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Prostaglandin E_2 induces the expression of functional inhibitory CD94/NKG2A receptors in human $CD8^+$ T lymphocytes by a cAMP-dependent protein kinase A type I pathway

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Abstract

The CD94/NKG2A heterodimer is a natural killer receptor (NKR), which inhibits cell-mediated cytotoxicity upon interaction with MHC class I gene products. It is expressed by NK cells and by a small fraction of activated T cells, predominantly of $CD8^+$ phenotype. Abnormal upregulation of the CD94/NKG2A inhibitory NKR on cytotoxic T cells (CTLs) could be responsible for a failure of immunosurveillance in cancer or HIV infection. In an attempt to identify the mechanisms leading to inhibitory NKR upregulation on T cells, we analyzed the expression of the CD94/NKG2A heterodimer on human CTLs activated with anti-CD3 mAb in the presence of PGE_2 or with 8-CPT-cAMP, an analogue of cyclic AMP. As previously described, anti-CD3 mAb-mediated activation induced the expression of CD94/NKG2A on a small fraction of $CD8^+$ T cells. Interestingly, when low concentrations of PGE_2 or 8-CPT-cAMP were present during the culture, the proportion of $CD8^+$ T cells expressing CD94/NKG2A was two- to five-fold higher. This upregulation was partially prevented by PKA inhibitors, such as KT5720 and Rp-8-Br-cAMP (type I selective). We also report that cAMP induces upregulation of NKG2A at the mRNA level. We further demonstrated that cross-linking of CD94 on $CD8^+$ T cells expressing the CD94/NKG2A heterodimer inhibits their cytotoxic activity in a bispecific antibody redirected lysis assay. Our findings clearly demonstrate that the PGE_2/cAMP/PKA type I axis is involved in the expression of CD94/NKG2A receptor on human $CD8^+$ T lymphocytes.

Keywords: Human; T lymphocytes; Cell surface molecules; Cytotoxicity

1. Introduction

Prostaglandin E_2 (PGE_2) is abundantly secreted by various types of cancer cells and by tumor-infiltrating macrophages (reviewed in [1]). PGE_2 interferes with the immune function at different levels and is therefore postulated to play a central role in the establishment of tumor-associated immunosuppression (reviewed in [2,3]). Several recent studies have shown the dramatic effect of PGE_2 on the differentiation and function of dendritic cells [4]. PGE_2 also selectively inhibits Th1 polarization of $CD4^+$ T helper cells [5–7]. With regard to the important role of cytotoxic T cells in the control of tumor growth, it is surprising that the effect of PGE_2 on CTL function has hardly been investigated so far.

The CD94/NKG2 heterodimer is a C-type lectin receptor formed by the covalent association of CD94, a type II integral membrane protein with a very short non-signaling intracytoplasmic tail [8] and one of the NKG2 molecules [9,10]. To generate a functional receptor, CD94 is disulfide linked with a member of the NKG2 family, namely NKG2A, -B, -C or -E [11]. In humans, CD94/NKG2A interacts with complexes of non-classical HLA-E molecules bound to a peptide derived from the leader sequence of a classical HLA class I protein [12,13]. The intracellular domain of NKG2A contains immunoreceptor tyrosine-based inhibition motifs (ITIMs), responsible for transducing inhibitory signals [14]. All other NKG2 members lack ITIMs and are linked to transmembrane proteins, such as DAP10 and DAP12 which contain immunoreceptor tyrosine-based activating motifs and therefore transduce activating signals [15–17].

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CD94/NKG2A is normally expressed on most NK cells whereas less than 5% of peripheral $CD8^+$ T cells are positive. In mice, various experimental infections with bacteria or viruses strongly increase the proportion of CD94/NKG2A positive cells within antigen specific $CD8^+$ T cells [18]. In humans, chronic infection with HIV-1 also increases the proportion of $CD8^+$ T cells expressing CD94/NKG2A [19,20]. Recent evidence suggests that Vδ1 γδ $CD8^+$ T cells could preferentially be involved in the process [21]. $CD8^+$ T cells infiltrating primary melanoma also upregulate CD94/NKG2A while T cells expressing identical Vβ variable regions do not express the inhibitory receptor when they are isolated from the sentinel lymph nodes [22].

The molecular mechanisms regulating CD94 and NKG2A expression in infection and cancer remain unclear. From in vitro experiments, it was suggested that TCR engagement is required for CD94/NKG2 expression [23] while cytokines, such as IL-10 and IL-15 as well as TGF-β could also positively influence the expression of the inhibitory receptor [24–26].

In view of the role of PGE_2 in immunosuppression associated with cancer and chronic viral infection, we have postulated that this mediator could be involved in the regulation of CD94/NKG2A expression by human $CD8^+$ T cells by a pathway involving cyclic AMP and type I protein kinaseA.

2. Results

2.1. PGE_2 and 8-CPT-cAMP, a cAMP analogue strongly increase the proportion of $CD8^+$ T cells which express the CD94/NKG2A inhibitory receptor after anti-CD3 mAb activation

$CD8^+$ T lymphocytes cells were negatively purified from fresh human PBLs then activated with an immobilized anti-CD3 mAb in the presence of IL-2 (50 U/ml) with or without PGE_2 (0.08 μM). At day 0, 1 to 6% of $CD8^+$ T cells expressed CD94/NKG2A. This proportion increased during the culture to culminate at day 7 around 8% in most experiments. Interestingly, when PGE_2 was present in the culture, there was a two- to three-fold increase of the proportion of CD94/NKG2A positive cells within $CD8^+$ T lymphocytes (Fig. 1A and B). This effect was clearly dose dependent with a proportion of CD94/NKG2A positive cells reaching a plateau for a concentration of PGE_2 around 1 μM. Above this concentration, toxic effects were observed in the culture (not shown). Since PGE_2 receptors (mostly EP2 in T lymphocytes [27,28]) belong to the seven-transmembrane-domain receptor family and are coupled via G proteins to the cAMP-signaling route, we investigated the effect of 8-CPT-cAMP, an analogue of cAMP on the expression of CD94/NKG2A. As previously reported [29], 8-CPT-cAMP inhibited cell proliferation in a dose dependent manner (data not shown). Considering this inhibition curve, we used for most experiments 8-CPT-cAMP at a concentration of 5×10^{-5} M which inhibits cell proliferation by 50% without affecting cell viability. At this concentration, 8-CPT-cAMP increased four- to five-fold the proportion of CD94/NKG2A positive $CD8^+$ T cells, twice as much as what was observed with PGE_2, suggesting that the induction of CD94/NKG2A is cAMP dependent (Fig. 1C and D). The fact that PGE_2 was less efficient than 8-CPT-cAMP to induce CD94/NKG2A could be due to the activity of the phosphodiesterases which affect the endogenous level of the cAMP induced by PGE_2 but not 8-CPT-cAMP which is not significantly hydrolyzed by the PDEs [30]. Interestingly, when $CD8^+$ T lymphocytes were activated with anti-CD3 and anti-CD28 mAb a significant decrease of the percentage of CD94/NKG2A positive cells was observed (Fig. 1E).

Since a small fraction of $CD8^+$ T cells was already positive for CD94/NKG2A at day 0, it was necessary to establish if the induction of the inhibitory receptor during the culture with cAMP or PGE_2 was due the preferential proliferation of this minor subset or to "recruitment" of CD94/NKG2A negative cells. To address this question, we depleted all CD94/NKG2A positive cells present within the $CD8^+$ T cell subset at day 0 and incubated them with the anti-CD3 mAb and 8-CPT-cAMP (5×10^{-5} M) (Fig. 2). After 7 days of culture, a large fraction of $CD8^+$ T cells had become CD94/NKG2A positive in the presence of the cyclic AMP analogue, demonstrating that expression of CD94 and NKG2A was induced on cells initially devoid of the inhibitory receptor.

Previous reports have shown that most peripheral Vγ9Vδ2 T cells express an intracellular pool of CD94/NKG2A receptors that is translocated to the cell surface upon activation by TCR triggering or IL-2 [31]. Furthermore, Vδ1 γδ $CD8^+$ T cells express CD94/NKG2A in patients infected with HIV-1 [32]. It was therefore possible that γδ $CD8^+$ T cells largely contributed to the amplification observed in our cultures in the presence of cAMP or PGE_2. We measured the proportion of γδ T cells within CD94/NKG2A $CD8^+$ T cells at day 0 and at day 7 when the proportion of T cells expressing the inhibitory receptor was culminating. At day 0, a large fraction of CD94/NKG2A positive cells present in the $CD8^+$ subset were γδ T cells but this proportion sharply declined at day 7, demonstrating that αβ $CD8^+$ T cells were induced to express the inhibitory receptor by cAMP analogues (Fig. 3 and Table 1).

2.2. Specific inhibition of c-AMP dependent protein kinase A type I partially blocks the effect of PGE_2 and 8-CPT-cAMP on CD94/NKG2A expression by $CD8^+$ T lymphocytes

To further demonstrate that the effect of PGE_2 and 8-CPT-cAMP on CD94/NKG2A expression is related to

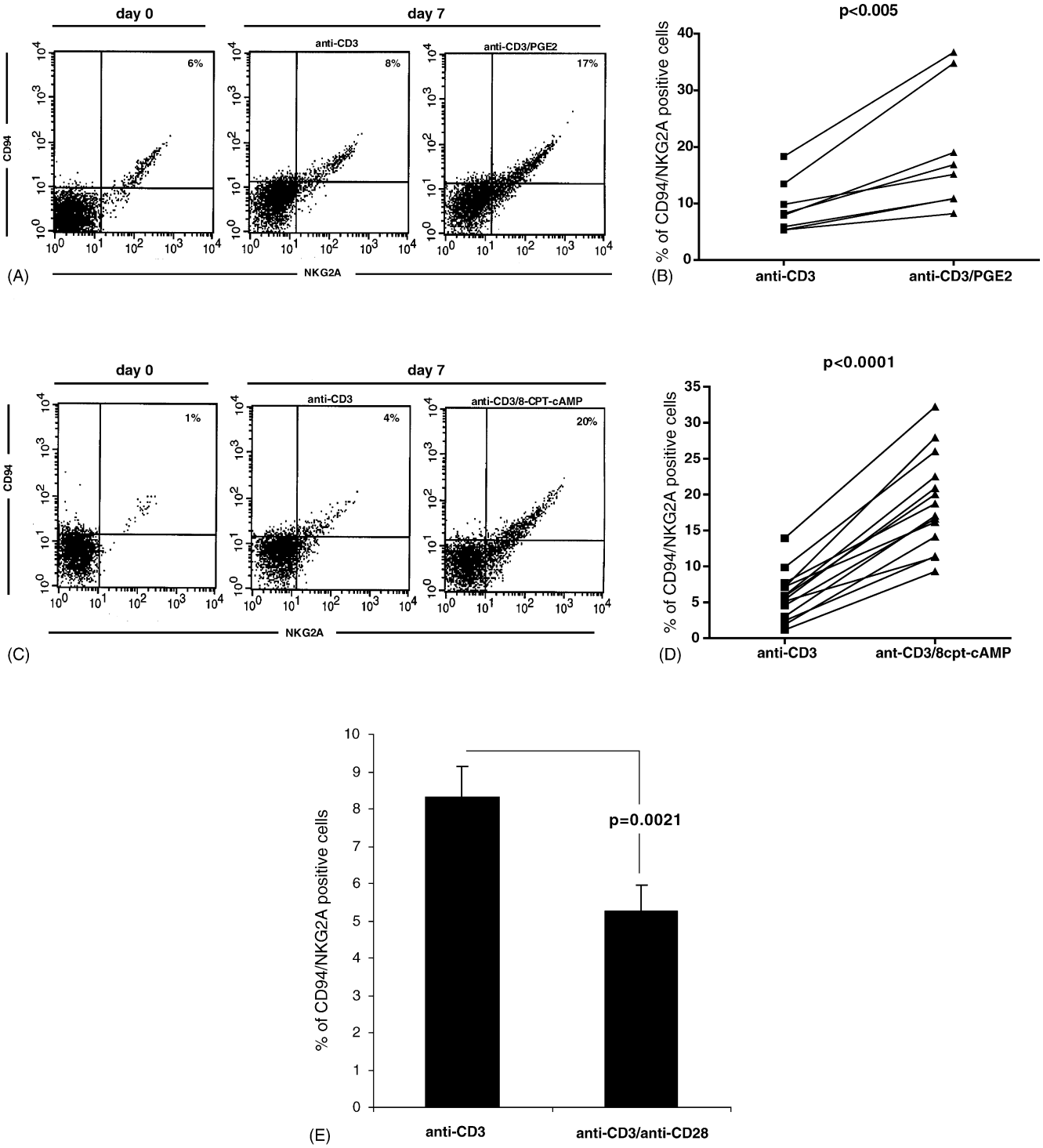

Fig. 1. PGE_2 and 8-CPT-cAMP, an analogue of cAMP, strongly increase the proportion of $CD8^+$ T cells expressing the CD94/NKG2A inhibitory receptor after anti-CD3 mAb activation. Representative flow cytometry diagrams showing the expression of CD94/NKG2A in human $CD8^+$ T lymphocytes activated with anti-CD3 in the presence or absence of PGE_2 (0.08 μM) (A) or the cAMP analogue, 8-CPT-cAMP (5×10^{-5} M) (C)). $CD8^+$ T cells were negatively sorted out of normal human PBL then triple stained for the surface molecules CD8, CD94 and NKG2A. The percentage of $CD94^+$ $NKG2A^+$ cells within gated $CD8^+$ T cells was calculated and is shown in each dot plot. Numbers in the upper right quadrants refer to the percentage of NKG2A/CD94 positive cells within the CD8 T^+ cell subset. Paired *t*-test was used to compare the percentage of $CD94^+/NKG2A^+$ cells at day 0 and at day 7 of culture with anti-CD3 and PGE_2 (eight different donors)(B) and with 8-CPT-cAMP (14 different donors) (D). (E) $CD8^+$ T lymphocytes were activated with anti-CD3 in the presence or absence of anti-CD28 mAb. After 7 days of culture, the percentage of CD94/NKG2A positive cells within the $CD8^+$ T cell fraction was determined by flow cytometry. A representative experiment from a single donor is shown, with means ± S.D. from triplicates. Similar observations were made in three other experiments.

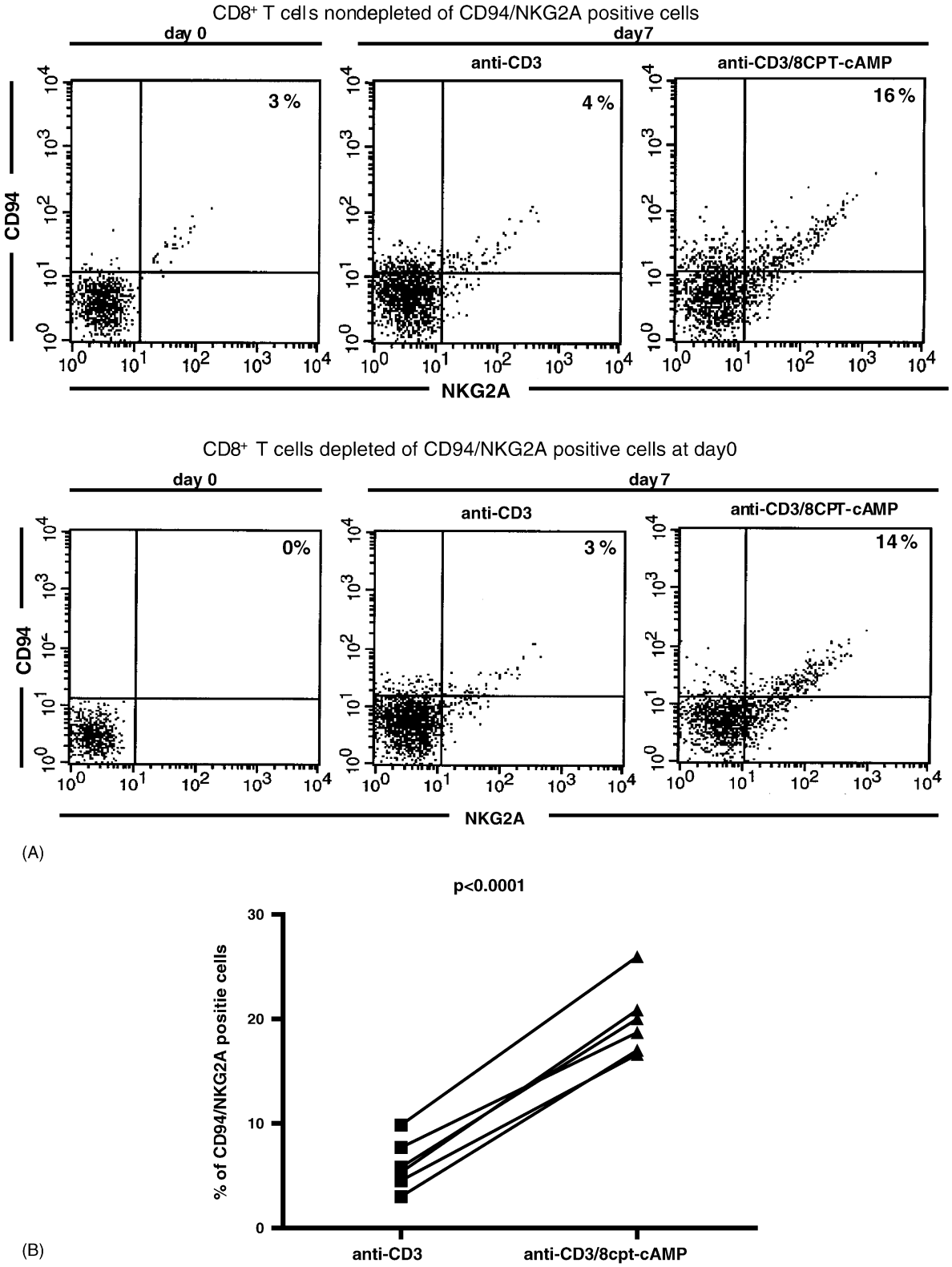

Fig. 2. 8-CPT-cAMP induces de novo expression of the CD94/NKG2A heterodimer. (A) $CD94^+/NKG2A^+$ cells were depleted from $CD8^+$ T lymphocytes by FACS sorting at day 0 then activated for 7 days with anti-CD3 mAb alone or in association with 8-CPT-cAMP (5×10^{-5} M). $CD8^+$ T cells were gated and were analyzed by flow-cytometry for CD94 and NKG2A expression after triple immunostaining with anti-CD8-Percp, anti-CD94-FITC and anti-NKG2A-PE. (B) Paired *t*-test comparing the percentage of $CD94^+/NKG2A^+$ cells among the $CD8^+$ T cells initially depleted (day 0) of CD94/NKG2A positive cells and kept in culture for 7 days with both anti-CD3 and 8-CPT-cAMP vs. anti-CD3 alone (six different donors).

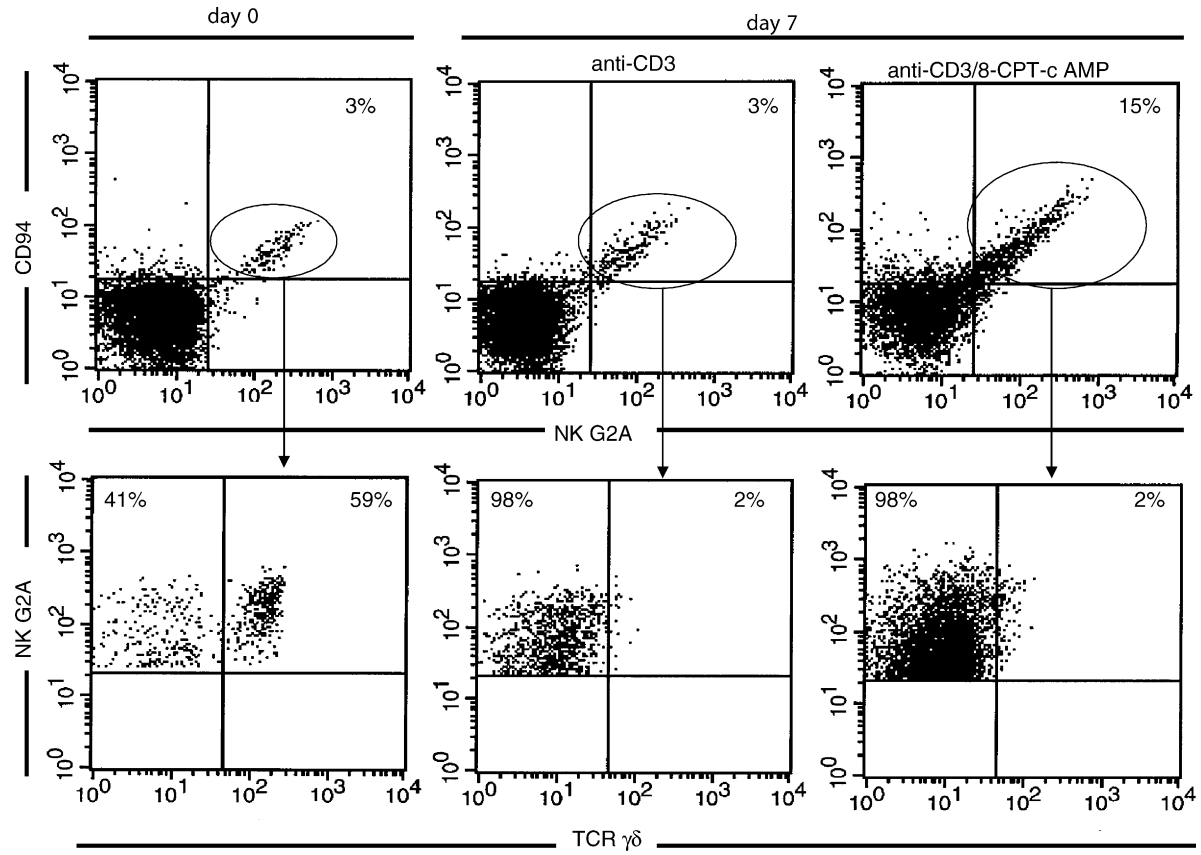

Fig. 3. 8-CPT-cAMP induced NKG2A/CD94 CD8$^+$ T cells express an αβ TCR. The proportion of γδ T cells within CD94$^+$/NKG2A$^+$ CD8$^+$ T lymphocytes was measured by flow cytometry at days 0 and 7 of culture in the presence of anti-CD3 alone or anti-CD3 and 8-CPT-cAMP (5×10^{-5} M). CD8$^+$ T cells were gated as described in Figs. 1 and 2 and the cells were analyzed for CD94 and NKG2A (upper panel) and for the TCR γδ (lower panel).

cAMP induction and to clarify whether PKA type I is involved in this pathway, we used Rp-8-Br-cAMP, an analogue of cAMP which inhibits cAMP-dependent protein kinase I [33]. CD8$^+$ T lymphocytes were incubated as above with anti-CD3 mAb and IL-2 and the expression of CD94/NKG2A was induced with PGE_2 (0.08 μM). When Rp-8-Br-cAMP (250 μM) was also present in the culture, the inducing effect of PGE_2 was much lower, corresponding in most experiments to an inhibition of more than 50% of the PGE_2 effect (Fig. 4A and B). In order to rule out a non-specific inhibitory effect of Rp-8-Br-cAMP on the induction of inhibitory receptors, we used KT5720 another inhibitor of PKA which acts on the catalytic site. KT5720 (2.5×10^{-5} M) strongly inhibited the induction of CD94/NKG2A by 8-CPT-cAMP (5×10^{-5} M) confirming the role of PKA in this induction (Fig. 4D).

Interestingly, both Rp-8-Br-cAMP and KT5720 also partially prevented the induction of CD94/NKG2A expression induced by TCR triggering alone in the absence of cAMP inducing agents (Fig. 4). This observation suggests that PKA type I also plays a role in the induction of CD94/NKG2A after physiological engagement of the TCR.

2.3. PGE_2 and cyclic AMP analogues induce an increase of NKG2A at the mRNA level

Previous reports have shown that surface homeostasis of CD94/NKG2A is influenced by recycling from intracellular compartments [34]. Furthermore, membrane translocation of CD94/NKG2A in response to TCR engagement has been shown for $V\gamma_9V\delta_2$ T cells [35]. It was therefore

Table 1
Percentage of αβ and γδ TCR$^+$ T cells among 8-CPT-cAMP induced CD94$^+$/NKG2A$^+$ CD8$^+$ T cells

	Day 0	Day 7	
		Anti-CD3	Anti-CD3/8cpt-cAMP
Percentage of CD3$^+$ CD8$^+$ αβ$^+$ NKG2A$^+$ cells ($n = 3$)	54 ± 13.8	95.77 ± 2.47	98.09 ± 0.85
CD3$^+$ CD8$^+$ γδ$^+$ NKG2A$^+$ cells ($n = 3$)	45 ± 13.8	4.22 ± 2.47	1.9 ± 0.85

Negatively sorted CD8$^+$ T cells were incubated with anti-CD3 alone or in combination with 8-CPT-cAMP (5×10^{-5} M). After 7 days of culture, the proportion of γδ T cells within the CD8$^+$ CD94$^+$/NKG2A$^+$ fraction was measured by flow cytometry at both days 0 and 7. Each value presented represents the mean ± S.D. of three independent experiments performed on three different healthy donors.

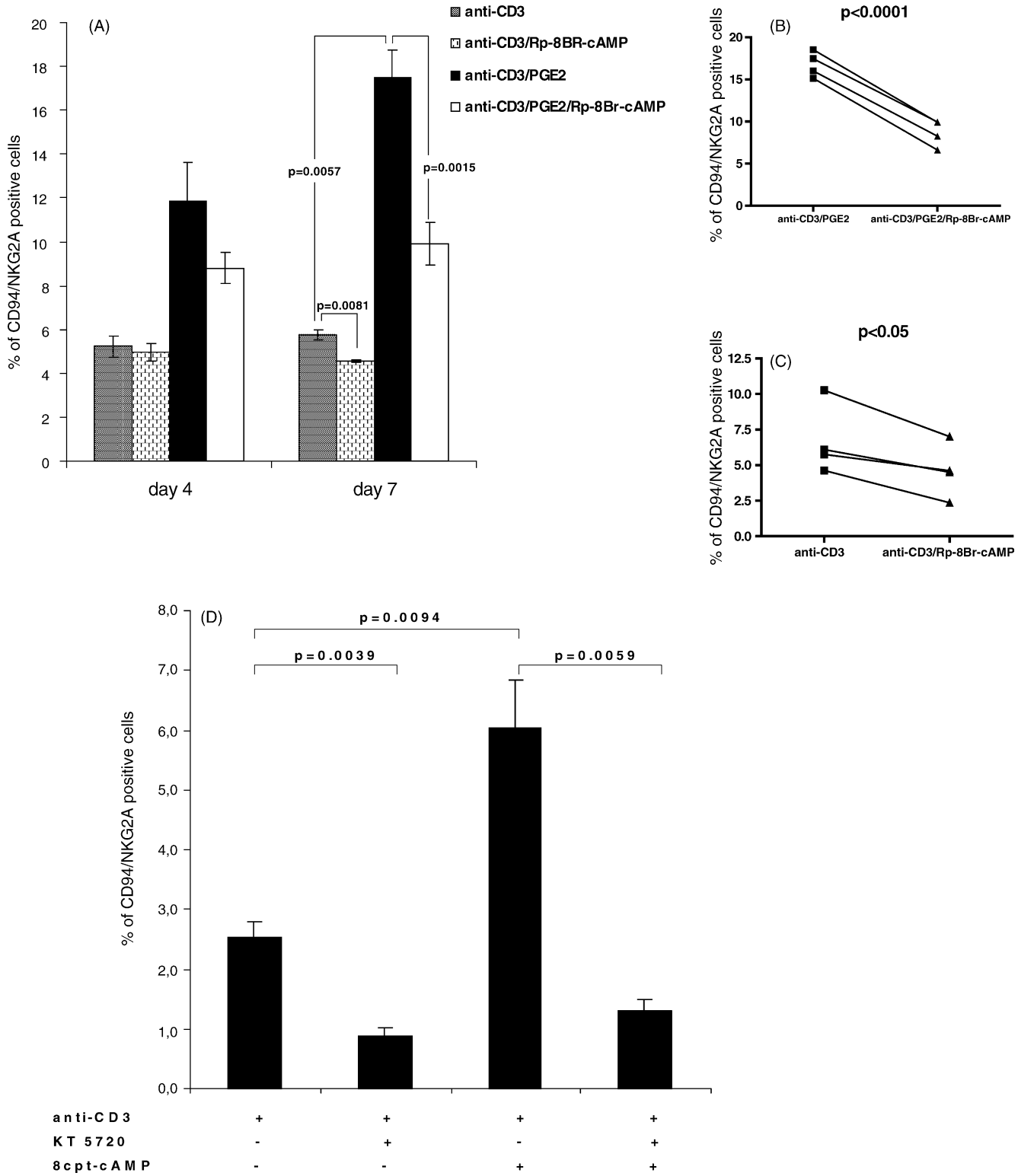

Fig. 4. Effect of PKA inhibitors on PGE_2 and 8-CPT-cAMP-induced CD94/NKG2A expression on $CD8^+$ T lymphocytes. (A) Purified $CD8^+$ T lymphocytes were activated in the presence of anti-CD3 alone, anti-CD3/PGE_2 or anti-CD3, PGE_2 (0.08 μM) and Rp-8-Br-cAMP (250 μM). After 4 and 7 days of culture, cells were analyzed by flowcytometry after triple immunostaining for CD8, CD94 and NKG2A. The percentage of NKG2A/CD94 positive cells among the $CD8^+$ T cell population is shown. A representative experiment from a single donor is shown with means ± S.D. from triplicates. The effect of Rp-8-Br-cAMP was observed in all donors tested. Paired *t*-test was used to compare the percentage of CD94/NKG2A positive cells in cultures with anti-CD3/ PGE_2 vs. anti-CD3/PGE_2/Rp-8-Br-cAMP (B) (four different donors) and anti-CD3 vs. anti-CD3/Rp-8-Br-cAMP (C) (four different donors). (D) $CD8^+$ T lymphocytes were activated as above with anti-CD3 and 8-CPT-cAMP (5×10^{-5} M) in the presence of KT5720 (2.5×10^{-5} M). The percentage of CD94/NKG2A positive cells was analyzed by flow cytometry. A representative experiment is shown with means ± S.D. from triplicates. Similar results were observed in two independent experiments performed with two different donors.

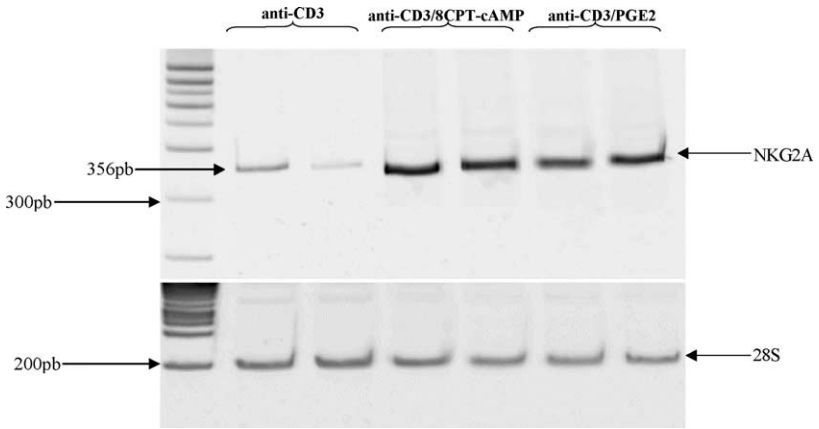

Fig. 5. PGE_2 and 8-CPT-cAMP induce an increase in the total mRNA level of NKG2A. A representative example of electrophoretic pattern of RT-PCR products of NKG2A mRNA performed with primers specific for NKG2A and for 28S rRNA. Total RNA was extracted from sorted $CD8^+$ T lymphocytes activated for 7 days with either anti-CD3, anti-CD3/8-CPT-cAMP (5×10^{-5} M) or anti-CD3/PGE_2 (0.08 μM).

important to determine if PGE_2 and cAMP analogues were influencing the mRNA level of CD94/NKG2A.

$CD8^+$ T lymphocytes were purified and activated as described above in the presence of PGE_2 (0.08 μM) or 8-CPT-cAMP (5×10^{-5} M). Total RNA was extracted and the level of expression of NKG2A mRNA was determined by semi-quantitative RT-PCR. As shown in Fig. 5, NKG2A mRNA levels were 8- to 10-fold higher in $CD8^+$ T lymphocytes incubated with 8-CPT-cAMP and anti-CD3 mAb compared to anti-CD3 alone and slightly lower with PGE_2 and anti-CD3 mAb (Fig. 5).

2.4. The CD94/NKG2A receptors induced by exposition to cyclic AMP analogues are functional and their engagement inhibits bispecific antibody-redirected cell lysis

Certain conditions are associated with the expression of non-functional CD94/NKG2A heterodimers which do not inhibit cytotoxic activity after engagement [36]. In order to evaluate the function of the CD94/NKG2A receptors expressed by $CD8^+$ T cells after exposure to PGE_2 or cyclic AMP analogues, we used bispecific antibody anti-CD3/anti-EGFR (M26.1) to redirect the cytotoxic activity against the CaSki cell line which expresses EGF receptors. Purified $CD8^+$ T cells were activated with anti-CD3 mAb and IL-2 together with 8-CPT-cAMP (5×10^{-5} M). After 5 days, bispecific antibody redirected cytotoxic activity was tested against Caski cells (Fig. 6A). There was no significant lysis of CaSki in the absence of the bispecific antibody. In contrast, addition of M26.1 induced a very strong lysis of CaSki by $CD8^+$ T cells with no significant difference between cells cultivated with or without the cyclic AMP analogue. This indicates that the cytotoxic machinery of CTLs is fully competent despite the fact that their differentiation occurred in the presence of 8-CPT-cAMP. Interestingly, a significant inhibition was obtained

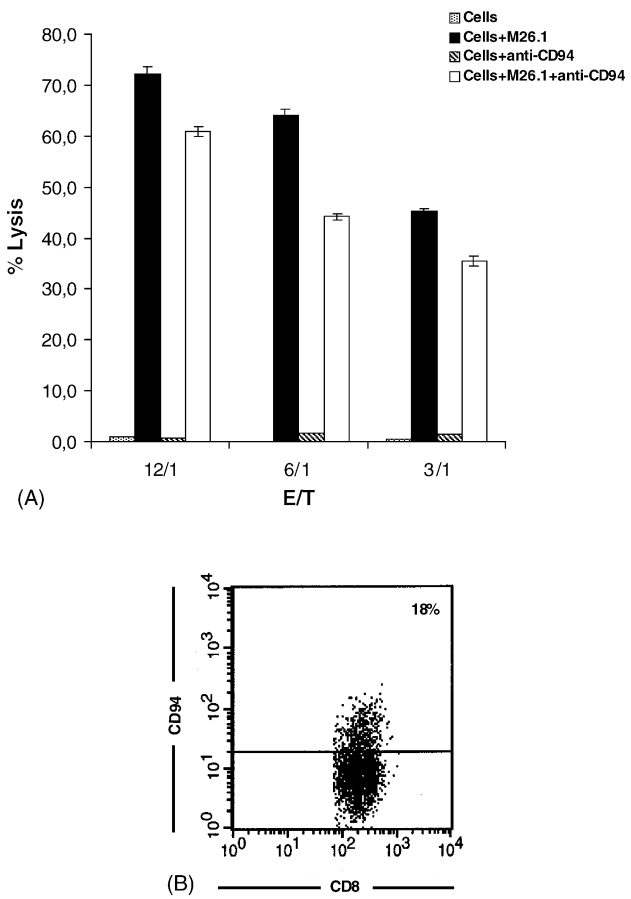

Fig. 6. CD94/NKG2A inhibits $CD8^+$ T lymphocytes function in a bispecific antibody mediated target cell lysis test. (A) Human $CD8^+$ T lymphocyte was activated with immobilized anti-CD3 mAb in the presence of 8-CPT-cAMP (5×10^{-5} M). The cells were harvested at day 5 and analyzed in a 4 h ^{51}Cr-release assay for their cytolytic activity against $EGFR^+$ CaSki target cells in the presence of anti-CD3/EGFR bi-mAb M26.1 and/or an anti-CD94 mAb. The data shown is a representative experiment with means ± S.D. from triplicates at 12:1, 6:1 and 3:1 $CD8^+$ T/CaSki ratio. (B) Dot plot showing the percentage of CD94 among the purified CD8 T cell population used for the experiment shown in panel A.

when the CD94/NKG2A heterodimer was engaged with an anti-CD94 mAb. The inhibition was partial but always correlated nicely with the proportion of CTLs expressing the heterodimer in culture (Fig. 6B).

3. Discussion

The role of PGE_2 in the host response to tumor growth is of paramount importance. PGE_2 has been shown to regulate cellular immune responses through different distinct EP receptors on different cell populations: EP2 receptors directly inhibit T cell proliferation while EP2 and EP4 receptors regulate antigen presenting cells functions [27,38]. Recent evidence demonstrates that mice lacking PGE_2 receptor EP2 exhibit significantly attenuated tumor growth and longer survival when challenged with MC26 or Lewis lung carcinoma cells due to stronger T cell responses directed against tumor antigens [37]. We describe for the first time that short-term culture of purified $CD8^+$ T cells with PGE_2 sharply increases the proportion of cells which acquire expression of CD94/NKG2A, an inhibitory receptor. This upregulation was a rather selective effect since the cytolytic potential of T cells was not affected unless the inhibitory receptor was engaged.

Since higher expression of COX-2 and increased secretion of PGE_2 have been described in various conditions, such as cancer [39–42] and viral infection (including HIV-1) [43], we suggest that the CD94/NKG2A upregulation on T cells documented in these conditions is at least in part related to the exposure of T cells to PGE_2 in tumor sites or lymphoid microenvironment. Obviously other mediators, such as TGF-β could be involved. In our experimental conditions, the effect of TGF-β on CD94/NKG2A induction was always modest and significantly lower than the one observed with PGE_2 (data not shown). In contrast, Bertone et al. observed that low concentrations of TGF-β could efficiently induce CD94/NKG2A in cultures of human unsorted PBL activated with bacterial superantigens. TGF-β has been shown to upregulate COX-2 expression in various conditions [44,45], we therefore postulate that part of the CD94/NKG2A inducing effects previously attributed to TGF-β could involve enhanced secretion of PGE_2 by macrophages.

Activation of PKA type I by increased intracellular levels of cAMP is involved in the normal regulation of antigen receptor signaling and contributes to the homeostasis of the immune responses (reviewed in [46]). PKA regulates the immune function at multiple levels: it inhibits TCR dependent proximal signaling and influences the expression of several genes involved in T cell activation [47,48]. Our results suggest that PKA also contributes to the homeostasis of cytotoxic responses by regulating CD94/NKG2A expression on the cell surface after TCR engagement.

Interestingly, not all $CD8^+$ T cells upregulate CD94/NKG2A after TCR engagement and it has been suggested that the ability to upregulate the inhibitory heterodimer is a clonal attribute [49]. This is consistent with our observations where some $CD8^+$ T cells remain negative for CD94/NKG2A even after several days of culture in the presence of anti-CD3 and PGE_2 or cAMP analogues. We propose a model in which the intracellular level of cAMP induced during the first TCR engagement of a naïve $CD8^+$ T cell clonally determines the commitment to re-express CD94/NKG2A after activation. CD28 costimulation induces recruitment of a cAMP phosphodiesterase, PDE4, leading to reduced cellular cAMP levels [50]. Initial TCR engagement of a naïve $CD8^+$ T cell in the absence of optimal CD28 costimulation would therefore lead to higher cAMP levels and to a clonal commitment to re-express CD94/NKG2A. Accordingly, $CD28^-$ T cell clones usually upregulate CD94/NKG2A [51]. Furthermore, we have observed that the proportion of $CD8^+$ T cells expressing CD94/NKG2A after TCR engagement was significantly lower if an anti-CD28 mAb was also present in the culture (Fig. 1E). Initial stimulation of $CD8^+$ naïve T cell in the presence of a high concentration of cAMP inducing agents, such as PGE_2 or catecholamines could also result in clonal inhibitory commitment.

Taken together, our results suggest that administration of COX-2 inhibitors or PKA type I antagonists could increase the therapeutic potential of tumor vaccines by limiting the fraction of CTLs committed to express inhibitory receptors after TCR engagement in tumor sites.

4. Materials and methods

4.1. Antibodies and reagents

The anti-CD3/anti-EGF-R bimAb M26.1 was produced as previously described [52]. Fluorescein isothiocyanate (FITC)-labeled: HIT3a (anti-CD3), HP-3D9 (anti-CD94), (anti-TCR γ/δ), peridinin chlorophyll protein (Percp)-labeled SK1 (anti-CD8) and purified UCHT1 (anti-CD3) were purchased from Pharmingen (BD, San Diego, CA). Phycoerythrin (Pe)-labeled Z199 (anti-NKG2A) and purified HP-3B1 (anti-CD94) were purchased from Immunotech (Immunotech, Marseille, France). Recombinant IL-2 was purchased from Biosource (Biosource, CA). 8-CPT-cAMP, PGE_2 and KT5720 were both from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Rp-8-Br-cAMP was from Biolog (Biolog Life sciences, Bremen, Germany).

4.2. Isolation of $CD8^+$ T lymphocytes and cell culture

Human peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density centrifugation from enriched lymphocytes preparations (buffy coats) obtained from Liège University hospital blood bank. $CD8^+$ T lym-

phocytes were negatively sorted by MACS using the StemCell Antibodies cocktail containing monoclonal antibodies to the following human cell surface antigens: CD4, CD14, CD16, CD19, CD56 GlycophorinA; (StemCell Technologies, Meylan, France) according to the instructions of the manufacturer. Purity of sorted populations was higher than 85% as monitored by flow cytometry using Percp-conjugated anti-CD8 and FITC-conjugated anti-CD3 (Pharmingen-BD, San Diego, CA).

5–7 $\times$ 10^5 $CD8^+$ T lymphocytes were then incubated in 24-well plates with RPMI medium supplemented with 10% FCS, 30U/ml of penicillin and streptomycin and 2 mM of L-glutamine. The cells were activated with human recombinant IL2 (50 U/ml) and immobilized anti-CD3 antibody. 8-CPT-cAMP (5×10^{-5} M), PGE_2 (0.08 μM) Rp-8-Br-cAMP (250 μM) and KT 5720 (2.5×10^{-5} M), when used, were added to the cells 30 min before the activation.

4.3. Proliferation assay

Proliferation of PBMC was measured 96 h after stimulation with anti-CD3 antibody in 96-well flat-bottom culture plates using 2×10^5 cells/well. 0.4 μCi/well of thymidine [methyl-^{3}H] (Amersham, Uppsala, Sweden) was added for the last 4 h. Cells were washed and harvested with a cell harvester onto glass fiber filters and counted in a scintillation analyzer (Packard Top Count, Meriden, CT).

4.4. Flow cytometric analysis

Triple-staining was performed with fluorescent conjugated antibodies. The following monoclonal antibodies were used: Percp-conjugated anti-CD8, FITC-conjugated anti-CD94 and PE-conjugated anti-NKG2A. The phenotype was performed on (1–5) $\times$ 10^5 cells following standard protocols. The cells were analyzed for fluorescence intensity on a FACSvantage with CellQuest software (BD Biosciences, San Diego, CA).

4.5. RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from $CD8^+$ T lymphocytes using the High Pure RNA Isolation Kit (Roche Diagnostics, Germany) according to the procedures supplied by the manufacturer. Reverse transcription was performed on 10 ng of total RNA using the RT-PCR kit (Applied Biosystem, Foster City, CA) in a total reaction volume of 20 μl following the conditions provided by the manufacturer's protocol. Briefly, RNA was mixed with the rTth DNA polymerase in the presence of reverse transcriptase buffer, $MnCl_2$ solution, dNTPs and primers for the reverse transcription. The reaction was stopped by placing the tubes on ice. The PCR reaction was performed by adding chelating buffer, $MgCl_2$ and primers for the PCR reaction to each sample. The primers used for PCR reactions were 5′-AGCAGTCATCATCCATGGGTG-3′ (sens); and 5′-AAAATGAGCCCGACACAAATG-3′ (anti-sens) corresponding to the nucleotides 731–751 and 1066–1086, respectively, of the published sequence of NKG2A mRNA [53]. Quantification of NKG2A mRNA included normalization to amplification of the 28S message. Primers used for the 28S sequence were 5′-GTTCACCCACTAATAGGGAAC GTGA-3′ (sense) and 5′-GGATTCTGACTTAGAGGC GTTCAGT-3′ (anti-sense). All primers were synthesized by Eurogentec (Eurogentec, Liège, Belgium). Cycling conditions were as follow: for NKG2A, 94 °C for 15 s, 60 °C for 20 s and 72 °C for 23 s; for 28S, 94 °C for 15 s, 66 °C for 22 s and 72 °C for 10 s. The number of cycles was determined experimentally and was 30 for NKG2A and 19 for 28S. A negative control without RNA was included for each RT-PCR reaction. Amplified PCR products were analyzed on a polyacrylamid gel electrophoresis and stained with Syber Green (Roche Diagnostic, Belgium). Transcript levels were analyzed by Fluor-STM Multimager analyzer (Bio-Rad).

4.6. ^{51}Cr-release assay for cytolytic activity

Target cells (CaSki) were labeled with ^{51}Cr (Amersham) (100 μCi/10^6cells) for 1 h then washed. Meanwhile, 5 days pre-activated (with anti-CD3, and IL-2 in the presence of 8-CPT-cAMP (5×10^{-5} M)) effector cells ($CD8^+$T selected cells), were incubated with 5 μg/ml Ab (M26.1 and/or anti-CD94) for 30 min in complete RPMI medium. The two cell populations were mixed at different E:T ratio and incubated for 4 h at 37 °C, 5% CO_2. A 100 μl of culture supernatant was collected for measurement of radioactivity in a gamma counter. Spontaneous and maximal release were measured in target cells supernatant alone and in the presence of 1% RBS, respectively. The percentage of specific lysis was determined using the following formula: ([experimental lysis − spontaneous lysis]/[maximal lysis − spontaneous lysis]) $\times$ 100.

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