being the most abundant cytokine detected in the supernatant (about 30,000 pg/ml) (Fig. 5(A and C)). Interestingly, cross-linking of the different NKRs under study produced contrasting outcomes in cytokine secretion. Thus, while cross-linking of CD56, NKp46 or NKG2D did not result in significant changes in the level of any of the cytokines analyzed when compared to control (mIgG-treated) cells, cross-linking of KIR2DL2/3, KIR2DL4 and NKG2A resulted in a statistically significant increase in the secretion of IFN-γ, TNF-α, IL-10 and IL-1β (Fig. 5(C)).

Discussion

The common γ chain cytokine IL-15 is considered a crucial factor for CD8+ T cell homeostasis and the maintenance and generation of memory CD8+ T cells (Alves et al. 2007; Kim et al. 2008; Mortier et al. 2009). Despite the existence of work documenting the existence of cell surface NKR acquisition by a subset of memory CD8+ T cells, the specific signals that control their expression have, to date, not been clearly defined. In this study, we have shown by using an *in vitro* model of human CD8+ T cell differentiation that IL-15 is a factor responsible for the acquisition of NKRs by CD8+ T cells, closely linked with the acquisition of an effector-memory T_{EM}/T_{EMRA} phenotype. Also, we have found that our *in vitro* IL-15-differentiated CD8+ CD56+ T cells express high levels of the anti-apoptotic protein Bcl-2 and large amounts of intracellular granzyme B and perforin, reinforcing the role of IL-15 as a cytokine responsible for CD8+ T cell survival (Alves et al. 2007; Oh et al. 2008), and suggesting a link between CD56 acquisition by CD8+ T cells and increased resistance to apoptosis concomitantly with increased cytotoxic potential. Interestingly, this phenotype is reminiscent of different "NK-like T cells" described *in vivo* (Anfossi et al. 2001; Lemster et al. 2008; Mingari et al. 1996; Norris et al. 1999; Pittet et al. 2000; Vella et al. 1998; Young et al. 2001). In line with these results, IL-15-cultured CD8+ CD56+ T cells have shown TCR-mediated and NK-like cytotoxicity, indicating that IL-15-induced differentiation was associated with acquisition of effector functions.

It is becoming increasingly apparent that IL-15 exerts its biological function in context of membrane-bound IL-15-IL-15Ra complexes transpresented by neighbor cells to the target cells. Accordingly, we believe that, *in vivo*, this should be the physiological way by which IL-15 exerts the effects reported in our study towards CD8+ T cells. Indeed, a recent article has shown that IL-15Rα expression on macrophages supports the early transition of antigen specific effector CD8+ T cells to memory cells. After memory CD8+ T cell differentiation, IL-15Rα expression on dendritic cells selectively supports central memory CD8+ T cells; whereas IL-15Rα expression on macrophages supports both central and effector memory CD8+ T cells (Mortier et al. 2009).

Importantly, in our study we have shown that IL-15-induced CD8+CD56+ T cells co-express several activating and inhibitory NKRs, including KIR members, NKG2A, NKG2D and the bona-fide natural killer receptor NKp46. Although the consequences of NKR expression for the function of CD8+ T cells after TCR engagement have been described (Ugolini and Vivier 2000), we wanted to ascertain the functional outcome of engagement of the acquired NKRs alone. Accordingly, their role in two important CD8+ T cell effector functions, release of cytotoxic granules and cytokine secretion, were dissected.The degranulation assays revealed three important facts. First, that CD3 triggering induced high levels of degranulation, indicating that IL-15-cultured CD8+ T cells are functional and capable to respond to TCR/CD3-mediated stimuli, as also shown by redirected cytolysis with OKT3. Second, that NKG2D triggering itself was capable of leading to a significant increase in the CD107a levels, which is of note since in previous studies NKG2D has been shown to function only as a co-stimulatory receptor in human CD8+ T cells, incapable of inducing cytotoxicity by itself (Bauer et al. 1999; Groh et al. 2001). In agreement with our results, Meresse et al. (2004) have shown that in the presence of IL-15, CD8+ T cells can acquire the ability to kill NKG2D ligand-bearing targets, which could be related with an increase in NKG2D and DAP10 levels after IL-15 culture and a coordinated IL-15 induction in PI3K and JNK pathways. Interestingly, Horng et al. (2007) have found that after activation by IL-15, Janus kinase 3 (Jak3) phosphorylated DAP10, suggesting an association between DAP10 and IL-15R. It was also consistent with the fact that, in our study, although almost all purified CD8+ CD56- T cells expressed NKG2D at day 0, they were not capable of inducing cytotoxicity. Third, and finally, contrarily to NKG2D, any of the other NKRs under study were unable to induce a significant effect in degranulation after triggering, which could be due either to the low percentages of CD8+ T cells expressing those NKRs and/or to the fact that they are instead involved in cytokine secretion in these conditions (see below).

In the past, CD8+ T cells have been regarded as a homogeneous population of cytotoxic cells producing only a limited number of cytokines, however, it is now becoming clear that CD8+ T cells have the potential to produce a wider array of cytokines. In this study, we have shown that IL-15 cultured CD8+ T cells were capable to secrete IFN-γ, TNF-α, IL-1β and predominantly MIP-1β without further re-stimulation, which agrees with the effector memory phenotype displayed (Alves et al. 2003; Hamann et al. 1997; Kim et al. 2009). The effect of NK receptor cross-linking had interesting results. Noteworthy, besides having a significant effect in increasing cytotoxicity of IL-15-cultured CD8+ T cells (see above), NKG2D triggering did not lead to an increase in IFN-γ production or any other cytokine studied. These results are in accordance with other studies showing that, contrarily to NK cells, NKG2D in human CD8+ T cells seems to be primarily involved in the regulation of cytotoxic activity and not cytokine secretion (Hayakawa and Smyth 2006; Meresse et al. 2004), which could be probably related with the absence of DAP12 in CD8+ T cells (Diefenbach et al. 2002; Hayakawa and Smyth 2006).

Remarkably, triggering of KIR2DL2/3, KIR2DL4 and NKG2A in IL-15-induced CD8+NKR+ T cells induced a strong and significant increase in the secretion of IFN-γ, TNF-α, IL-1β and IL-10. Even though KIR2DL4 triggering does not induce cytotoxic activity, it has been pointed as a more activating than inhibitory KIR in NK cells due to its involvement in IFN-γ, and other cytokines, secretion (Rajagopalan et al. 2001; Rajagopalan et al. 2006). However, we

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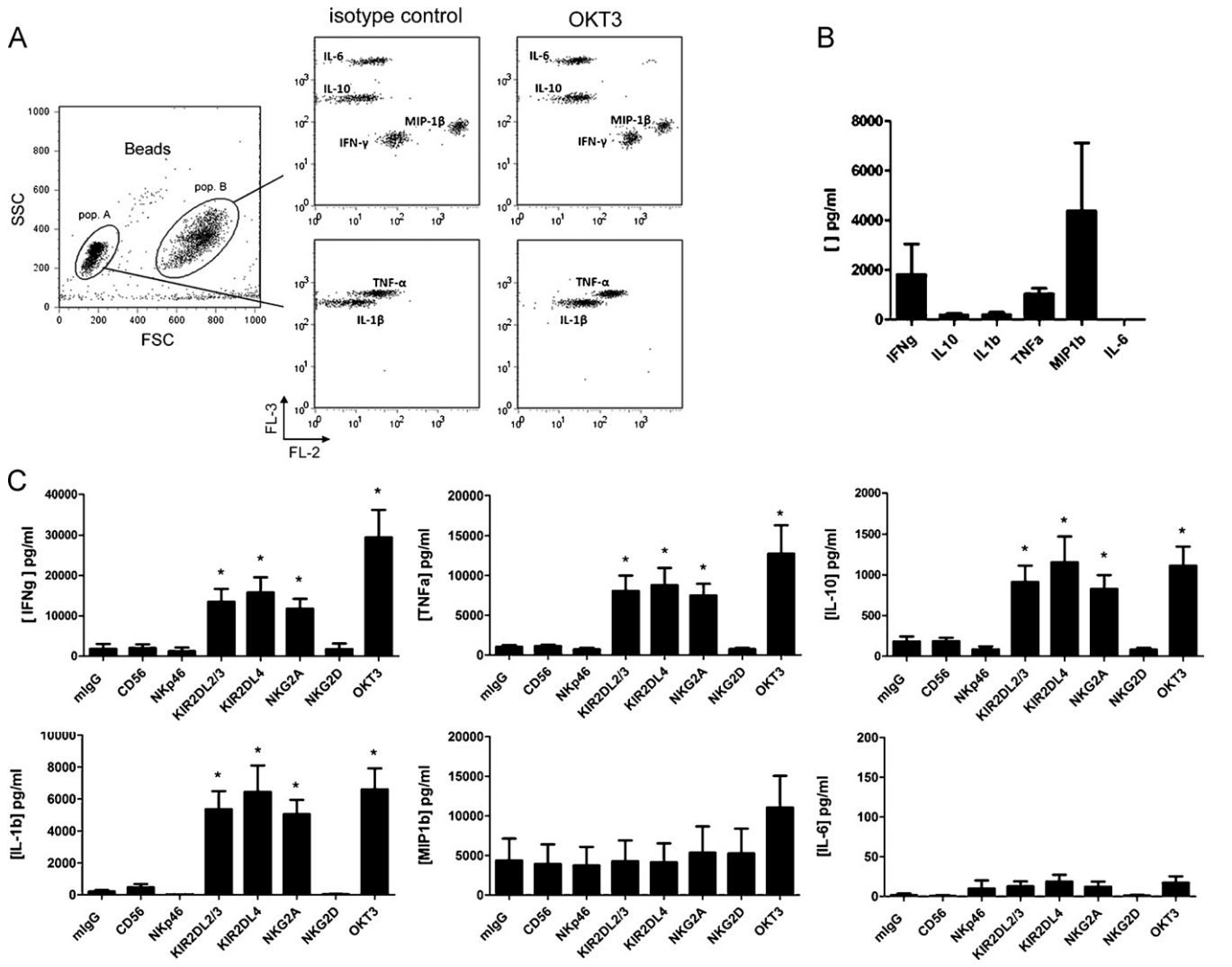

Fig. 5. NK receptors induced by IL-15 are functional and capable of modulating cytokine secretion (A–C) Cytokine secretion was assessed by fluorescent bead immunoassay (Flowcytomix), as described in Material and Methods. (A) Dot-plot illustrating a representative experiment of how the different cytokines were quantitated (see Material and Methods) without cross-linking (isotype control) or after CD3 cross-linking (OKT3). (B) Graph showing the quantification of each cytokine described (pg/ml) in the absence of NKR triggering. (C) Graphs representing the quantification of each described cytokine (pg/ml) after the cross-linking of each NKR (with mIgG and OKT3 included). Data is presented as mean ± SEM, $n = 5$ independent experiments, $^{*}p < 0.05$.

have shown that similarly to KIR2DL4, triggering of the inhibitory KIR2DL2/3 and NKG2A receptors also lead to the secretion of those cytokines, which represents a novel and intriguing finding. Indeed, to our knowledge, this is the first description that the triggering of inhibitory NK receptors on IL-15-induced CD8+NKR+ T cells could by themselves trigger such cytokine secretion pattern. It is known that KIR2DL4 exhibits structural features of both activating and inhibitory KIR, and IFN-γ secretion has been shown to be dependent on its charged arginine residue (Miah et al. 2008). Considering that the other inhibitory NKRs lack the charged aminoacid residue, it is possible to speculate that they may use alternative signaling pathways leading to cytokine production, namely involving ERK and PI3K, based on the integration of some previous knowledge (Wang et al. 2005; Wu et al. 2001; Kim et al. 2007; Ogasawara et al. 2002; Parihar et al. 2005; Young and Uhrberg 2002). Importantly, IL-15 has been shown to activate the ERK and PI3K-Akt pathways (Budagian et al. 2006), pointing to the possibility that the inhibitory NKRs expressed by CD8+ T cell in the presence of IL-15 could make use of alternative pathways that will lead in turn to cytokine secretion.Noteworthy, the cytokine secretion pattern observed in this study is reminiscent of a Tc1 (IFN-γ and TNF-α) and Tc2 (IL-10) profile (Vukmanovic-Stejic et al. 2000), suggesting that IL-15-induced CD8+ NKR+ T cells could display an immunoregulatory role. While IL-10 is a cytokine with well known anti-inflammatory and immunoregulatory functions (Moore et al. 2001), involved in the generation of T regulatory cells (Horwitz et al. 2003), IFN-γ is strongly associated with pro-inflammation and immune activation (Billiau and Matthys 2009). However, IFN-γ has also been reported to be a cytokine capable of suppressing T cell responses and inducing regulatory T cells (Billiau and Matthys 2009). In addition, a relationship between NKG2A and IFN-γ has been previously described (Sheu et al. 2005). Furthermore, exogenously added IFN-γ has been shown to protect ovarian carcinoma cell lines from CTL lysis via a NKG2A-dependent mechanism, by increasing the expression of HLA-E ligands on the target cells (Malmberg et al. 2002), and to facilitate viral evasion from NK killing by a similar mechanism (Cerboni et al. 2001). In this context, it is tempting to speculate that triggering of inhibitory NKRs on IL-15-induced CD8+NKR+ T cells could lead to a robust IFN-γ production that in turn would lead to an increase in the amount of their cognate ligands in target cells, pointing to inhibitory NKRs themselves as part of an intricate mechanism that could ultimately result in impaired antiviral

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and anti-tumoral CD8+ T cell function. Moreover, a population displaying a memory phenotype characterized by lack of CD28 and expression of CD56 and capable to produce IFN-γ, TGF-β and IL-10, has been described within suppressor CD8+ T cells (Niederkorn 2008; Suzuki et al. 2008). Also, TNF-α and IL-1β have been shown to induce apoptosis and suppression of immune cells, suggesting that they may also display a suppressor activity (Lenardo et al. 1999; Vukmanovic-Stejic et al. 2001). Accordingly, the possibility that IL-15 is generating effector-memory CD8+ CD56+ T cells co-expressing inhibitory NKRs and endowed with suppressor activity is a plausible scenario that deserves further investigation. Indeed, a recent report describing that the IL-10/IFN-γ pathways are essential to the potent immunosuppressive activity of cultured CD8+ NK-like T cells towards naïve responder T cells (Zhou et al. 2008) supports this assumption.Finally, our results strengthen our previous hypothesis that IL-15-differentiated CD8+CD56+ T cells could account for the high percentage of NK-like T cells present in human livers. Indeed, we have found that they have phenotypic and functional similarities, namely being effector-memory CD8+CD56+ T cells expressing other NKRs, (Norris et al. 1999; Tu et al. 2007) endowed with capacity to display TCR-mediated and NK-like cytotoxicity (Doherty et al. 1999; Norris et al. 1999) and to produce Tc1 and Tc2 cytokines after stimulation (Doherty et al. 1999). In this context, it can be proposed that IL-15 could function as a putative regulator of NKR expression which in turn may shape CD8+ T cell functional phenotype in a tissue-specific manner.In summary, it is possible to speculate that IL-15 enriched microenvironments could induce CD8+ T cell differentiation into effector NK-like T cells by the acquisition of functional NKRs capable of modulating both cytotoxicity and cytokine release. The outcome of cytokine secretion by triggering of inhibitory NKRs could suggest that CD8+NKR+ T cells might also have regulatory functions, bridging innate and acquired immunity through an intricate NK receptor triggering balance.

Conflict of interest

The authors declare no competing financial interests.

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