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Regulation of T Cell Lymphokine Production by Killer Cell Inhibitory Receptor Recognition of Self HLA Class I Alleles

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Summary

The killer cell inhibitory receptors (KIRs) are surface glycoproteins expressed by natural killer (NK) and T cells that specifically recognize defined groups of polymorphic human histocompatibility leukocyte antigen (HLA) class I molecules. Interactions between KIRs on NK or T cells and major histocompatibility complex (MHC) class I molecules on potential target cells inhibit cell-mediated cytotoxicity, presumably by delivering a negative signal preventing lymphocyte activation. In this study we examined whether KIRs also regulate cytokine production induced in response to T cell receptor–dependent T cell activation. $CD4^+$ and $CD8^+$ T cell clones were stimulated by bacterial superantigens in the presence or absence of monoclonal antibodies (mAbs) against the KIR NKB1 or MHC class I molecules, and production of tumor necrosis factor α and interferon γ was evaluated. When bacterial superantigen was presented by an autologous antigen-presenting cell (APC) to a KIR^+ T cell clone, cytokine production was always enhanced in the presence of anti-MHC class I mAb. Similarly, anti-KIR mAb also augmented cytokine production, provided that the APC expressed a HLA class I allele recognized by the KIR. These results suggest that recognition of autologous MHC class I molecules by KIR+ T cells provides a regulatory mechanism acting to modulate the potency of their responses to antigenic challenge.

The killer cell inhibitory receptors (KIRs) are a family of structurally related cell surface molecules that are expressed on subsets of human NK and T cells (1–3). These receptors bind to polymorphic HLA class I glycoproteins (4), and this interaction prevents NK cells from killing potential target cells expressing certain HLA-B or HLA-C alleles (5–7). Recent studies suggest that the Src homology region 2-domain phosphatase (SHP) tyrosine phosphatase may be responsible for the KIR-mediated inhibition of cytotoxicity (8). Although structurally unrelated, the Ly49 receptors expressed on mouse NK cells also recognize H-2 class I molecules and inhibit NK cell–mediated cytotoxicity (9).

While initially discovered on NK cells, KIRs are also present on subsets of T lymphocytes, including both $CD4^+$ and $CD8^+$ T cells (10–12). The expression of KIR by T cells is intriguing because this suggests that T cell responses are regulated by two distinct, opposing receptors for MHC/peptide ligands. Whereas the TCR transmits positive signals after engaging MHC/peptide complexes, the KIR may serve to dampen the extent of cellular activation, thereby regulating the signaling threshold required for effector cell function. Previously, we demonstrated that KIRs can inhibit the lytic function of CTLs activated by bacterial superantigens (11). Here we examine the ability of the KIR to regulate other T cell responses and demonstrate that disruption of KIR recognition of self–class I molecules leads to significant increases in T cell cytokine production in response to superantigen stimulation.

Materials and Methods

T Cell Clones and B Cell Lines. T cell clones were generated and maintained as described previously (13, 14). The HLA class I–negative B lymphoblastoid cell line (B-LCL) 721.221 was generously provided by Dr. Robert DeMars (University of Wisconsin, Madison, WI) (15). 721.221 transfectants expressing HLA-B*5801 were generated as described previously (14).

Antibodies and Flow Cytometry. mAbs were generously provided by Becton Dickinson Immunocytometry Systems (San Jose, CA). The anti-KIR-NKB1 mAb DX9 and anti-HLA-A,-B,-C mAb DX17 were produced and characterized in our laboratory (6, 11). The anti-KIR mAbs GL183 and EB6 mAbs were kindly provided by Dr. Lorenzo Moretta (University of Genoa, Genoa, Italy), and HP-3E4 by Dr. Miguel Lopez-Botet (University of Madrid, Madrid, Spain). Methods of immunofluorescence staining and flow cytometry are described elsewhere (16).

Cytokine Assays. T cell clones were cultured in 96-well flat-bottom microtiter plates (Becton Dickinson Labware, Lincoln Park, NJ) with or without *Staphylococcus* enterotoxin B (SEB, Toxin Technology Inc., Sarasota, FL). In some assays, 721.221 cells or 721.221 cells transfected with HLA-B*5801 were precoated with SEB, washed to remove residual superantigen, and used as APCs at a stimulator to responder ratio of 1:1. After overnight incubation, triplicate 50-μl samples of cell-free supernatant

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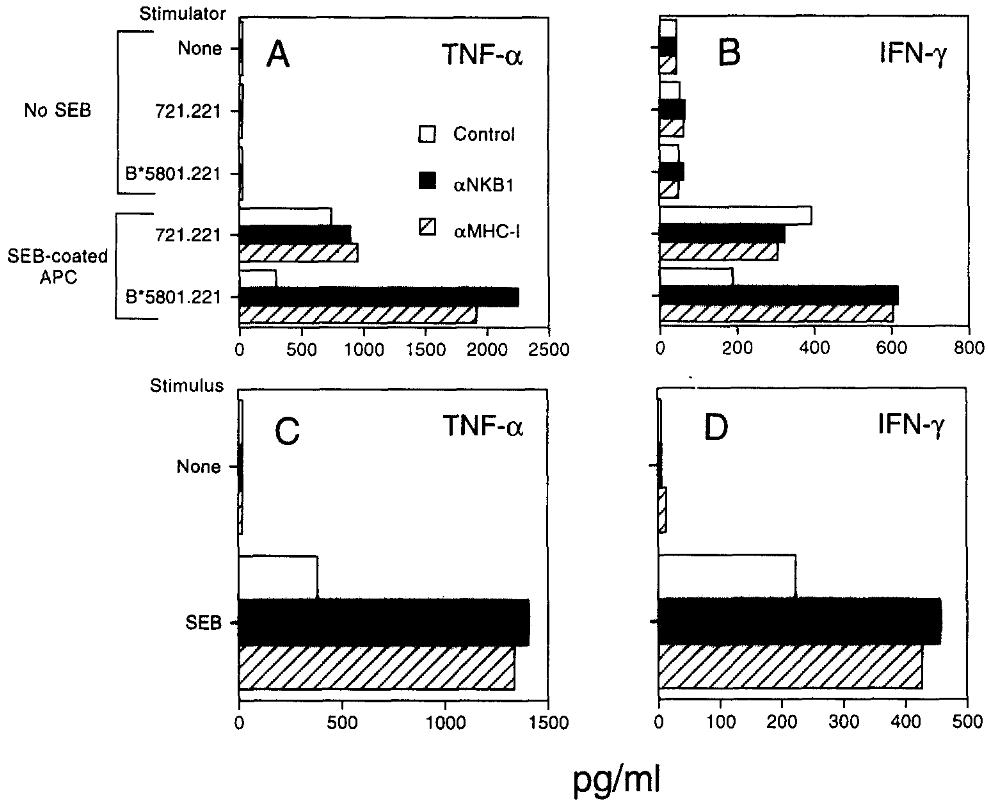
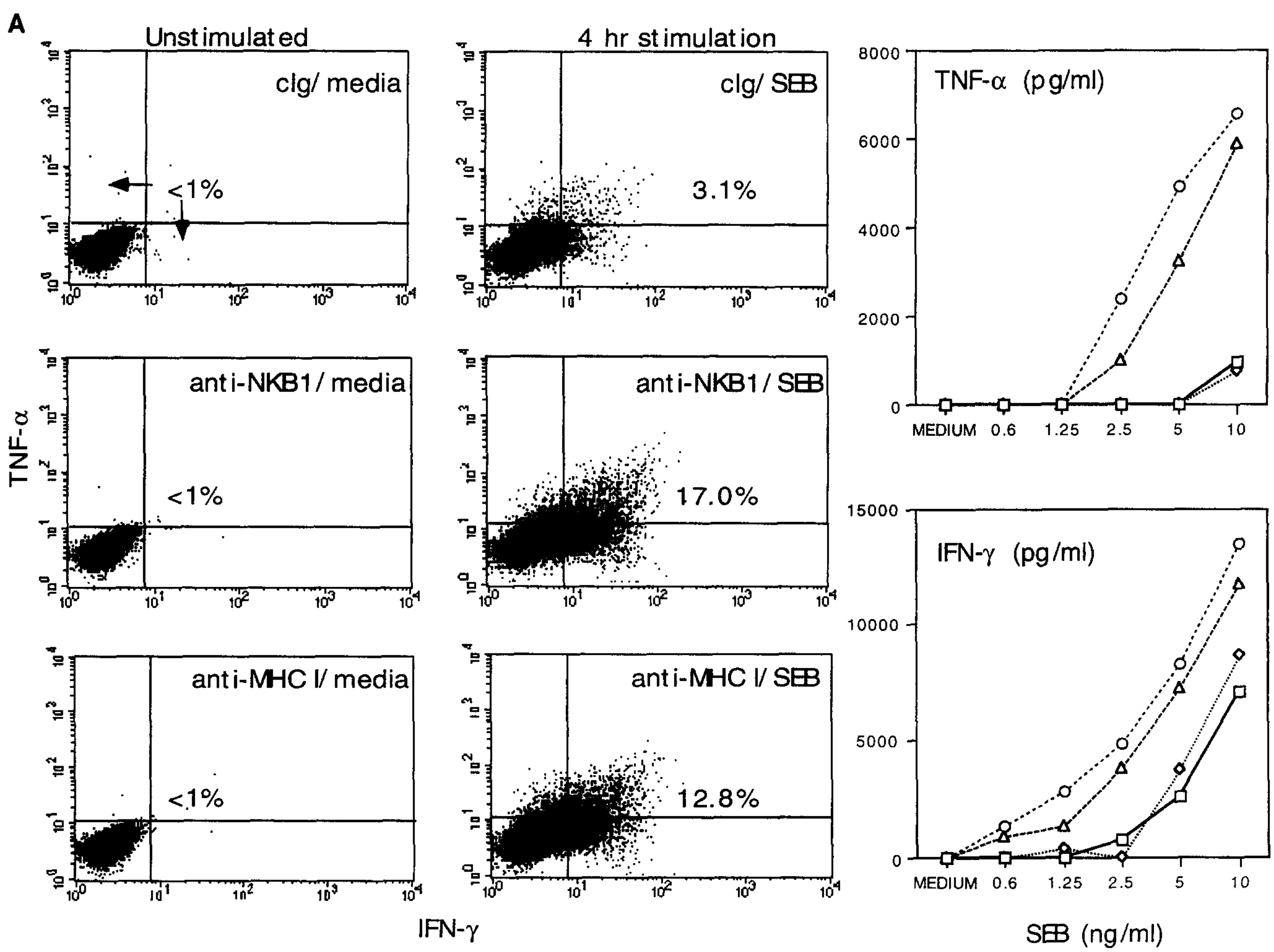


Figure 1. KIR regulation of cytokine production by T cell clones. (*A* and *B*) A NKB1$^+$, CD8$^+$ T cell clone (TC867.9) (10^6 cells/well) was cultured with 721.221 cells or 721.221 cells transfected with HLA-B*5801 (10^6/well) in the presence of control IgG1 (anti-Leu 19, CD56), anti-NKB1 (DX9), or anti-HLA-A,B,C (DX17) (10 μg/ml). The 721.221 cells and transfectants were preincubated with or without 10 ng/ml of SEB (30 min at 37°C) and washed, before coculture with T cells. (*C* and *D*) The T cell clone was incubated (without 721.221 cells) in the presence or absence of 10 ng/ml of soluble SEB and anti-NKB1 or anti-HLA-A,-B, -C (10 μg/ml), as indicated. After 20-h culture at 37°C, cell-free supernatants were collected and assayed for TNF-α (*A* and *C*) or IFN-γ (*B* and *D*) by ELISA (mean of triplicate cultures, SD <10%).



were collected from each well and IFN-γ and TNF-α were measured by ELISA, as previously described (17, 18).

Intracellular Staining. Intracellular cytokines were detected by immunofluorescent staining, as described previously (19). PE-labeled anti-TNF-α and FITC-labeled anti-IFN-γ mAb were generously provided by Drs. Ken Davis and Skip Maino (Becton Dickinson Immunocytometry Systems).

Northern Blot Analysis. Northern blot analysis was performed as previously described (20). RNA (10 μg/lane) was transferred to nylon membranes and hybridized with a ^{32}P-labeled KIR-NKB1 cDNA probe (2). Radioactivity was detected using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results and Discussion

Superantigens are viral and bacterial proteins that activate T lymphocytes by binding to MHC class II molecules on APCs and to the V region of TCR β chains on the responding T cells (21). We previously demonstrated that KIR$^+$ CTL kill superantigen-coated class I–negative B-LCL, but are inhibited when the potential target cells express a HLA class I allele reactive with the KIR. Whereas KIRs have been implicated in regulation of cytotoxicity in both NK and T cells, less is known about the role of these inhibitory receptors in the regulation of other antigen-induced T cell functions, in particular, cytokine synthesis. To this end, we generated KIR$^+$, TCR-α/β$^+$ T cell clones (both CD4$^+$ and CD8$^+$) and selected them for further study based on their ability to produce cytokines in response to stimulation with SEB presented either by the HLA class I–deficient B-LCL 721.221 cell line or 721.221 transfectants stably expressing certain HLA class I alleles. The results presented in Fig. 1 are from a representative CD8$^+$ T

B

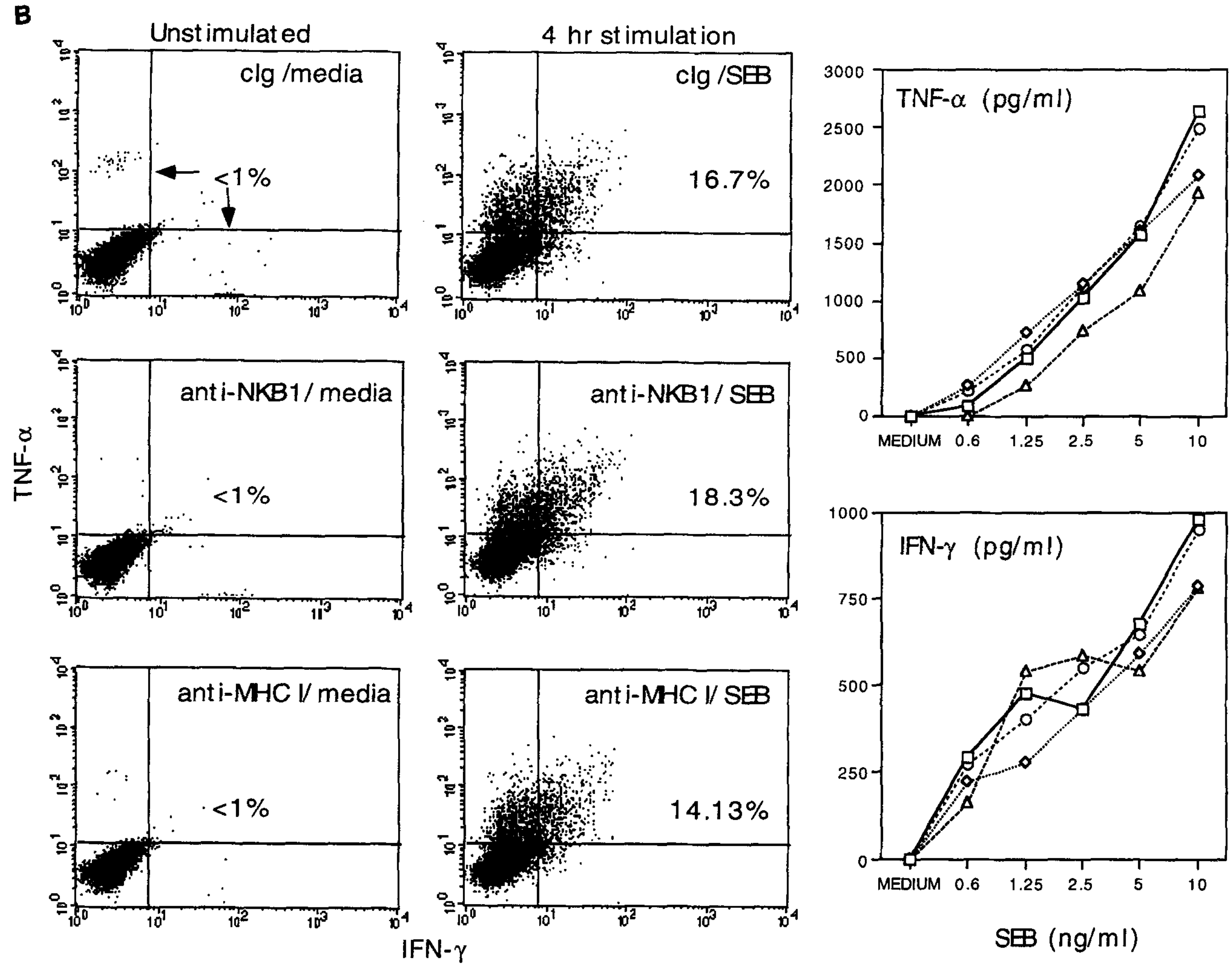


Figure 2. SEB-induced cytokine production by KIR$^+$ and KIR$^-$ T cell clones. For detection of intracellular cytokines, NKB1$^+$ (AD130.2) (*A*) and NKB1$^-$ (T81) (*B*) T cell clones were either untreated or stimulated with 10 ng/ml of soluble SEB (4 h) in the presence or absence of the control IgG1, anti-NKB1, or anti-HLA-A,-B,-C (10 μg/ml). Cells were harvested after 4 h, fixed, and stained with fluorochrome-conjugated control Ig, PE anti-TNF-α, or FITC anti–IFN-γ. Data are displayed as bivariate dot plots (*x* and *y* axes, 4-decade-log scales). In samples stained with FITC- and PE-conjugated IgG control mAb, >98% of cells were in the lower right quadrant (not shown). The total percentage of cells secreting TNF-α, IFN-γ, or both cytokines (sum of upper right, upper left, and lower right quadrants) is indicated in each panel. For detection of secreted cytokines, T cell clones were cultured in the presence of increasing concentrations of SEB for 20 h at 37°C with 10 μg/ml of control IgG1 mAb (◇), anti-HLA-A,-B,-C (DX17) (○), anti-NKB1 (DX9) (△), or medium (□). TNF-α and IFN-γ in the cell-free supernatants of the NKB1$^+$ (*A*) or NKB1$^-$ (*B*) T cell clones were measured by ELISA (mean of triplicate cultures, SD <10%).

cell clone (Tc867.9) expressing the KIR NKB1 which recognizes HLA-Bw4 ligands (7). In the absence of SEB, no cytokines were produced in response to coculture with 721.221 or a 721.221 transfectant expressing HLA-B*5801 (a HLA-Bw4 allele). Higher levels of TNF-α and INF-γ were secreted by the T cell clone when SEB was presented by the class I–negative APC, compared with the HLA-B*5801$^+$ transfectant (Fig. 1, *A* and *B*). Suppression of cytokine production by the HLA-B*5801$^+$ APC apparently is regulated by the KIR present on the T cell clone because anti-NKB1 mAb or anti-MHC class I mAb enhanced cytokine production to a level equal to or higher than when the SEB is presented by the HLA class I–negative APC. In parallel with experiments measuring cytokine secretion, this T cell clone was assayed for its ability to kill SEB-coated 721.221 or HLA-B*5801$^+$ 721.221 cells, and consistent with prior findings, the NKB1 KIR also inhibited superantigen-induced cytotoxicity against targets expressing HLA-B*5801 (not shown). These results demonstrate that binding of the KIR to its class I ligand is not only capable of modulating T cell–mediated cytotoxicity but also has inhibitory effects on T cell activation pathways leading to lymphokine production. These results are consistent with a prior study demonstrating effects of anti-KIR mAb on cytokine production (10).

Whereas studies with MHC-deficient EBV-transformed B-LCL cell lines transfected with a single class I allele are useful to analyze the specificity of KIRs, they do not address the role of these inhibitory receptors in immune responses initiated by normal, autologous APCs. Because activated human T cells express MHC class II, they are able to serve as APCs for superantigens. Therefore, this provides the opportunity to study KIRs regulation of a T cell response in a completely autologous system with SEB presented on autologous class II molecules and KIRs interacting with self-class I ligands. Preliminary studies demonstrated that HLA-DR$^+$ human T cell clones are capable of autopresentation of SEB and that the response is blocked by anti-HLA-DR mAbs (data not shown). To examine the role of KIRs in the regulation of cytokine production induced by autologous APCs, we selected a NKB1$^+$ T cell clone from a HLA-Bw4$^+$ individual so that the KIR is capable of interacting with a self-MHC class I ligand. These KIR$^+$ T cells were cultured with SEB in the presence or absence of anti-NKB1 or anti-HLA class I mAbs, and cytokines were measured (Fig. 1, *C* and *D*). When the T cell clone autopresented SEB, lymphokine production (TNF-α and INF-γ) was substantially increased if self–class I recognition by the KIR was disrupted by either anti-NKB1 or anti-class I mAb. These results demonstrate that interactions between KIRs and self-HLA class I molecules may regulate TCR-induced immune responses initiated by autologous APCs. It should be noted that we used the same NKB1$^+$ T cell clone for the experiments shown in Fig. 1, *A*–*D* to directly compare SEB presentation by the 721.221 APC (Fig. 1, *A* and *B*) and the autologous T cell as APC (Fig. 1, *C* and *D*). Similar results were obtained in both situations.

Further experiments were performed to evaluate the kinetics and the effects of superantigen concentration on KIR regulation of cytokine production. As shown in Fig. 2 *A*, a NKB1$^+$ CD4$^+$ T cell clone (AD130.2) was stimulated with SEB in the presence or absence of anti-NKB1 or anti-HLA class I mAb. To examine early time points in the SEB-induced response, cytokines were detected by using immunofluorescence staining of intracytoplasmic lymphokines. Substantially more TNF-α and INF-γ were present in the cytoplasm of KIR$^+$ T cells stimulated in the presence of anti-NKB1 or anti-HLA class I mAb even within 4 h after SEB activation. After overnight culture, the ability of KIRs to suppress cytokine secretion by SEB-activated T cells was evident at all concentrations of superantigen tested, although at higher antigen doses the inhibition was not absolute, implying a quantitative regulation of activation. In general, KIR regulation of TNF-α production was more profound than control of IFN-γ secretion. IL-2 was not detected in these cultures and superantigen stimulation did not induce substantial proliferation under the conditions studied.

Whereas KIRs are present on a subset of T cells, many T cell clones lack expression of these molecules. For comparison with the KIR$^+$ T cell clones, we selected T cell clones that were unreactive with all existing anti-KIR mAbs (including EB6, GL183, HP-3E4, and DX9) and did not possess KIR transcripts as determined by Northern blot analysis (Fig. 3). These KIR$^-$ T cells were evaluated for SEB responsiveness in the presence or absence of anti-HLA class I mAb. A representative KIR$^-$ CD4$^+$ T cell clone (T81) is shown in Fig. 2 *B*. In this case, anti-HLA class I mAb had no effect on cytokine production induced by SEB, consistent with the lack of inhibitory receptor for self-HLA class I.

Whereas the NKB1 KIRs recognize HLA-Bw4 ligands, other members of the KIR family react with HLA-C or HLA-A molecules (5, 22, 23). KIR reactive with HLA-C may also regulate SEB-induced cytokine production. For example, the CD8$^+$ T cell clone TC867.4 expresses a GL183 KIR that presumably can interact with this individual's HLA-Cw*0703 allele. Consistent with this possibility, anti-HLA class I mAb enhanced SEB-induced cytokine production by this T cell clone (Table 1). An individual NK cell clone can simultaneously express two or more KIRs on the cell surface (22, 24). Moreover, often KIRs are present on NK cells of individuals who apparently lack self–class I alleles able to bind the receptor (25). Similarly, we have observed T cell clones expressing more than one KIR and

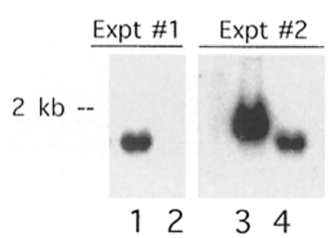


Figure 3. Northern blot analysis of KIR transcripts in T cell clones. Total RNA was extracted from a KIR$^+$ NK cell line (lanes *1* and *4*), from the KIR$^-$ T81 T cell clone (lane *2*), and from the AD130.2 NKB1$^+$ T cell clone (lane *3*). The Northern blot was hybridized with ^{32}P-labeled NKB1 probe. Equivalent amounts of RNA were loaded into each lane as determined by visual inspection of ethidium bromide–stained gels (not shown).

Table 1. *Regulation of TNF-α and IFN-γ Production by KIR Recognition of Self-class I Alleles*

Clone	Phenotype	HLA type	KIR expression	IFN-γ Medium	IFN-γ αNKB1	IFN-γ αHLA-I	TNF-α Medium	TNF-α αNKB1	TNF-α αHLA-I
				pg/ml					
TC867.9	$CD8^+$ TCR-α/β	A*0101, A*2902, B*0801 (Bw6), B*1302 (Bw4) Cw*0603, Cw*0703	NKB1	223	456	427	385	1,405	1,338
AD130.2	$CD4^+$ TCR-α/β	A3, A30, B49 (Bw4), B62 (Bw6), CW3, CW3	NKB1	2,566*	8,315*	7,202*	944	6,548	5,880
TC867.4	$CD8^+$ TCR-α/β	A*0101, A*2902, B*0801 (Bw6), B*1302 (Bw4) Cw*0603, Cw*0703	GL183	113	168	342	137	174	1,332
AD169.5	$CD8^+$ TCR-α/β	A2, A3 B35 (Bw6), B50 (Bw6) Cw4, Cw6	NKB1, GL183	589	474	1,287	153	102	687
T81	$CD4^+$ TCR-α/β	A1, A29 B8 (Bw6), B13 (Bw4) Cw6, Cw7	None	978	946	778	2,634	2,490	1,927

T cell clones were analyzed by flow cytometry for their KIR expression. IFN-γ and TNF-α were analyzed by ELISA after stimulation for 20 h with 10 ng/ml of SEB in the presence or absence of anti-NKB1 (DX9, 10 μg/ml) or anti-MHC class-I (DX17, 10 μg/ml) mAbs. IgG1 control mAb had no effect on cytokine production (not shown).
*SEB concentration = 5 ng/ml.

receptors apparently unable to interact with self-class I ligands. T cell clone AD169.5 represents a cell expressing two KIRs, NKB1 and GL183; however, this individual does not express a HLA-B or HLA-C allele known to interact with NKB1 or GL183 (Table 1). Nonetheless, in functional assays, anti-HLA class I mAb augmented cytokine production, suggesting that yet another KIR or inhibitory receptor for a self-class I allele may be expressed by this clone. Results from these studies indicate that the distribution and function of KIRs on T cells are quite similar to NK cells and reveal the complexity of analyzing T cell clones able to simultaneously express multiple KIRs, only some of which may interact with self-class I ligands.

In summary, our findings suggest that KIRs affect not only NK cell–mediated cytotoxicity, but also cytokine production by $CD4^+$ and $CD8^+$ T cells, implying a more general regulatory role in the immune system. The exact mechanisms governing KIR expression on NK and T cells are unclear at present and an understanding of how the KIR repertoire of an individual is shaped by self-HLA is limited. We have observed that KIRs are preferentially expressed on peripheral T cells with a "memory" phenotype and are rare in thymus and cord blood, suggesting that these inhibitory receptors may be expressed late during differentiation and serve to regulate responses against antigens, although this remains to be proven. It is tempting to speculate that in the T cell lineage, KIR may provide a novel mechanism to increase the threshold of activation for effector T cells, thereby prohibiting stimulation due to encounters with low affinity, self-peptide/MHC complexes. That these receptors, upon binding to their class I ligands, are able diminish the potency of T cell responses, suggests a mechanism for limiting T cell activation possibly operative in the maintenance of tolerance or prevention of autoimmunity.

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