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Research Article

Role for NKG2-A and NKG2-C surface receptors in chronic CD4$^+$ T-cell responses

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Summary The participation of CD94 and NKG2 gene family members in the function of NK cells and CD8$^+$ cytolytic cells has recently been addressed in detail. However, the role that these molecules play in the key CD4$^+$ regulatory cells remains largely unexplored. This study has examined the expression and regulation of CD94 and NKG2 genes in purified human peripheral CD4$^+$ cells stimulated with several agents. We found a constitutive expression of NKG2-E in CD94-depleted resting peripheral CD4$^+$ cells, whereas inductions of NKG2-A and NKG2-C required chronic cell activation and occurred after expression of CD94. We found that CD3-mediated stimulation induces the expression of CD94 first by day 5 of culture, followed by NKG2-A by day 15 and finally NKG2-C, which is not detected until 20 days after repeated stimulation. This pattern of gene expression differs sharply from that observed in purified CD8$^+$ T cells, where mRNA from all NKG2 gene family members are detected after 5 days of stimulation. Selective activation of TCR Vβ_2-bearing cells with toxic shock syndrome toxin-1 superantigen reveals that mRNA induction of NKG2-A and NKG2-C genes is significantly influenced by the presence of cytokines (IL-10 and TGF-β) and by the restimulation of the cells. In addition, the occupancy of the CD94/NKG2-A receptor expressed on these superantigen-stimulated CD4$^+$ T lymphocytes abrogates TNF-α and IFN- γ production, whereas NKG2-C enhances production of these cytokines. Taken together our results reveal strict gene regulatory mechanisms for CD94 and NKG2 gene expression on CD4$^+$ cells that are different from those governing the expression of these same genes in CD8$^+$ cells. The results suggest that these genes also participate in chronic CD4$^+$ T-cell responses.

Key words: C-type lectin receptors, cytokine regulation, interleukin-10, tumour growth factor-β, tumour necrosis factor-α.

Introduction

CD94/NKG2 receptors are C-type lectin heterodimers composed of CD94 covalently associated to one of the NKG2 molecules.[1–3] CD94 is a type II integral membrane protein with a very short non-signalling intracytoplasmic tail and acts as the invariant chain of the receptors.[4] The variable component is one of the members of the NKG2 family located on human chromosome 12p12–p13[5] (namely NKG2-A, -B, -C, -E, -F and -H; with A/B and E/H being splice variants of the same genes). The CD94/NKG2 association gives rise to a functional molecule that is able to transduce either activating (NKG2C and probably -E, -F and -H) or inhibitory (NKG2A/B) signals.[6–8] The ligand for CD94/NKG2-A and -C heterodimers is the non-classical HLA-E molecule.[9] Expression of CD94/NKG2 molecules was initially found on NK cells and some subsets of α/β$^+$ or γ/δ$^+$ CD8$^+$ T peripheral lymphocytes.[10,11] Functional studies revealed the participation of CD94/NKG2 receptors in cytotoxicity processes mediated by NK and CD8$^+$ T cells, leading to either inhibition or enhancement of cell lysis.[9,12,13] The expression and function of NKG2 receptors on T-cell populations is dependent on cellular activation and is positively regulated by certain cytokines, such as IL-15,[14] IL-12[15] and TGF-β.[16] In this context, we found that IL-10 contributes to the expression of CD94/NKG2-A on long-term activated human CD8$^+$ T cells.[17] Regarding the CD4$^+$ T lymphocytes our studies have revealed that activated CD4$^+$ T cells express functional CD94/NKG2-A receptors in response to CD3-mediated stimulation and that IL-10 enhances the expression of CD94.[18] Triggering of the NKG2-A receptors strongly inhibits Th1 cytokine production by CD4$^+$ T lymphocytes, although it does not affect production of Th2-type cytokines.[18]

In general terms, the fate and functional activity of T lymphocytes depend largely on the precise dynamics of gene expression and protein production. In this study we have addressed the dynamics and factors influencing the expression of CD94 and individual NKG2 genes in total lymphocyte populations as well as in highly purified human peripheral

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$CD4^+$ T cells stimulated by anti-CD3 mAb or superantigens. In addition, we have determined the influence of cytokines in the timing of this process. We found that $CD4^+$ cells can acquire expression of all NKG2 gene family members in a time-dependant fashion that is influenced by the stimulation used and the cytokines added to cultures. The orderly appearance of CD94 and NKG2 genes on $CD4^+$ cells differs from that observed in unfractionated peripheral T cells, and it is also different from that reported for developing NK cells[19] and $CD8^+$ cells,[20] suggesting distinct regulatory mechanisms of CD94/NKG2 genes in the $CD4^+$ lymphocytic lineage.

Materials and Methods

Isolation and culture of T lymphocytes

Peripheral blood lymphocytes were obtained by density gradient centrifugation and purified by adherence and immunomagnetic negative selection as previously described in detail.[18] T lymphocytes were cultured in RPMI-1640 media in the presence of 25 U/mL of rIL-2[21] (a kind gift from Hoffman-LaRoche obtained through the National Institute of Health, AIDS Research and Reagent program, Rockville, MD, USA) and stimulated in 24-well plates precoated with purified anti-CD3 mAb.[18] Cells were re-stimulated every 5 days in freshly OKT3-coated plates with replacement of culture media and rIL-2. Toxic shock syndrome toxin-1 (TSST-1) (Sigma Chemicals, St. Louis, MO, USA) was used at 5 ng/mL. In some experiments the cells received a second challenge of the relevant toxin after extensive washing at day 5. When required, IL-10 at a concentration of 10 ng/mL (BD-Pharmingen, San Diego, CA, USA) or TGF-β at 1 ng/mL (Sigma) were added.

Primers and retrotranscriptase–polymerase chain reaction of CD94 and NKG2 genes

Ribonucleic acid from the cytoplasms of indicated T-cell populations, either resting or activated, were extracted at designated time points (Rneasy; Qiagen GmbH, Hilden, Germany) and reverse-transcribed using the AMV-Reverse transcription system (Promega, Madison, WI, USA). Equal amounts of cDNA templates were amplified by PCR using specific NKG2-A, NKG2-C, NKG2-E and CD94 primer oligonucleotides and cycling conditions previously described in detail.[18,22] Internal loading and amplification controls were carried out using previously characterized primers[23] of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GADPH), which yielded a 579 bp DNA product.

Antibodies and reagents

The following picoerythrin (PE)- or FITC-labelled mAb were purchased from Becton Dickinson (Mountain View, CA, USA): Leu4-SK7 (anti-CD3), Leu-3a-SK3 (anti-CD4) and a goat antimouse IgG (GAM) second antibody. FITC-labelled HP-3D9 (anti-CD94) was obtained from BD-Pharmingen. OKT3 (anti-CD3) was purified from tissue culture supernatants using Protein-A binding.[18] The unconjugated, purified mAb used were: Z199 (anti-NKG2-A) (Immunotech, Marseille, France) and mAb HP3D1 (anti-CD94), which was kindly donated by Dr M López-Botet (Universitat Pompeu Fabra, Barcelona, Spain). PE-conjugated anti NKG2C mAb (clone 134591) was purchased from R & D Systems (Minneapolis, MN, USA).

Proliferation assays

$CD4^+$ lymphocyte proliferation assays were performed as previously described.[24] Briefly, $CD4^+$ T cells restimulated with TSST-1 for 20 days were cultured in flat-bottomed 96-well tissue culture plates (Nunc, Roskylde, Denmark; 10^5 cells/well) for 72 h in the presence of either 2 μg/well of HP-3D9 (anti-CD94), 2 μg/well of 134591 (anti-NKG2-C) or 2 μg/well of an isotype-matched irrelevant mAb. Culture wells were pulsed for an additional period of 16 h with 1 μCi/well of ^{3}H-thymidine (ICN Pharmaceuticals, Costa Mesa, CA, USA), harvested onto glass fibre paper and ^{3}H-thymidine incorporation determined using liquid scintillation counting.

Flow cytometry

For direct immunofluorescence assays, 10^5 cells were incubated with mAb on ice for 30 min, washed with PBS and analysed on a FACScalibur flow cytometer (Becton Dickinson). For indirect immunofluorescence, a second incubation with the isotype-specific FITC-labelled GAM antibody was carried out. Live cells were selectively gated on forward and side scatter parameters and for double fluorescence experiments the flow cytometer was appropriately compensated with Calli-Brite beads (Becton Dickinson) and FACS Comp 4.1 Software.

Detection of intracellular cytokines

$CD4^+$ cells were stimulated at days 0 and 5 with TSST-1 (5 ng/mL), harvested on day 10 of culture, washed and resuspended in complete media lacking IL-2 with the additional presence of 10 μg/mL of Brefeldin-A (Sigma). Cells were stimulated for 6 h in 24-well plates coated with 5 μg/well of OKT3 plus either 2 μg/well of mAb Z199 (anti-NKG2-A), 2 μg/well of the 134591 mAb (anti-NKG2-C) or 2 μg/well of HP-3D9 (anti-CD94). After stimulation the cells were treated with FACS permeabilizing solution (Becton Dickinson), incubated for 10 min at room temperature in the dark with labelled anticytokine antibodies (0.15 μg/10^6 cells) and analysed using flow cytometry.

Results

Analysis of the expression of CD94 and NKG2 gene family members in purified resting T-cell subpopulations

The expression of CD94 and NK2G gene family members was initially investigated in different populations of resting T lymphocytes. For this purpose, fresh peripheral Nylon-Wool T cells (NWT cells) were depleted of $CD94^+$ and $CD56^+$ cells by immunomagnetic negative selection, yielding a population that was consistently >99% $CD3^+CD94^-CD56^-$. In most experiments these cells were further fractionated by positive selection to obtain highly purified $CD4^+$ T cells that were typically >99% $CD4^+CD94^-$ as detected by double immunofluorescence flow cytometry (data not shown). Where indicated, purified $CD8^+$ cells were also studied. We undertook a molecular approach using RT-PCR gene amplification with well-defined specific oligonucleotides[18,22] to detect transcription of individual NKG2 genes because there are no serological probes available that specifically define all members of the NKG2 family. As a positive control we carried out RT-PCR amplifications of RNA from the human NK-like cell line NKL, which is known to express CD94 as well as all members of the NKG2 gene family. We observed strong

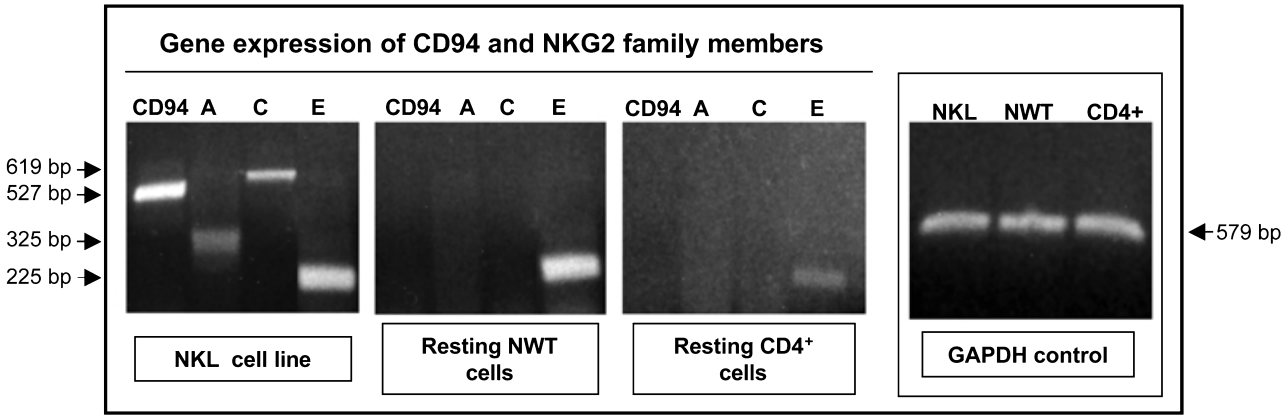

Figure 1 Gene expression profile of CD94 and NKG2 gene family members in freshly isolated peripheral resting T-cell populations. Equivalent amounts of cDNA retrotranscribed from RNA extracted from the NK-like cell line (NKL) as positive control (left panel), CD94/CD56-depleted Nylon Wool T cells (centre panel) or highly purified CD4⁺ T cells (right panel) were amplified by PCR using specific primers for CD94, NKG2-A, NKG2-C and NKG2-E genes. A representative experiment out of four is shown. Internal retrotransciptase–polymerase chain reaction (RT-PCR) loading controls were included by amplification of the housekeeping gene GAPDH.

amplification bands of expected sizes for all genes analysed (Fig. 1; left panel). By contrast, we detected in both resting CD3⁺ NWT cells and in highly purified CD4⁺ resting lymphocytes the constitutive expression of only the NKG2-E gene (Fig. 1; centre panels). In all cases, equivalent amounts of RT-PCR products were loaded as shown by the amplification products of the GADPH housekeeping gene (Fig. 1; right panel). The identity of amplified PCR products from CD4⁺ T cells was confirmed by capillary sequencing after purification using an ABI-PRISM (Applied Bipsystems, Foster City, CA, USA) automatic DNA sequencer (data not shown).

Time-course analysis of the expression of CD94 and NKG2 gene family members in highly purified CD4⁺ cells stimulated with OKT3 mAb

CD94 and some members of the NKG2 gene family are not expressed in resting T cells, therefore, we determined whether stimulation through the CD3/TCR complex leads to the de novo expression of some or all members of the family. Five days after CD3-mediated stimulation of purified CD4⁺ T cells we detected a faint CD94 amplification band (Fig. 2; upper panel). However, flow cytometric analysis indicated that CD94 mRNA expression at this early stage of cell stimulation does not result in surface protein expression of either homodimeric or heterodimeric CD94 molecules (Fig. 3b). The CD94 amplification bands detected after 10 days of CD3-mediated stimulation are significantly stronger, correlating with a progressive protein surface expression of CD94 as detected by immunocytometry (Fig. 3b). Expression of NKG2-A remained undetected until day 15 of culture, both at the mRNA level (Fig. 3a) and as surface expression (Fig. 3b). We only found NKG2-C amplification products after 20 days of OKT3-stimulation of CD4⁺ cells (Fig. 3a), which correlated with the detection of protein expression on the cell membrane by flow cytometry (Fig. 3b). This late-activation expression of NKG2-A and NKG2-C in purified CD4⁺ cells is in sharp contrast to that found in unfractionated NWT cells (data not shown) and in highly purified CD8⁺ cells (Fig. 3a), where strong amplification bands corresponding to all members of the NKG2 gene family are detected as early as day 5 after OKT3 stimulation. To ensure that the purity of the CD4⁺ population is maintained throughout the culture, we carried out double immunofluorescece experiments (Fig. 3c) showing 100% of CD4⁺ cells at day 20 of culture. In addition, we did not detect by RT-PCR CD8α gene transcripts at any time point of our study (data not shown).

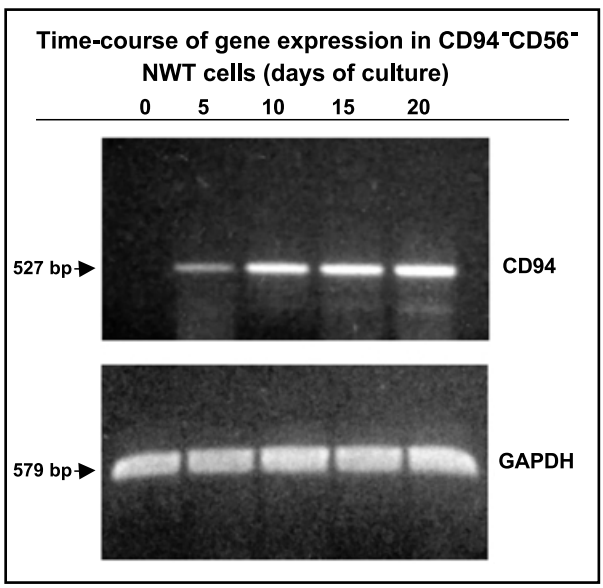

Figure 2 Time-course of CD94 gene expression in CD94⁻CD56⁻ Nylon Wool T cells (NWT) after CD3-mediated stimulation. RNA from peripheral NWT depleted of CD94⁺ and CD56⁺ cells by negative immunoselection were extracted at the indicated time points after CD3-mediated stimulation and amplified by RT-PCR. A representative experiment out of three is shown.

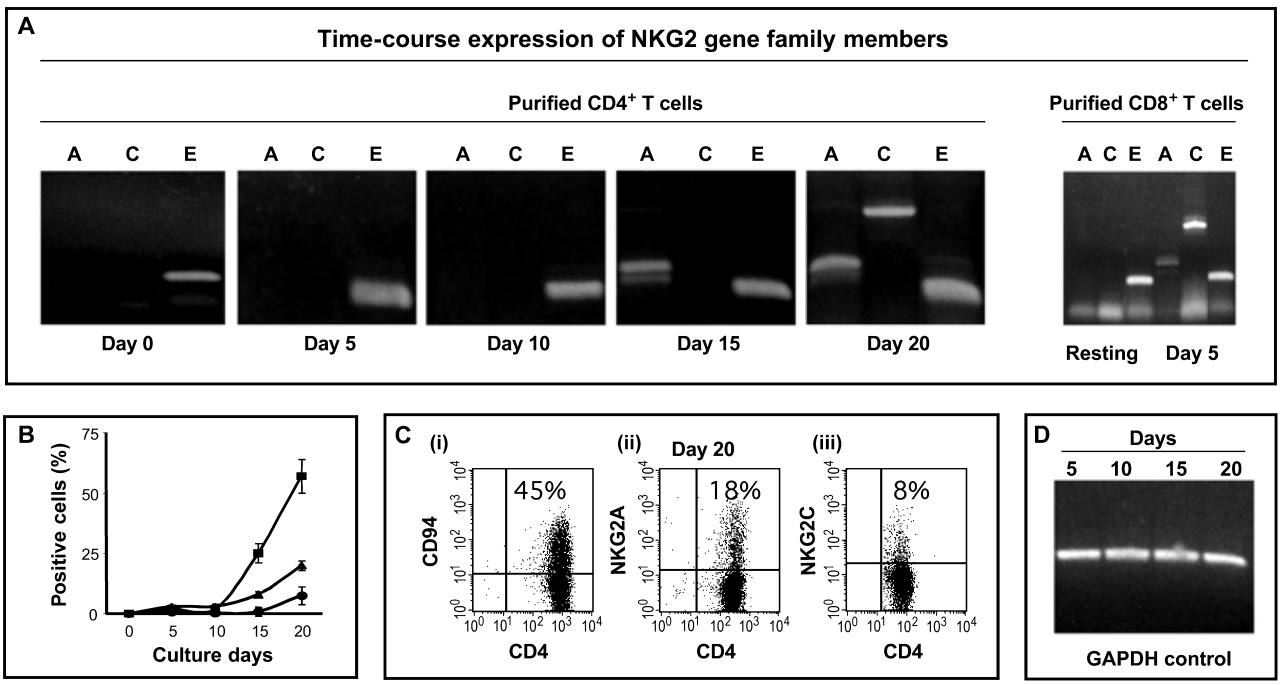

Figure 3 Time-course of NKG2 gene family members expression in purified $CD4^+$ T cells after CD3-mediated stimulation. (A) Equivalent amounts of RNA extracted from $CD4^+$ cells at indicated time points after OKT3 stimulation were retrotranscribed and amplified by PCR using specific oligonucleotides. NKG2-E is detected in non-stimulated cells, whereas NKG2-A appears by day 15 after OKT3-stimulation and NKG2-C appears at day 20. However, all NKG2 gene family members are detected as early as 5 days after OKT3-stimulation in highly purified $CD8^+$ cells. A representative experiment out of four is shown. (B) Percentage of cells expressing, at the indicated time points, surface CD94 (■), NKG2-A (▲) or NKG2-C (●) in highly purified $CD4^+$ T lymphocytes after OKT3-mediated stimulation, as detected by flow cytometry. Means and SD from three independent experiments are shown. (C) Double immunofluorescence flow cytometric analysis of purified $CD4^+$ cells at day 20 of culture. CD4 expression is represented in ordinates, whereas expression of CD94 (i); NKG2-A (ii); or NKG2-C (iii) is represented in abscises. (D) RT-PCR loading control with amplification bands of the GADPH housekeeping gene.

Because we had previously reported that IL-10 upregulates the surface expression of CD94 and NKG2-A in OKT3-stimulated $CD4^+$ cells,[18] all the above experiments were also carried out in the presence of this cytokine. We found that, as previously described, IL-10 increases the percentage of cells expressing CD94 and NKG2-A, but the presence of this cytokine does not modify the timing of CD94 and NKG2-A gene expression (data not shown). Internal loading controls of RT-PCR amplifications carried out with the GAPDH housekeeping gene are shown in Fig. 2d. Two independent experiments were carried out to test the influence of different doses of IL-10 on the timing of CD94 and NKG2 receptor expression. Increasing doses of IL-10 do not influence the timing of receptor appearance on the cell surface because we obtained similar results to those described above (data not shown).

Appearance of NKG2-A and NKG2-C gene expression in $CD4^+$ cells is influenced by the type of stimulation and the cytokines added to the culture

We further examined whether different CD3/TCR-mediated stimulations result in equivalent patterns of CD94 and NKG2 gene expression. Purified $CD4^+$ cells were exposed to an alternative stimulation via TCR using the TSST-1 superantigen, which selectively activates cells bearing $V\beta_2$ T-cell receptors.[25] CD94 is detected after 5 days of TSST-1 superantigen stimulation alone (Fig. 4a; left upper panel), whereas NKG2-A and NKG2-C remain unexpressed even after 10 days of the primary stimulation. These results are in sharp contrast to those found in experiments using highly purified $CD8^+$ cells because we detected amplification products from all genes examined by day 5 of culture and amplification was steadily maintained throughout the culture (data not shown). Analysis of the $CD4^+$ cell populations present in the culture at that time point indicates a vigorous activation and expansion of $V\beta_2$-bearing cells because almost 100% of cultured cells express this TCR receptor family (Fig. 4b). Interestingly, restimulation of cells at day 5 of culture with TSST-1 allows detection of both NKG2-A and NKG2–C genes at day 10 (Fig. 4; right lower panel). The presence of cytokines significantly influences the kinetics of expression of NKG2-A and NKG2-C. Thus, TSST-1 superantigen stimulation in the presence of TGF-β induces rapid expression of all genes studied, which are then readily detected at day 5 after stimulation (Fig. 4; upper right panel). Interleukin-10 has an early effect on the expression of NKG2-A, which is detected in the presence of this cytokine at day 5 of stimulation (Fig. 4; upper centre panel), and a late effect on the NKG2-C expression because

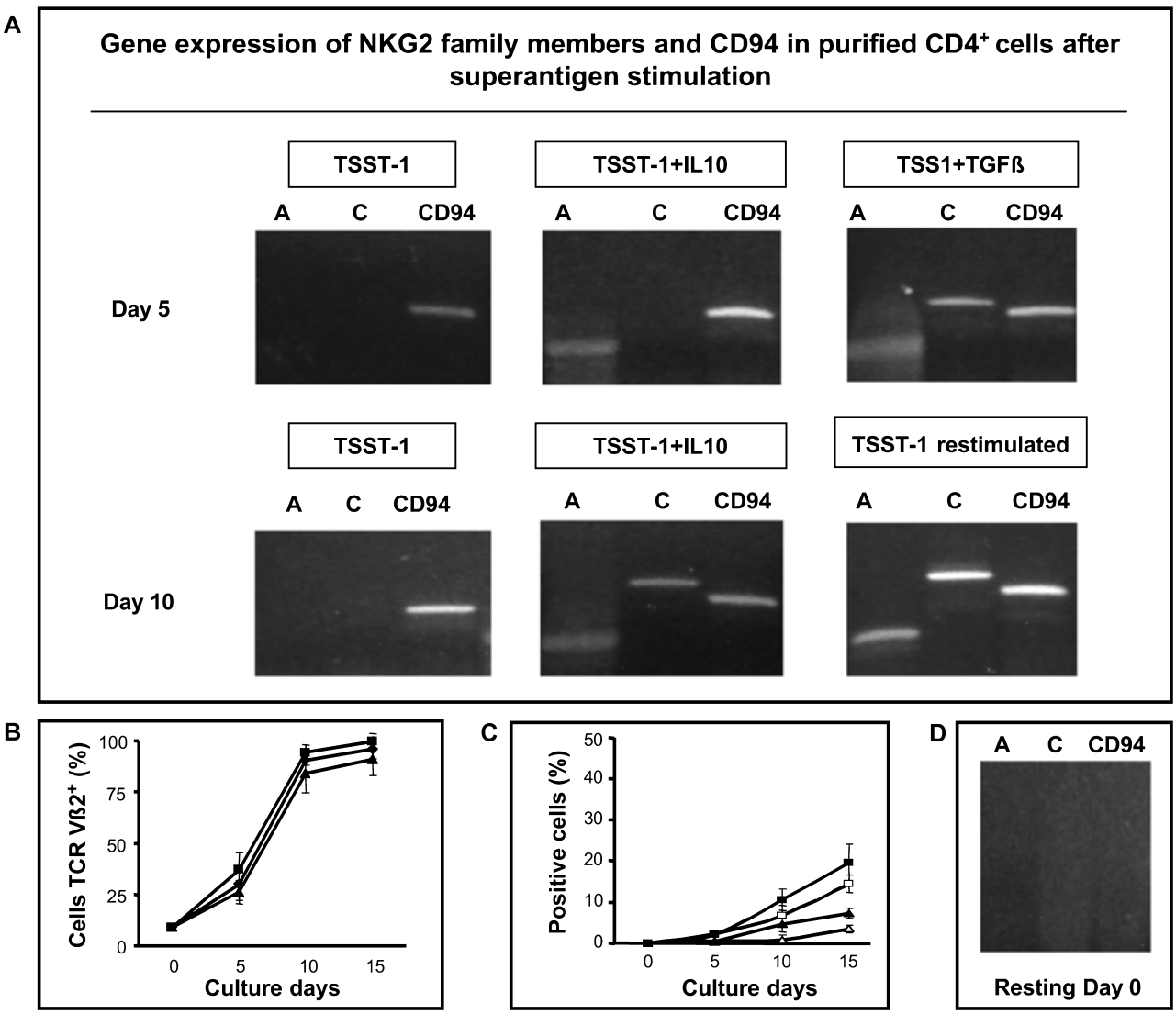

Figure 4 Gene expression of CD94, NKG2-A and NKG2-C in purified CD4⁺ cells after toxic shock syndrome toxin-1 (TSST-1) superantigen stimulation and expansion of specific clones. (A) RNA from purified CD4⁺ cells stimulated with TSST-1 alone (left column) or in the additional presence of IL-10 (centre column) or TGF-β (right column) were amplified by RT-PCR at days 5 and 10 after stimulation. The right lower panel represents cells stimulated at day 0 and again at day 5 with TSST-1 alone, and analysed at day 10. Bands of expected sizes were detected. Equal amplification of CD94 in all samples served as the internal loading control. (B) Clonal expansion of $V\beta_2$-bearing cells: TSST-1-responding cells were analysed using flow cytometry with anti-$V\beta_2$-specific mAb at the indicated times of culture after stimulation with superantigen alone (●) or in the presence of IL-10 (■) or TGF-β (▲). Points represent the mean and SD obtained from three independent experiments. (C) Surface expression of CD94 (□) and NKG2-A (△) after TSST-1 stimulation of CD4⁺ cells. Cells were also stimulated with TSST-1 plus IL-10, and the expression of CD94 (■) and NKG2-A (▲) was determined at the indicated time points. Data represent means and SD from three independent experiments. (D) PCR amplification products of NKG2-A, NKG2-C or CD94 are not detected in resting cells at the initiation of culture.

its mRNA is not amplified until day 10 (Fig. 4; lower centre panel). The flow cytometric analysis of the surface expression of CD94 and NKG2-A is consistent with the results obtained by RT-PCR (Fig. 4c). Similar results to those described for TSST-1 were obtained in response to stimulation of cells with the *Staphylococcus aureus* enterotoxin B toxin (SEB) (data not shown), a superantigen that expands cells belonging to several TCR Vβ families.

Functional regulatory role of the NKG2-A and NKG2-C surface receptors in superantigen-stimulated CD4⁺ T cells

We had previously reported that signals delivered by the NKG2-A molecule expressed on OKT3-stimulated CD4⁺ cells strongly influence cytokine production[18] and, in particular, result in a marked inhibition of both TNF-α and IFN-γ production. Therefore, we examined whether either CD94/NKG2-A or NKG2-C surface receptors are able to regulate

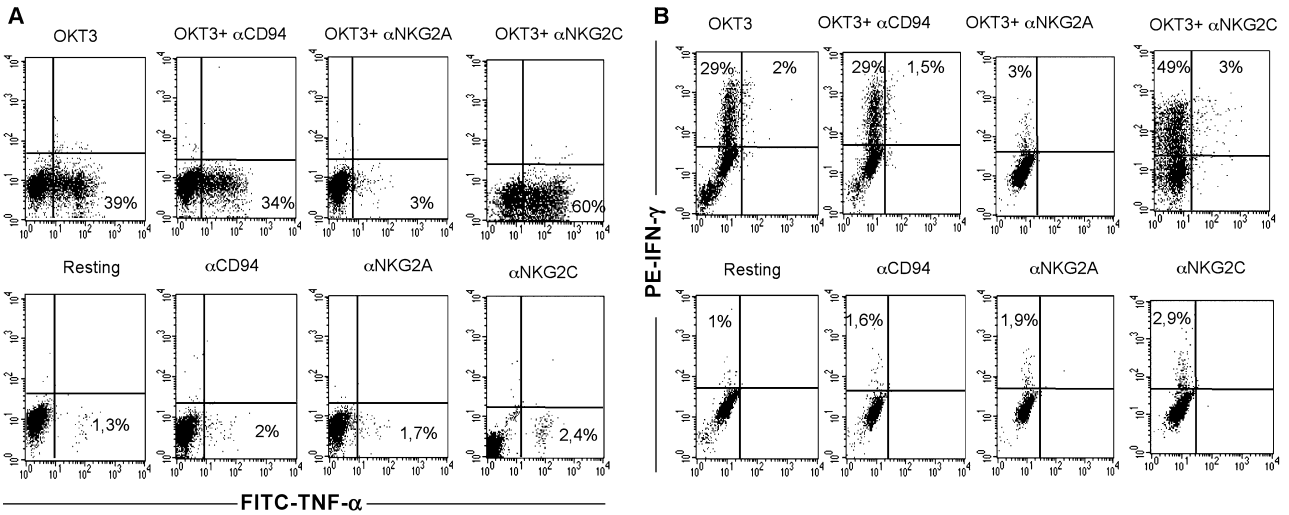

Figure 5 Modulation of TNF-α and IFN-γ production by engagement of the NKG2-A receptor on superantigen-stimulated cells. Intracytoplasmic cytokine production of toxic shock syndrome toxin-1 (TSST-1)-stimulated cells was detected using flow cytometry. Cells were crosslinked (from left to right) with OKT3 mAb alone; OKT3 plus anti-CD94 mAbs; OKT3 plus anti-NKG2-A mAb; or OKT3 plus anti-NKG2-C mAb. Cells treated with an isotype-matched irrelevant antibody or with anti-CD94, anti-NKG2-A or anti-NKG2-C mAb alone are shown in the lower panels. Production of TNF-α was detected using (a) a FITC-labelled specific antibody, whereas IFN-γ production was determined using (b) a picoerythrin (PE)-labelled antibody.

cellular functions in TCR-restricted, superantigen-responding cells by means of studying the modulation of their cytokine production. Intracytoplasmic staining of TSST-1-stimulated cells revealed that occupancy by agonistic mAb of the NKG2-A surface receptor results in strong inhibition of the TNF-α production triggered after TCR engagement (Fig. 5a; right upper panel). Occupancy of the NKG2-A molecule does not modulate the production of other cytokines such as IL-5, IL-7, IL-10 and IL-13 (data not shown). We also found that engagement of the NKG2-A receptor in superantigen-driven cells also inhibits the production of IFN-γ, an observation that is consistent with our previous findings[18] (Fig. 5b). As expected, binding of specific antibodies to the non-signalling molecule CD94 does not modify the cytokine production profile (Fig. 5a,b; upper centre panels). Crosslinking of cells with an irrelevant antibody or binding of anti-CD94 mAb or anti-NKG2-A mAb in the absence of a previous TCR engagement does not induce TNF-α or IFN-γ production (Fig. 5; lower rows). Ligation of NKG2-C by its specific mAb enhances the production of TNF-α and IFN-γ (Fig. 5; right panels). Consequently, the NKG2-C receptor was tested for its ability to modulate the proliferative response of cells chronically stimulated with TSST-1. Ligation of the NKG2-C receptor by immobilized anti-NKG2-C mAb enhances proliferation of $CD4^+$ T cells precultured in the presence of TSST-1 (Fig. 6). Because it occurs when IFN-γ and TNF-α production were determined, ligation of the CD94 molecule by its agonistic mAb did not trigger or inhibit proliferation of cells (Fig. 6).

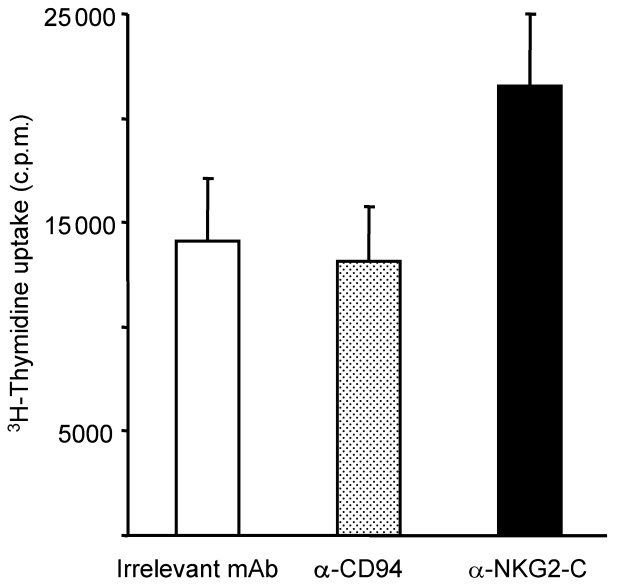

Figure 6 Ligation of NKG2-C receptor increases proliferation of superantigen-stimulated T cells. Superantigen activated T cells (10^5 cells/well) were restimulated with toxic shock syndrome toxin-1 (TSST-1) in the presence of immobilized irrelevant antibody (□), anti-CD94 (▨) or NKG2-C mAb (■) for 72 h, pulsed with 1 μCi of ^{3}H-thymidine for 16 h, harvested on fibreglass paper and thymidine uptake measured in a liquid scintillation counter. The figure shows the mean ± SD of results obtained from three independent experiments.

Discussion

The expression of genes belonging to the NK complex has attracted attention because their encoded products apparently play relevant roles in regulating the Th1 and cytolitic arms of the immune response.[3] In NK cells and $CD8^+$ CTL these molecules act as receptors that either initiate or inhibit target cell lysis. However, very little is known about the expression

and function of this family of genes and their products within the $CD4^+$ compartment of T lymphocytes. In a previous study, we provided evidence that cell activation of $CD4^+$ lymphocytes leads to de novo expression of CD94 and NKG2 gene products.[18] In the present study we revealed that: (i) expression of CD94 in T lymphocytes is independent of the stimulation agent and the subpopulation studied; (ii) expression of NKG2-A and NKG2-C genes is achieved in $CD4^+$ T cells during the chronic phase of the response; (iii) that such expression is modified by the concurrent presence of cytokines in the media; and (iv) that CD94/NKG2-A and NKG2-C receptors are functional in $CD4^+$ cells.

The induction of CD94 on T lymphocytes at early activation stages indicates that this is probably a non-related molecular event to the expression of NKG2 genes. We revealed that neither NKG2-A nor NKG2-C are expressed on resting cells and their appearance upon stimulation indicates that these genes are positively regulated following lymphocyte activation, rather than representing the expansion of cell clones already expressing NKG2 receptors. Interestingly, the profile of NKG2 gene expression in resting cells is distinctive according to lymphocyte locations, and it is known that resting intestinal intraepithelial $CD8^+$ cells constitutively express NKG2-C.[20] Consistent with these data, we found that CD3-mediated stimulation of unfractionated peripheral T cells (a mixture of $CD4^+$ and $CD8^+$ lymphocytes) results in prompt expression of the NKG2-C gene. In sharp contrast, expression of this same gene in peripheral $CD4^+$ lymphocytes is detected by RT-PCR and flow cytometry in 10% of the cells only after 20 days of culture, representing a very late activation event. These findings reflect differences in the function of those molecules in $CD8^+$ and $CD4^+$ lymphocytes rather than representing differences in the role of NKG2 gene products in $CD8^+$ cells from distant origins and/or locations. Indeed, the precise timing of expression of NKG2 molecules on $CD4^+$ lymphocytes suggests that these receptors may play either activating or inhibitory functions, which in turn would result in the maintenance of the chronic responses or its interruption depending on the dominant receptor engaged.

We have also observed that the orderly appearance of NKG2 gene family members on $CD4^+$ cells is not the same as that detected in developing NK cells[19] or $CD8^+$ T lymphocytes.[20] Recent data indicate that the precise nature of the antigen and its recognition (TCR specificity) dictates CD94/NKG2-A expression by human CTL.[20] Thus, TCR repertoires of $CD8^+$ cells precommitted to express the inhibitory NKG2-A receptor are different from those that can be induced to express the activating NKG2-C complex.[20] However, we observed that only a fraction of $CD4^+$ cells responding to TSST-1 express CD94 and NKG2-A, despite the fact that all cells in the culture ($NKG2\text{-}A^+$ and $NKG2\text{-}A^-$) express the same $V\beta_2^+$ TCR. This indicates that on $CD4^+$ T cells NKG2 molecules do not follow a dictated TCR rule. This observation is consistent with data previously described showing differential requirements for inducing expression of C-type lectin receptors in activated lymphocytes.[26] Nevertheless, it remains to be addressed whether the observed differential requirements for induction of NKG2 are the consequence of triggering specific regulatory elements, such as the nuclear transcription machinery involved, or whether they reflect alternative responses based on upstream signalling elements and/or the intensity of the same stimulation signal(s) delivered by different agents.

We have observed in resting purified $CD4^+$ T lymphocytes a constitutive expression of NKG2-E transcript. Because it occurs with NKG2A/B, a double band corresponding to split alleles E and H was detected. Unfortunately, the presence of NKG2-E on the cell membrane cannot be confirmed because of the current lack of specific serological probes. However, it appears likely that NKG2-E plays a relevant role from the early stages of $CD4^+$ T-cell activation because its transcripts are detected in resting cells.

The role that cytokines play in regulating the expression of NKG2 gene family members on T cells and vice versa, that is, the role that NKG2 receptors play in the control of cytokines production by T lymphocytes, are beginning to be appreciated. Expression of NKG2 gene family members is greatly influenced by the concurrent presence of cytokines in the media. Thus, we found that the expression of NKG2-A is detected in a temporal range from 5 to 15 days, depending on the stimulus and cytokines present in the medium. Differences are even more pronounced with the NKG2-C gene, whose expression ranges from 5 to 20 days. Taken together, our results reveal that the upregulatory activity of either IL-10 or TGF-β is required for adequate expression of those genes. In this context, the observation that restimulation of cells with superantigen drives the expression of NKG2-A and NKG2-C in the absence of added cytokines probably results from the endogenous overproduction of relevant cytokines.

In the light of previous findings[14,16] and our observations that IL-10 and TGF-β are able to induce mRNA and protein expression of NKG2-A (inhibitory) and at least mRNA of NKG2-C (activating) genes, which represent receptors with opposite functions, an important question arises: what is the functional significance of the concomitant expression of NKG2-A and NKG2-C receptors within the same polyclonal $CD4^+$ T-cell population responding to a given stimulus? This question becomes even more relevant when considering that our responding $CD4^+$ cells bear the same TCR (i.e. $V\beta_2^+$ cells stimulated with TSST-1 toxin). Of special interest is the finding that coupling of the NKG2-A receptor leads to abrogation of production of some Th1 cytokines by a population of $CD4^+$ T lymphocytes responding to TSST-1 superantigen (responsible for toxic shock syndrome in humans), whereas triggering of NKG2-C results in increased T-cell proliferation at the very least. Hence, we suggest that NKG2 receptors might also regulate dual functions in $CD4^+$ cells. Depending on the NKG2 receptor predominantly engaged (activating or inhibitory), the functional result would be either enhancement or inhibition of the production of Th1 cytokines by $CD4^+$ cells, in a similar mechanism to the one that triggers in $CD8^+$ lymphocytes either activation or inhibition of cell lysis. Occupancy of the NKG2-A receptor (expressed on 20% of cells) results in a selective and complete inhibition of TNF-α and IFN-γ (produced by up to 40% of cells). This finding suggests extensive intercellular communications that probably also occur in the case of NKG2-C. However, because Brefeldin A was used during the assays and protein secretion was prevented, the signal(s) leading to a down-regulation or enhancement of cytokine production could not be delivered by a secreted protein. Thus, the prime candidates for mediating those effects are receptor-ligand pair molecules

expressed on the membranes. Finally, and unlike in other cells, cytokine production was not modified by receptor engagement with the anti-CD94 mAb. This finding might reflect a different usage of signalling messengers in $CD4^+$ cells or that the effect of coupling CD94 alone may require more time to mediate its effect. However, other possibilities cannot be excluded. CD94 has been proposed as a molecule identifying Th1 lymphocytes in mice,[27] but given the differences in the number of cells expressing NKG2 receptors and the number of cells producing both cytokines, our data do not support these receptors as a marker for all cells producing Th1 cytokines, but rather a subset of those cells.

Understanding the contribution of the NKG2 receptors to the immunobiology of $CD4^+$ lymphocytes and the functional characterization of $NKG2^+$ populations are questions that need to be further addressed. We found that NKG2 molecules appear orderly and sequentially, suggesting that each type of receptor is probably required at different stages of the immune response. In this context, it could be speculated that the expression of CD94/NKG2 receptor(s) on human $CD4^+$ cells, which preferentially occurs during late stages of cell activation, may contribute to the regulation of chronic immune responses (i.e. certain viral infections; chronic allograft rejection). Considering that CD94/NKG2 receptors are actively involved in the regulation of cytotoxic responses, the functional role of these molecules in $CD4^+$ cells could be related to the control of cytotoxicity by modulating the cytokine production. Thus, it is conceivable that inhibitory and activating receptors of the NKG2 gene family may help to adjust the overall Th1 cytokine levels on interaction of the NKG2 surface receptor expressed on $CD4^+$ cells with its ligand, the HLA-E molecule, thereby leading to either inhibition or enhancement of cytotoxic function of effector $CD8^+$ and NK cells. Our finding that the production of TNF-α in cells responding to superantigens is inhibited by coligation of NKG2-A deserves attention and requires further investigation to ascertain its therapeutic potential in toxic-shock syndrome patients. Thus, the data reported here, showing a strict regulation of the CD94/NKG2 gene expression in $CD4^+$ cells, together with their functional diversity as either inhibitory or activating receptors, provide new insights into the resources available to $CD4^+$ cells to control and drive human immune responses.

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