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Natural killer activating receptors trigger interferon γ secretion from T cells and natural killer cells

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ABSTRACT **Proliferation of human CD4+ αβ T cells expressing a natural killer cell activating receptor (NKAR) has been shown to be enhanced, particularly in response to low doses of antigen, if the target cells present appropriate human class I major histocompatibility complex (MHC) molecules. Here, we show that NKAR also enhance proliferation and killing of target cells by subsets of CD8+ αβ and CD8+ γδ T cells, as well as by NK cells. Strikingly, interferon γ secretion from all of these types of lymphocytes was markedly increased by interaction of the NKAR with their MHC class I ligands, independently of enhancement of proliferation. Thus, the recognition of class I MHC molecules by NKAR on both T cells and NK cells may provide a regulatory mechanism that affects immune responses through the secretion of interferon γ and possibly other cytokines. It represents a signal for cytokine secretion alternative and/or augmentative to that through the T cell receptor.**

Natural killer (NK) cells, as well as cytotoxic T cells, are major components of the mechanism by which an immune response leads to the destruction of foreign or infected tissue (1). NK cells and T cells arise from a common progenitor and express several cell surface molecules in common (2, 3). Among these molecules are the NK cell inhibitory receptors (NKIR) (CD 158a and b, p58 proteins) that are members of the Ig superfamily (4–6). Binding of CD 158 on human NK cells to their HLA-C ligands leads to the recruitment of protein tyrosine phosphatases such as PTP1C (SHP-1) and PTP1D (SHP-2) and the generation of a negative signal that blocks NK cell killing (7–10). CD 158 members also are expressed on some T lymphocytes, including both CD4+ and CD8+ αβ T cells and double-negative γδ T cells (11–14). Production of the cytokines interferon γ (IFN-γ) and tumor necrosis factor α is reduced when antigen is presented to T cells expressing the inhibitory receptor CD 158 on antigen-presenting cells that express MHC class I proteins (15). An alternative CD 158-like receptor protein also was identified that can activate NK cell lysis [NK cell activating receptor (NKAR)] (16, 17). In contrast to CD 158 proteins, which have an uncharged transmembrane anchor and a long cytoplasmic tail containing two immunoregulatory tyrosine-based inhibitory motifs, NKAR proteins contain a transmembrane domain with a lysine residue and a truncated cytoplasmic domain lacking the two immunoregulatory tyrosine-based inhibitory motifs of CD 158 (5, 16).

NKAR also can be expressed on CD4+ T cells, where they may serve as costimulating receptors, i.e., engagement of NKAR by an appropriate major histocompatibility complex (MHC) class I ligand results in T cell proliferation to otherwise nonstimulatory low doses of superantigen. This effect is mediated by an as yet uncharacterized signaling pathway (18). Here, NKAR are shown also to regulate responses of both γδ and αβ T cells and NK cells through the binding of class I MHC ligands, resulting in secretion of interferon γ independent of T cell proliferation.

MATERIALS AND METHODS

Cell Lines and mAbs. NK clones, TANK (*T* cells expressing *A*ctivating *NK* receptors) and other T cell clones, were prepared from adult donors HTR and DP as described (18). The MHC class I-negative human B cell line 721.221 (19) was obtained from the American Type Culture Collection. Stable 721.221 transfectants expressing HLA-C molecules were generated previously (20). The following mAb against the following cell surface molecules were used: αβ T cell receptor (TCR) (T1039.1A-31), γδ TCR (B1.1), CD3 (UCHTI), CD56 (B159), CD94 (HP3D9), CD16 (3G8) (all purchased from PharMingen), CD158a (NKR1:HP3E4, a gift of M. Lopez-Botet, Madrid, and EB6, Immunotech, Westbrook, ME), CD158b (NKR2:GL183, Immunotech), and NKR3 (NKB1, a gift of L. Lanier, Palo Alto, CA).

Assays. The cytolytic activity of T cell clones and NK clones against the various HLA-C transfectants was assessed in 5-h ^{35}S release assays (20). In all presented experiments, the spontaneous release was <25% of maximal release. Each experiment was repeated at least three times. The range of triplicates was under 5% of their mean. In experiments in which NKR-specific mAbs were used to block MHC/NKR interaction, mAb was included in the medium to a final concentration of 5 μg/ml.

Proliferation assays were performed as described (18). In brief, 2.5×10^4 irradiated (6,000 Rad) target cells were mixed with 5×10^4 T cells and various amounts of superantigen. After 2 days, ^3H-thymidine was added, and cells were incubated further overnight. Cells than were harvested and counted on a liquid scintillation counter. The background cpm from wells in which identical reagents and target cells were placed in the absence of any T cells was subtracted. Each experiment was repeated three to six times. The range of the triplicates was under 5% of their mean.

For cytokine production, 5×10^4 effector cells (TANK or other T cell or NK clones) were mixed with 2.5×10^4 irradiated (6,000 Rad) target cells and incubated for 72 h in RPMI 1640 medium supplemented with 10% fetal calf serum and glutamine in round-bottomed, 96-well plates. In some experiments in which TANK were used, various concentrations of superantigen were added. Three days later, supernatants were collected and assayed for cytokine content by ELISA. To

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Abbreviations: NK, natural killer; NKIR, NK cell inhibitory receptor; IFN-γ, interferon γ; NKAR, NK cell activating receptor; MHC, major histocompatibility complex; TANK, *T* cells expressing *A*ctivating *NK* receptors; TCR, T cell receptor.

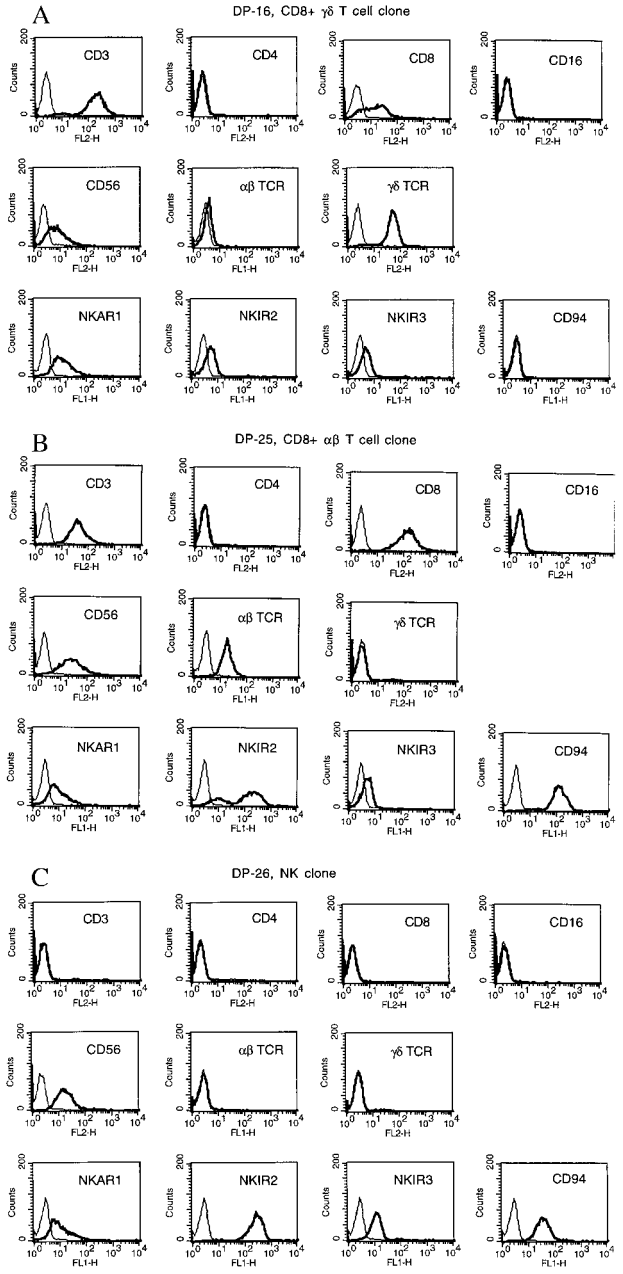

FIG. 1. Expression of CD56 and NK receptors on different types of lymphocytes from DP. (*A*) DP-16, CD8+ γδ T cell clone; (*B*) DP-25 CD8+ αβ T cell; (*C*) DP-26 NK cell clone. (*A–C*) Generated and stained for the surface proteins shown by using fluorescein isothiocyanate goat anti-mouse IgG, as described in *Materials and Methods* (bold lines). Control cells were stained only with fluorescein isothiocyanate anti-mouse IgG (light lines). The phenotypes of NK receptors (NKR) are indicated as NKAR or NKIR based on other data shown in this paper.

measure IFN-γ, a capture ELISA was used in which microtiter plates (Immulon 4, Dynatech) were coated with 100 μl of 1 μg/ml capture antibody (mouse anti-human IFN-γ, Endogen, Cambridge, MA) in 0.1 M $NaHCO_3$ overnight at 4°C. Plates then were washed and blocked with 1% BSA (Sigma) in PBS for 3 h at 37°C. After washing, samples (50 μl) and standards were added to the plates for 18–20 h at 4°C followed by 1 μg/ml rabbit anti-human IFN-γ and 1:15,000 dilution of goat anti-rabbit Ig coupled to horseradish peroxidase (BioSource International, Camarillo, CA). Plates were developed with TMB one component peroxidase substrate (Kirkegaard & Perry Laboratories) and stopped by addition of 1 M phosphoric acid. Absorbance was measured at 450 nm by using a microplate reader (Bio-Rad). Similar procedures were used to measure IL-2 and IL-4 by using reagents obtained from Endogen or PharMingen, respectively. The assay sensitivity for IFN-γ and IL-4 was 20 pg/ml and for IL-2 was 50 pg/ml.

Table 1. Surface markers of CD3+ and CD56+ T cells from donor DP

	Surface markers					
Clones, *n*	CD3	CD56	NKR 1	NKR 2	NKR 3	CD94
3	+	+	−	−	−	−
3	+	+	−	−	+	+
4	+	+	−	+	−	+
5	+	+	+	+	+	−
15	+	+	+	+	+	+

T cell clones were prepared from donor DP and stained for cell surface markers and NK receptors as described in *Materials and Methods.* Only the T cell clones that were positive for both CD3 and CD56 proteins are shown.

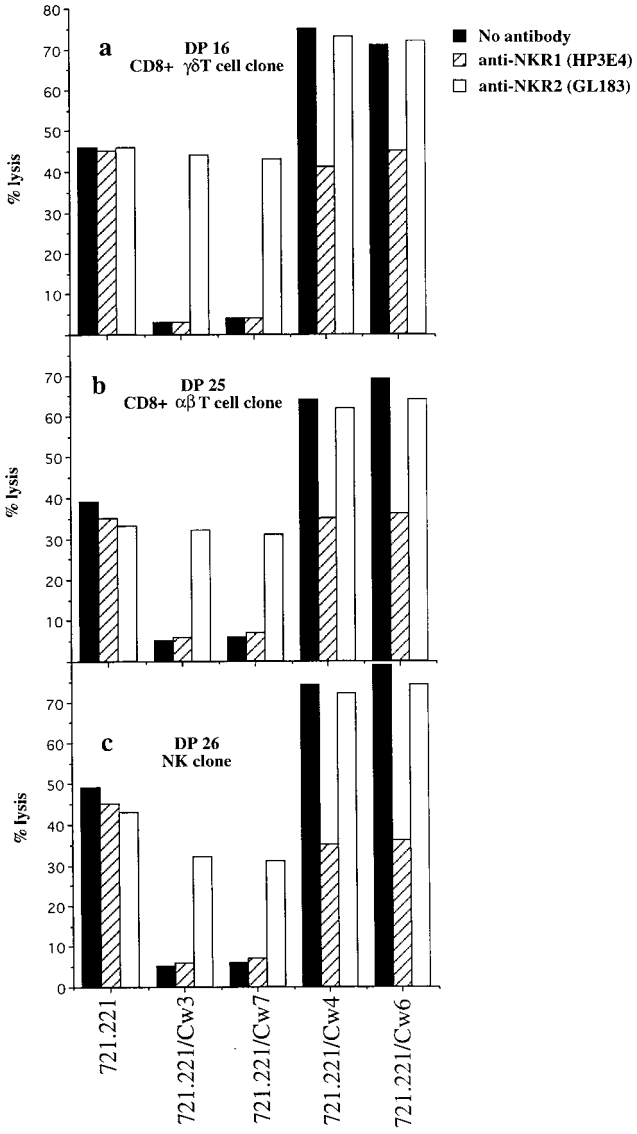

FIG. 2. NK receptors on both NK cell and T cell control target cell lysis. Different types of lymphocyte clones, CD8+ γδ T cells (*a*), CD8+ αβ T cells (*b*), or NK cells (*c*) prepared from donor DP, were incubated with various [^{35}S]-methionine-labeled target cells (721.221 or its transfectants) for 5 h at various effector-to-target ratios, with or without antibodies (GL183 or EB6, 5 μg/ml). Only the experiment using an effector-to-target ratio of 1:1 is shown.

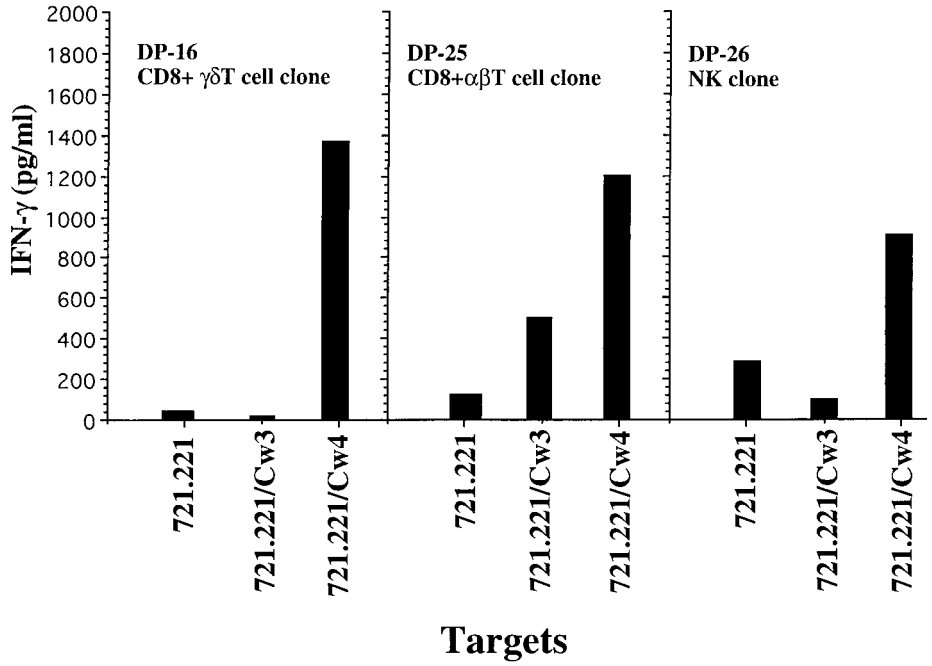

FIG. 3. Enhancement of IFN-γ production mediated by NK activating receptors. Effector cells (DP-16, DP-25, and DP-26) were incubated for 48 h with irradiated target cells (721.221 or its transfectants), and IFN-γ assays were performed as described in *Materials and Methods*. No secretion of IFN-γ was detected in the absence of effector cells.

RESULTS

Inhibitory and Activating NK Receptors Found on Both γδ and αβ T Cell and NK Cell Clones Regulate Lysis of a Class I-Negative Target Cell. Several T and NK cell clones were established from an individual (DP, subject 7a in ref. 21) with consistently elevated numbers of circulating CD56+ NK cells (15–20%). In addition, a relatively large portion of T cell clones obtained from DP were CD56+. Among 30 T cell clones, 27 (90%) also tested positive for at least one of the NK receptors (i.e., NKR including NKIR and/or NKAR) when fluorescence-activated cell sorter staining analysis was used; 20 of these 27 (74%) were positive for NKR1, NKR2, and NKR3 proteins as assayed by staining with EB6, GL183, and NKB1 mAb (Table 1). Three representative clones are (*i*) DP-16, a CD8+ γδ T cell clone that expresses both NKR1 (EB6+) and NKR2 (GL183+) as well as NKR3 (NKB1+) (Fig. 1*a*), (*ii*) DP-25, a CD8+ αβ T cell clone that expresses four NK receptors (NKR1, NKR2, and NKR3, together with the C-type lectin receptor CD94, detected by mAb HP3D9) (Fig. 1*b*), and (iii) DP-26, an NK clone that also expresses all four NK receptors (Fig. 1*c*).

All three DP clones tested lysed the class I-negative target cell 721.221 to almost the same extent (40–50% lysis) at an effector-to-target ratio of 1:1 (Fig. 2), i.e., the two T cell clones (DP-16 and DP-25) behaved as NK clones, possibly the result of culturing in high doses of IL-2 or of the coexistence of a still unidentified "NK lysis receptor" on these cells. Lysis of 721.221 cells transfected with either HLA-Cw3 or HLA-Cw7 (NK2 ligands) was reduced to background level with all effector cells tested (Fig. 2 *a–c*). This protective effect was mediated by NKIR2 proteins (CD158b) because lysis of the HLA-Cw3 and HLA-Cw7 transfectants could be restored by using the NKR2 mAb GL183 but not by using the NKR1 mAb EB6 (CD158a). In contrast, lysis of 721.221 target cells transfected with either Cw4 or Cw6 (NK1 ligands) was enhanced markedly, and the enhancement could be blocked by the EB6 antibody, indicating the existence of NKAR1. The coexistence of both NKAR1 and NKIR2 in all three clones tested also was confirmed by sequencing of reverse transcriptase–PCR products (data not shown).

Triggering of the NKAR by Class I MHC Proteins Leads to Enhancement of IFN-γ Secretion from a CD8+ αβ T Cell, a CD8+ γδ T Cell, and an NK Cell Clone. To test whether NKAR plays a role in cytokine production and in particular IFN-γ secretion, the T cell and NK clones from DP were incubated with 721.221 and 721.221 transfected with either HLA-Cw3 or HLA-Cw4. The CD8+ γδ T cell clone DP-16 did not produce any IFN-γ unless cultured with 721.221 cells transfected with HLA-Cw4, whereas production by either DP-25, the CD8+ αβ T cell clone, or DP-26, the NK clone, was enhanced 10- or 3-fold, respectively, when cultured with 721.221/Cw4 cells (Fig. 3). Secretion of IL-2 or IL-4 could not be detected (i.e., <50 or 20 pg/ml, respectively, data not shown). Thus, triggering of NKAR proteins found on three different types of killer lymphocytes led to significant enhancement in IFN-γ production.

Triggering of Cytokine Secretion from CD4+ αβ T Cell Clones. To ask whether ligation of activating receptors found on T cells cloned from a second donor also would lead to enhancement of IFN-γ production, CD4+ TANK clones from donor HTR (18) were used. TANK 1 express the NKAR clone 39 and proliferates in response to the superantigen SEB (18). As shown previously, proliferation of TANK 1 was enhanced when SEB was presented by 721.221 transfected with either HLA-Cw4 or HLA-Cw7 (Fig. 4*a*). The proliferation to both HLA-Cw4 (which has αLys80) and HLA-Cw7 (αAsn80) is unusual in that it does not correspond to the specificity of NK receptors for the amino acid at α80 (20) and was noted previously (18). The highest proliferation was observed with 0.5 ng/ml SEB. When no superantigen was present during the assays, TANK 1 did not proliferate significantly (<500 cpm) in the presence of either HLA-Cw4 or HLA-Cw7, indicating that the activating receptors expressed on these cells serve as costimulatory HLA-C binding receptors with respect to proliferation.

To ask whether triggering of the costimulating NK receptors on TANK 1 would result in increased IFN-γ secretion, supernatants were collected in parallel in the absence or presence of

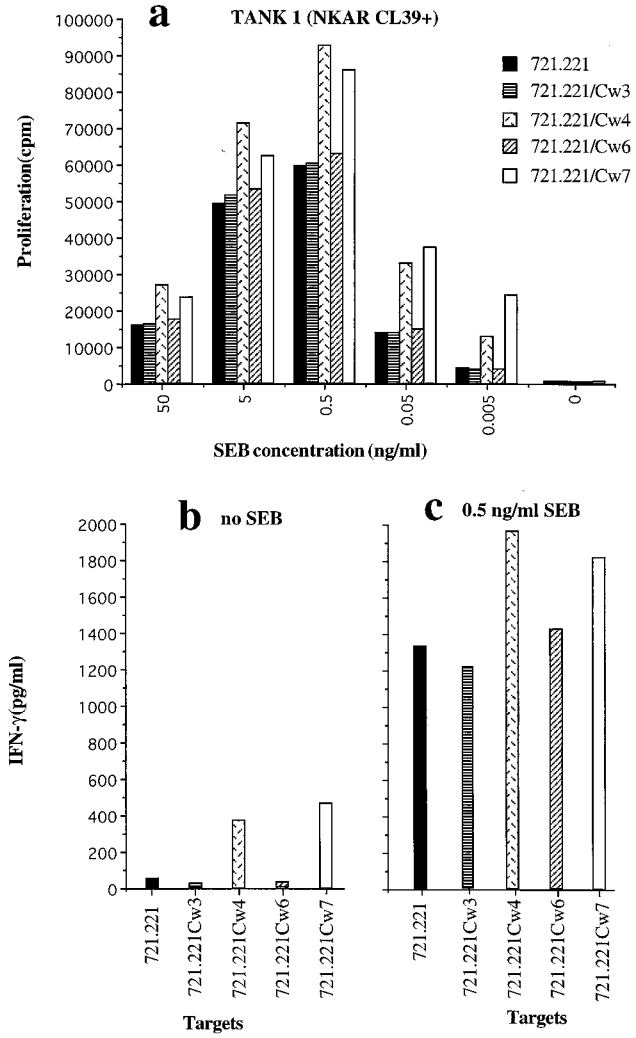

FIG. 4. Enhancement of proliferation and triggering of IFN-γ production from TANK 1 by class I MHC ligands for NKAR proteins. (*a*) TANK 1 were incubated with various concentrations of SEB and with the various irradiated target cells. Proliferation assays (72 h) were performed as described in *Materials and Methods*. Each data point was measured in triplicate. The range in each measurement was <5% of the mean of the triplicates. In the absence of SEB, the cpm was <200 regardless of the type of B cell or T cell present. One of six representative experiments is shown. (*b*) TANK 1 were incubated with irradiated target cells for 72 h, and IFN-γ secretion was measured as described in *Materials and Methods*. No secretion of IFN-γ was detected in the absence of either TANK 1 or the various targets. (*c*) TANK 1 were incubated for 72 h with irradiated target cells and 0.5 ng/ml SEB. IFN-γ secretion was measured (in parallel to the proliferation assays) as described in *Materials and Methods*. No secretion of IFN-γ was detected in the absence of either TANK 1 or the various targets.

0.5 ng/ml SEB coated on 721.221 cells. TANK 1 secreted elevated levels of IFN-γ when triggered through its NK-activating receptor specific for HLA-Cw4 and HLA-Cw7, even in the absence of SEB (Fig. 4*b*); in this circumstance, little or no proliferation of TANK 1 was detected (Fig. 4*a*). TANK 1 produced 10-fold more IFN-γ when incubated with either 721.221/Cw4 or 721.221/Cw7 and SEB compared with 721.221/Cw3, 721.221/Cw6, or the parental cell line 721.221 (Fig. 4*b*). Peak proliferation for TANK 1 was achieved in the presence of 721.221 cells and 0.5 ng/ml SEB (Fig. 4*a*). Even at this concentration of SEB, enhancement of IFN-γ production in the presence of 721.221/Cw4 or 721.221/Cw7 (Fig. 4*c*) was evident. Again, secretion of IL-2 or IL-4 could not be detected either in the absence or in the presence of SEB (data not shown).

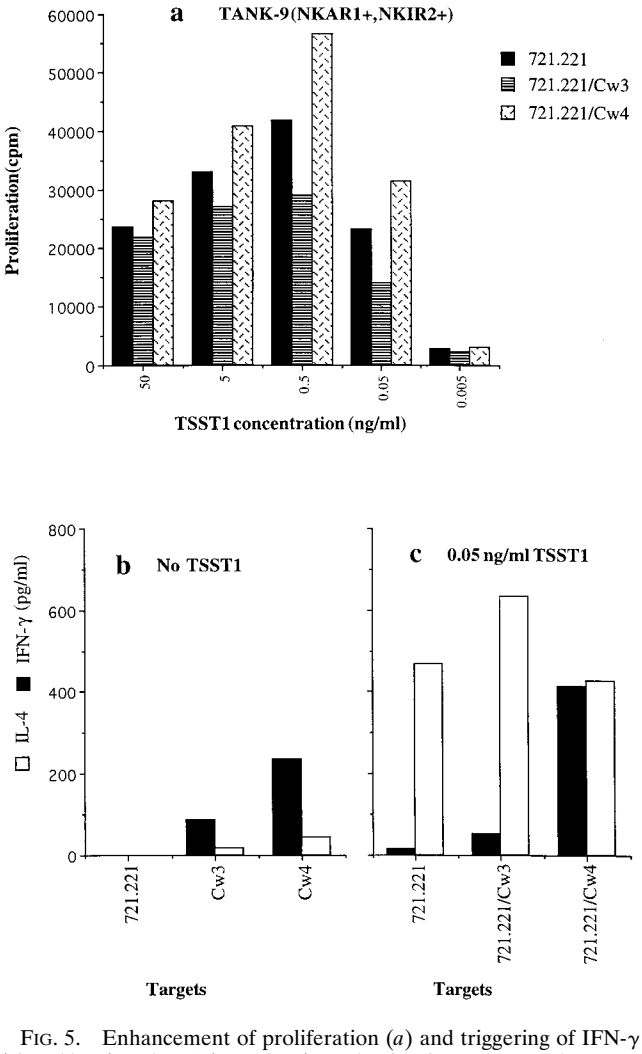

FIG. 5. Enhancement of proliferation (*a*) and triggering of IFN-γ (closed bars) and IL-4 (open bars) production from TANK 9 by MHC class I ligands for NKAR protein without (*b*) or with 0.05 ng/ml TSST1 (*c*). The experiment was carried out as described in the legend for Fig. 4.

A more complex example is provided by TANK 9. These cells express both an activating receptor (NKAR1) and an inhibitory receptor (NKIR2). They proliferate in response to the superantigen TSST1 coated on 721.221 cells (Fig. 5*a*) [but because they have a different T cell receptor from TANK 1 (18) not to SEB]. The proliferation was enhanced by group 1 HLA-C ligands (αLys80) such as HLA-Cw4 and HLA-Cw6 but not by group 2 ligands (αAsn80) such as HLA-Cw3 and HLA-Cw7. In the absence of TSST1 (and thus the absence of superantigen-stimulated proliferation), secretion of IFN-γ was enhanced markedly by HLA-Cw4, a ligand for NKAR1 (Fig. 5*b*), but little or no effect on IL-4 secretion was observed. Even in the presence of TSST1, a marked affect of HLA-Cw4 on IFN-γ secretion was observed. The enhancement of IFN-γ secretion was, therefore, similar to that found with TANK 1. In the presence of TSST1 in an amount sufficient to induce maximum proliferation (18), maximum IL-4 secretion (at the 72-h time point at which most experiments were done) was observed in the absence of the HLA-C ligands and could not be enhanced further or inhibited by HLA-Cw3 or HLA-Cw4 (Fig. 5*c*). Thus, in these experiments, IL-4 secretion appeared to be coupled to TSST1-induced proliferation.

Class I MHC Protein Is the Ligand that Enhances Both IFN-γ Secretion and Cell Proliferation. Addition of the Fab′

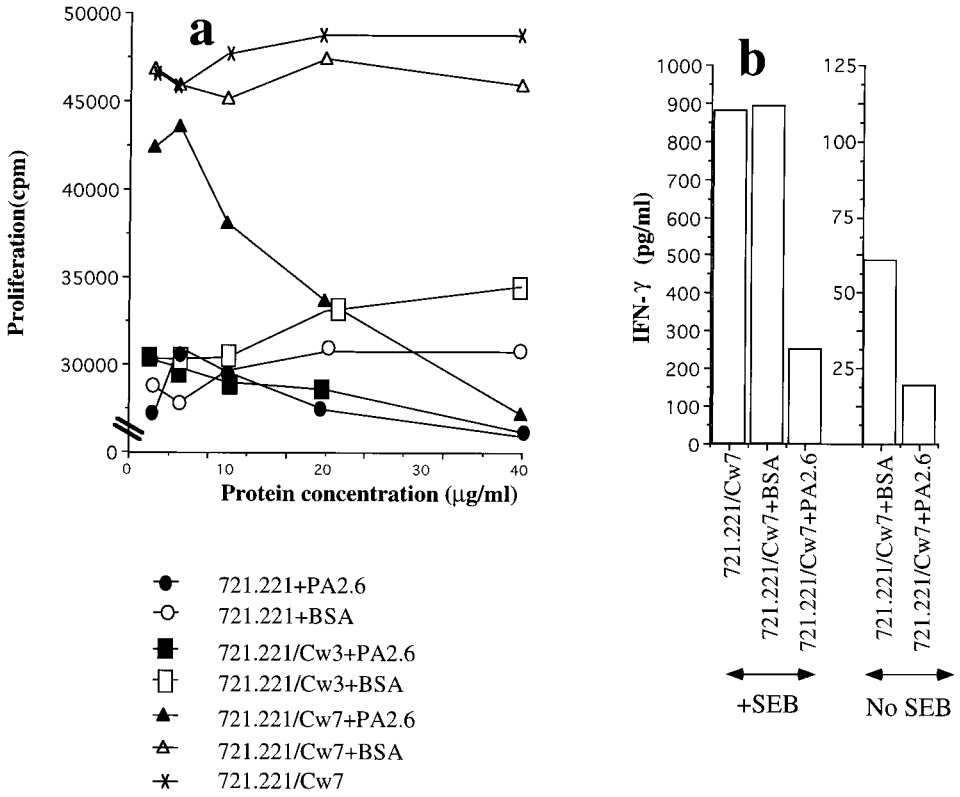

FIG. 6. Anti-class I MHC mAb block the enhancement of TANK 1 proliferation and IFN-γ production. (*a*) Proliferation of TANK 1 was measured in the presence of 0.5 ng/ml SEB, irradiated 721.221 cells, or 721.221 transfected cells and with increasing concentrations of either Fab′ fragments of the anti-class I mAb PA2.6 or control BSA protein. In the absence of superantigen, the cpm was <500 regardless of the type of B cell target or T cell present. Note that the proliferation scale is truncated and that the enhancement of proliferation by HLA-Cw7 is ≈50% (compare with Fig. 4). (*b*) IFN-γ secretion in the presence of 0.5 ng/ml SEB, irradiated 721.221/Cw7 cells, and 20 μg/ml either Fab′ fragments of PA2.6 mAb or the BSA protein as control. Results are presented as the extra secretion of IFN-γ after the reduction of basal level secretion derived from TANK 1 incubated either with irradiated 721.221 or 721.221/Cw7 cells.

fragment of PA2.6 (a pan class-I mAb) inhibited in a dose-dependent manner both proliferation of TANK 1 induced by 721.221/Cw7 and 0.5 ng/ml of SEB and IFN-γ secretion (Fig. 6 *a* and *b*). When no SEB was present, the secretion of IFN-γ was reduced to background levels by PA2.6. Addition of human serum albumin, BSA, or anti-CD8 (data not shown) had no effect.

DISCUSSION

Thus, triggering of NKAR found on a subset of CD8+ αβ and CD8+ γδ T cells, as well as on NK cells, from the individual DP by appropriate MHC class I ligands led to enhancement of IFN-γ secretion. Enhancement of IFN-γ production after triggering of NKAR also was observed on the CD4+ αβ T cells TANK 1 and TANK 9 derived from a second donor, HTR. TANK 1 were characterized previously and have been shown to express the co-activating NK receptor clone 39 (18). In the presence of an appropriate class I MHC ligand IFN-γ (but not IL-4, characteristics of a Th1 cell) was secreted by TANK 1 in the absence of superantigen and thus without proliferation. IFN-γ also was secreted by TANK 9, which expresses NKAR 1, in the presence of an appropriate MHC class I ligand in the absence of superantigen. TANK 9 also secreted IL-4 (i.e., its phenotype might be described as Th0), but secretion of this cytokine appeared to be induced by superantigen along with proliferation and independent of NKAR triggering. However, for both TANK 1 and TANK 9, no correlation was observed between IFN-γ secretion and cell proliferation, suggesting that for these T cell clones, secretion of this cytokine and proliferation are two independent events. Similarly, an altered murine Hb peptide with a single amino acid substitution induced IL-4 secretion without proliferation of the T cells (22). Hence, NKAR proteins are coactivating receptors with regard to proliferation or killing (16, 18), but they can be triggering receptors for IFN-γ secretion, delivering an unknown signal to the cell to produce this cytokine. In the mouse, T cells expressing another activating NK receptor, NKR-P1, have been identified (NK1.1 T cells). These cells have been shown to have an important role in regulation of the immune response, partially through the secretion of IFN-γ upon NKR-P1 ligation (23). Because NKR-like receptors have not been identified in the mouse, TANK may represent a subset of cells homologous to the mouse NK1.1+ T-cells (24).

Cytokine secretion normally is stimulated by engagement of TCR on both CD4+ and CD8+ αβ T cells by MHC/peptide ligands leading to production of distinct patterns of cytokines from CD4+ Th1 or Th2 and from CD8+ T cells. Alteration of the TCR contact residues of the MHC-bound peptide ligands can result in changes in the pattern of cytokine secretion (see, for example, ref. 25). Costimulatory molecules such as CD28 may augment cytokine production induced through the TCR but are not known to induce cytokine secretion independently. Cytokine secretion also is induced through autocrine loops involving the cytokine receptors (e.g., IL-2 or IL-4), and IL-12 produced by macrophages and other cytokines also can induce IFN-γ secretion through the appropriate cytokine receptors. NK cells also release cytokines on recognition of targets (1, 26), but a mechanism has not been established clearly; indeed, the recognition event that leads to lysis by NK cells also remains unclear. Moreover, cross-linking of CD16 or of the activating forms of CD94 and of CD158-like proteins (i.e., NKAR) on NK cells leads to production of granulocyte–macrophage colony-stimulating factor, tumor necrosis fac-

tor-α, and IFN-γ, at least (17, 27–30) As shown in the present paper, interaction of NKAR on T cells with their MHC class I ligands on other cells also leads to secretion of IFN-γ. Stimulation through engagement of NKAR is thus an alternative to TCR engagement for secretion of this and probably other cytokines from several types of T cells. Reciprocal to these findings, NKIR appear to down-modulate cytokine secretion because an increase in production of IFN-γ and tumor necrosis factor-α, at least, was observed from T cells expressing NKIR on addition of blocking anti-class I or anti-NKIR mAb (15). Moreover, because cytokine secretion after NKAR engagement on T cells (in the absence of superantigen) may be additive to that which occurs after TCR engagement by superantigen (Fig. 4), the signaling pathways for these two different processes could be distinct.

Note Added in Proof. IFN-γ production in NK cells independently from cytotoxicity also has been reported recently (31). Moreover, the association of DAP-12 with NKARs provides a first clue to understanding the mechanism by which NKARs may directly trigger cytokine production (32).

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