Immunity, Vol. 17, 487–499, October, 2002, Copyright ©2002 by Cell Press

TCR Specificity Dictates CD94/NKG2A Expression by Human CTL

Bana Jabri,[1] Jeanette M. Selby,[2] Horia Negulescu,[1] Leanne Lee,[1] Arthur I. Roberts,[3] Andrew Beavis,[1] Miguel Lopez-Botet,[4] Ellen C. Ebert,[3] and Robert J. Winchester[2,5]

[1]Department of Molecular Biology
Princeton University
Princeton, New Jersey 08544

[2]Division of Autoimmune and Molecular Diseases
College of Physicians and Surgeons
Columbia University
New York, New York 10032

[3]Department of Medicine
University of Medicine and Dentistry of New Jersey
New Brunswick, New Jersey 08903

[4]CEXS-Immunologia
Universitat Pompeu Fabra
Barcelona 08003
Spain

Summary

Activating and inhibitory CD94/NKG2 receptors regulate CTL responses by altering TCR signaling, thus modifying antigen activation thresholds set during thymic selection. To determine whether their expression was linked to TCR specificity, we examined the TCR repertoire of oligoclonal CTL expansions found in human blood and tissues. High-resolution TCR repertoire analysis revealed that commitment to inhibitory NKG2A expression was a clonal attribute developmentally acquired after TCR expression and during antigen encounter, whereas actual surface expression depended on recent TCR engagement. Further, CTL clones expressing sequence-related TCR, and therefore sharing the same antigen specificity, invariably shared the same NKG2A commitment. These findings suggest that TCR antigenic specificity dictates NKG2A commitment, which critically regulates subsequent activation of CTL.

Introduction

The innate immune response to signals triggered by pathogens and necrotic cell products plays a critical role in the activation of naive T cells (Matzinger, 1994; Medzhitov and Janeway, 2000; Steinman et al., 2000). The activation and survival of memory/effector CTL can also be regulated by innate signals, through a set of inhibitory and activating NKR which critically modulate the ability to clear viruses and reject tumors in vivo (Bellon et al., 1999; Groh et al., 2001; Lanier and Phillips, 1996; Moser et al., 2002; Roberts et al., 2001; Speiser et al., 1999; Ugolini and Vivier, 2001; Zajac et al., 1999). NKR might also be implicated in autoimmune T cell-mediated injury (Jabri et al., 2000; Namekawa et al., 2000; R.J. Winchester et al., 2001, Arthritis Rheum., abstract).

Two prominent NKR expressed by CTL are the C-type lectins NKG2D and CD94/NKG2. In humans, NKG2D is expressed by all naive and memory CD8 T cells and binds MICA/B and ULBP proteins (Bauer et al., 1999; Cosman et al., 2001), whereas the CD94/NKG2 family is preferentially expressed by a subset of $CD45RO^{+}$ CD8 T cells (Moretta and Moretta, 1997) and recognizes HLA-E (Braud et al., 1998; Lee et al., 1998). Engagement of NKG2D costimulates CTL activation (Groh et al., 2001; Roberts et al., 2001). However, the functional consequence of ligation of the CD94/NKG2 heterodimer is more complex, because it depends on whether the heterodimer contains an activating (NKG2C, NKG2E/H) or inhibitory (NKG2A/B) isotype (Lopez-Botet et al., 2000). The potential role of NKG2D and CD94/NKG2 and their ligands in the regulation of intraepithelial CTL in normal and diseased tissues has been recently suggested (Jabri et al., 2000; Roberts et al., 2001). By sensing local stress and inflammation through altered levels of their MHC class I and MHC class I-like ligands, they may locally up- or downregulate the adaptive CTL response.

The expression of such receptors on memory/effector CTL raises several important, unanswered questions. Is expression of a given activating or inhibitory NKR a clonal attribute; i.e., is it uniform within the progeny of a T cell clone or is it stochastically determined? Is NKR expression stable or is it a function of the activation status? How is the expression of different isotypes of the same NKR family, which have the same ligand specificity but opposing activating and inhibiting functions, regulated at the clonal level? Given that T cells undergo negative selection during thymic development based on TCR signaling by MHC/self-peptide complexes in the absence of NKR, expression of NKR at the memory/effector stage in the periphery is predicted to profoundly alter the activation thresholds set in the thymus and may thus promote or prevent immunopathological responses and autoimmunity (Lanier and Phillips, 1996). Such considerations suggest that expression of NKR might be tightly regulated and coordinated with individual TCR specificities.

Some of these issues have also been raised for NK cells, but, in the absence of clonal markers, the developmental regulation of NKR expression and the integration of their combined signaling remain poorly understood (Colonna et al., 2000; Long et al., 1997; Raulet et al., 2001; Schatzle et al., 1999; Smith et al., 2000). In fact, because T cells express a well-defined clonal marker, the TCR, which is also the dominant activating signaling force, these issues might be easier to address in T cells than in NK cells. Furthermore, because natural expansions of memory/effector T cells driven by the same antigen often share structural similarities in their TCR, as detected by spectratyping and sequencing (Bourcier et al., 2001; McHeyzer-Williams et al., 1999), it is possible to identify at least some of the CTL clones driven by the same antigen without precise knowledge of this antigen

[5]Correspondence: bjabri@bsd.uchicago.edu

(Arstila et al., 1999; Casanova and Maryanski, 1993; Costello et al., 2001; Yang et al., 1996).

Here, we examined the expression of the inhibitory CD94/NKG2A receptor and its relationship with TCR clonotypic specificity in fresh human CTL using large-scale, high-resolution analysis of their clonotypic repertoire. We identified and studied multiple naturally expanded CTL clones in vivo in the intestinal tissue, as well as in the blood. We found that expression of CD94/NKG2A was a clonal attribute identifying memory/effector CTL with an entirely distinct TCR repertoire. Further, CTL clones expressing distinct but sequence-related TCR, thus presumably of identical antigen specificity, invariably shared the same NKG2A pattern. Finally, our study revealed that NKG2A participates in a negative feedback loop in which TCR stimulation upregulates NKG2A expression, and in turn, NKG2A downmodulates TCR activation.

Results

CD94 and NKG2 Isotype Expression Defines Distinct Subsets in CTL

As previously reported, 30%–45% of intestinal intraepithelial T lymphocytes (T-IEL) expressed CD94, >90% of which were $CD8^+TCR\alpha\beta^+$ (Jabri et al., 2000). Analysis of 15 freshly isolated T-IEL preparations using anti TCR$\alpha\beta$, CD94, and NKG2A mAbs revealed two major populations of $CD94^+$ $TCR\alpha\beta^+$ IEL according to the expression of NKG2A (Figure 1A). The $NKG2A^+$ cells represented on average 40% (range 25%–75%) of the $CD94^+$ population and, as for NK cells (Perez-Villar et al., 1995), they expressed a higher level of CD94 ($CD94^{High}$) than the $NKG2A^-$ cells ($CD94^{Low}$). In PBL, 8%–18% of $CD3^+$T cells expressed CD94 receptors (Jabri et al., 2000), 30%–80% of which were $NKG2A^+$.

Because mAbs specific for the activating NKG2 isotypes are currently not available, we examined mRNA expression by PCR on freshly purified $CD94^-$, $CD94^{High}$ $NKG2A^+$, and $CD94^{Low}NKG2A^-$ subsets of $TCR\alpha\beta^+$ IEL and PBL (Figure 1B) and in clones derived from these subsets (Figure 1C). The NKG2A transcripts were selectively expressed in the $CD94^{High}NKG2A^+$ population and not detectable in the $CD94^{Low}$ or $CD94^-$ subset, whereas the activating isotypes NKG2C, NKG2E, and its splicing form NKG2H were expressed in all subsets. All $CD94^{High}$ $NKG2A^+$ clones expressed NKG2A, and many also expressed NKG2C (98%–100%) and E/H (50%–90%) transcripts, in agreement with results obtained with fresh populations. In contrast, none of the 19 IEL and 24 PBL $CD94^{Low}NKG2A^-$ clones obtained from three healthy individuals expressed detectable levels of NKG2A, whereas 78%–90% contained NKG2C and/or NKG2E transcripts. The remaining clones lacked any detectable transcripts for NKG2A, C, E, or H, suggesting that they expressed CD94 homodimers. This finding was further confirmed at the protein level by CD94 coimmunoprecipitation experiments of ^{35}S-labeled cells (data not shown). Altogether, these results suggest that the $CD94^{Low}NKG2A^-$ population is composed of a major subset defined by the expression of activating CD94/NKG2C, E/H heterodimers, and a minor population expressing nonfunctional CD94 homodimers. Furthermore, the data indicate that NKG2A is selectively present in the subpopulation of $CD94^{High}$ T-IEL and T-PBL, whereas the activating NKG2C and E/H isotypes, or at least their transcripts, are broadly expressed.

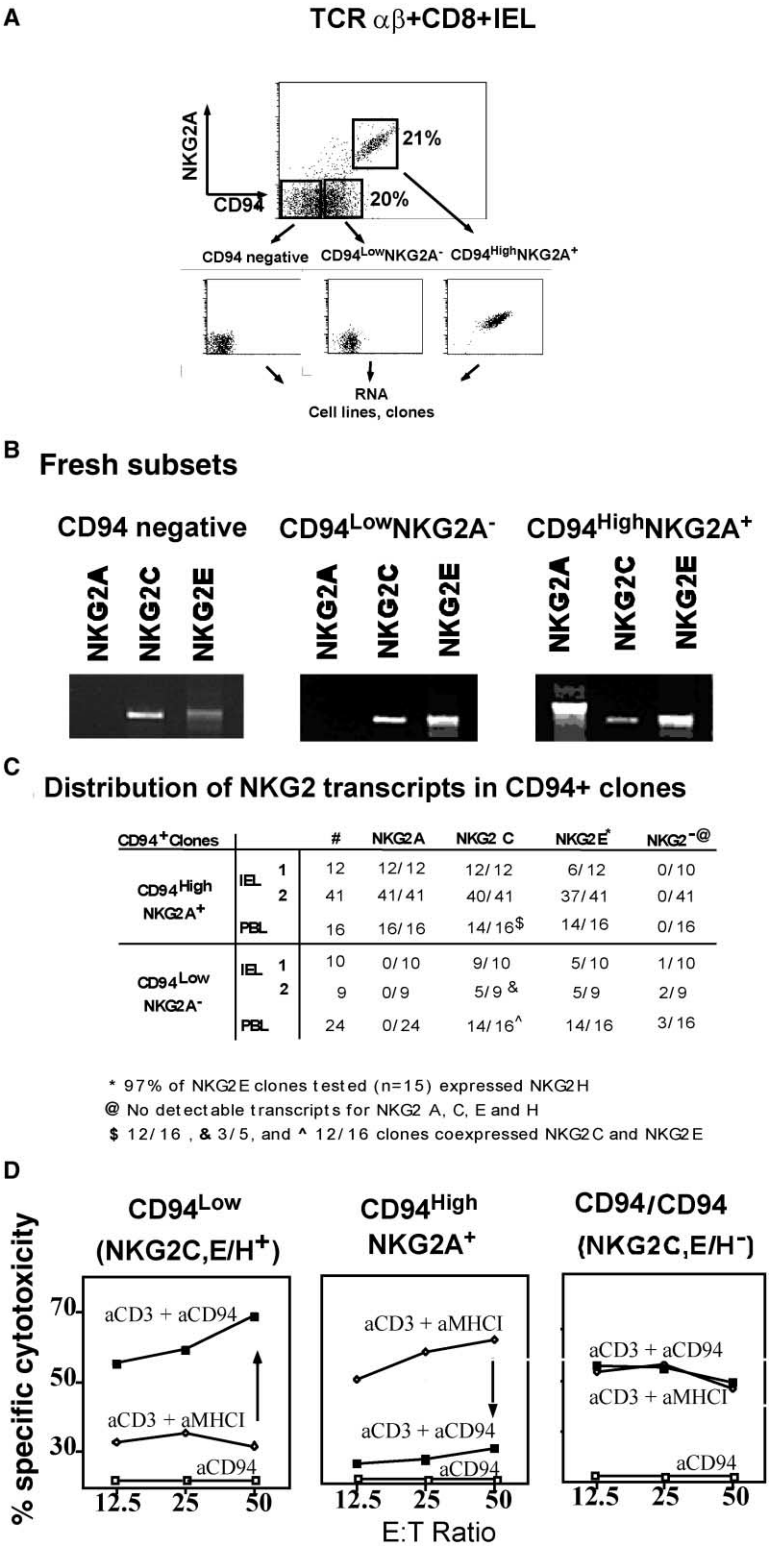
CD94 Receptors in $CD94^{High}NKG2A^+$ and $CD94^{Low}NKG2A^-$ CTL Have Functionally Opposite Properties

Using a redirected lysis assay (Perez-Villar et al., 1995), we examined the effect of co-crosslinking CD94 with the TCR. In these experiments, an anti-MHC class I mAb served as control. Crosslinking CD94 alone on $CD3^+$ $CD94^+$ T-IEL cell lines did not induce target lysis (Figure 1D), whereas, in control experiments with NK cells expressing $CD94^{Low}NKG2A^-$ receptors purified from peripheral blood, this treatment induced specific target cell lysis (data not shown). However, in TCR/CD94 costimulation experiments, crosslinking CD94 markedly enhanced TCR-mediated cytolysis in the $CD94^{Low}NKG2A^-$ ($NKG2C,E/H^+$) cell lines. In contrast, it inhibited TCR-mediated cytolysis in the $CD94^{High}NKG2A^+$ cell lines and had no effect on a cell line expressing CD94 homodimers ($NKG2A,C,E/H^-$) (Figure 1D). Similar results were obtained with multiple IEL lines and clones expressing NKG2C/E/H or NKG2A. These findings suggest that $CD94^+$ T-IEL can be subdivided into three functionally distinct subsets based on the expression of inhibitory $CD94^{High}NKG2A$ receptors, activating $CD94^{Low}$ NKG2C/E receptors, or nonfunctional CD94 homodimers. Because CD94 engagement in clones expressing both inhibitory NKG2A and activating NKG2C, E resulted in TCR inhibition, the inhibitory phenotype appears to be dominant (data not shown).

Distinct Oligoclonal TCR Repertoires in $CD94^{High}NKG2A^+$ and $CD94^{Low}NKG2A^-$ CTL

Taking advantage of the presence of large oligoclonal expansions of memory/effector CTL among T-IEL, we compared the TCR repertoire of the functionally distinct CD94/NKG2 subsets. The IEL preparations obtained from 15 different individuals were four-color stained for $\alpha\beta$ TCR, CD94, NKG2A, and each available BV family. The $\alpha\beta$ TCR repertoires of most individuals were dominated by a few markedly enlarged BV families, which often differed between individuals (Figure 2A). Importantly, the predominant BV families in the $CD94^{High}$ $NKG2A^+$ cells largely differed from that of the $CD94^{Low}$ $NKG2A^-$ cells. For example, in IEL sample H, BV7 was expressed by 17% of the $CD94^{Low}NKG2A^-$ cells but only 1.3% of the $CD94^{High}NKG2A^+$ cells. Conversely, in sample E, BV7 was expressed by 20% of the $CD94^{High}$ $NKG2A^+$ cells and barely detectable in the $CD94^{Low}$ $NKG2A^-$ cells. Interestingly, the BV3 family, which was found to be a major family in several (5 of 15) individuals, as reported by others (Blumberg et al., 1993; Van Kerckhove et al., 1992), always predominated in the $NKG2A^+$ subset (Figure 2A).

Next, we performed a higher resolution analysis of the TCR repertoire by determining the CDR3 β chain length distribution. This analysis demonstrated that the clonal composition of the $CD94^{High}NKG2A^+$ and $CD94^{Low}$ $NKG2A^-$ repertoires differed markedly from one another. Figure 2B contains a representative example from



CD94+Clones		#	NKG2A	NKG2 C	NKG2E*	NKG2-@
CD94High NKG2A+	IEL 1	12	12/12	12/12	6/12	0/10
	IEL 2	41	41/41	40/41	37/41	0/41
	PBL	16	16/16	14/16$	14/16	0/16
CD94Low NKG2A-	IEL 1	10	0/10	9/10	5/10	1/10
	IEL 2	9	0/9	5/9 &	5/9	2/9
	PBL	24	0/24	14/16^	14/16	3/16

* 97% of NKG2E clones tested (n=15) expressed NKG2H
@ No detectable transcripts for NKG2 A, C, E and H
$ 12/16 , & 3/5, and ^ 12/16 clones coexpressed NKG2C and NKG2E

Figure 1. Functional Subsets of CD94+ Memory/Effector CTL According to NKG2 Isotype Expression

(A) Freshly isolated IEL and PBL were stained with anti-TCRαβ, anti-CD8, anti-CD94, and anti-NKG2A antibodies to sort CD8+TCRαβ+ with a CD94HighNKG2A+, CD94LowNKG2A−, or CD94− phenotype. Statistical quadrants were set based on isotype control staining (data not shown).

(B and C) The pattern of NKG2 isotype expression defined by RT-PCR with NKG2 isotype-specific primers is shown in (B) freshly sorted T-IEL subsets and (C) CD94High NKG2A+ and CD94HighNKG2A− clones directly derived from the corresponding purified IEL and PBL subsets.

(D) The function of CD94 receptors was studied in subsets expressing CD94HighNKG2A+, CD94LowNKG2A−, and CD94/CD94 homodimers using a redirected cytolytic assay of P815 cells in the presence of various doses (10–20 ng/ml) anti-CD3 and 1 μg/ml of anti-CD94 mAb or isotype control (anti-MHC class I mAb).

IEL sample D showing that a single BV12 expansion of CDR3 length = 11 was present in the CD94LowNKG2A− subset, whereas reciprocally a BV18 expansion of CDR3 length = 11 was exclusively detected in the CD94High NKG2A+ cells. Likewise, the BV repertoire of the CD94High NKG2A+ and CD94LowNKG2A− subsets of PBL were also found to be composed of major oligoclonal expansions that differed between the two populations by flow cytometry (Figure 2A) and β chain length distribution analysis (Figure 2B).

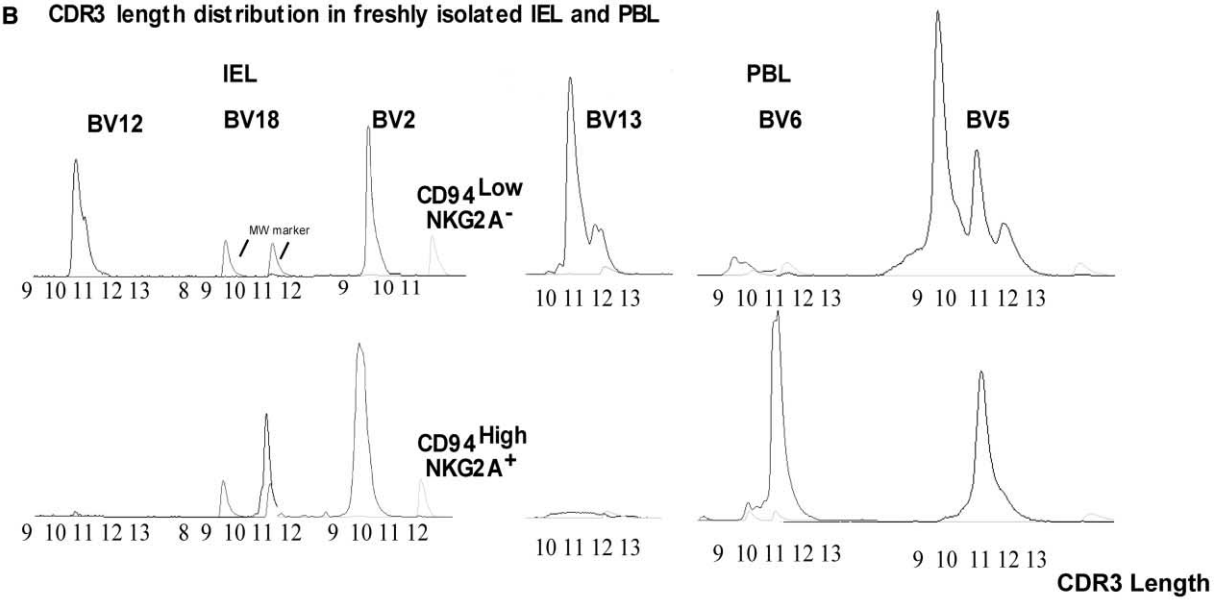
Using the polyclonal CD4+ PBL repertoire as a reference, we measured a marked degree of oligoclonal nar-

A **Quantitative determination of BV family usage in freshly isolated CD8+TCRαβ+CD94+IEL and PBL**

IEL Sample		A	B	C	D	E	F		G		H	I		J		K	L
Predominant BV family		1	1	2	3	3	3	7	3	17	13.1	3	17	7	13.1	14	14	21.3	21.3	21.3
% purified subset	$CD94^{High}NKG2A^{+}$	8	32	13	18	12	12	20	12	7	5	12	1.3	1.3	31	4	16	17	1.5	5
	$CD94^{Low}NKG2A^{-}$	2.7	10	11	0.1	1.2	3	0.1	2	0.1	0.1	0.6	7	17	3.2	17	0.3	0.3	12	2

PBL Sample		P			Q		
Predominant BV family		1	2	5	2	8	12
% purified subset	$CD94^{High}NKG2A^{+}$	1	13	7	10	11	1
	$CD94^{Low}NKG2A^{-}$	17	11	0.5	6	2	8

B **CDR3 length distribution in freshly isolated IEL and PBL**



C **Number of oligoclonal expansions and distances between TCR repertoires in IEL subsets**

	Oligoclonal expansions 10% of BV or greater				Average Hamming distance between		
	Number of oligoclonal expansions				CD94 + IEL subsets	Reference and CD94+ IEL subsets	
	Restricted NKG2A+	Restricted NKG2A-	Shared NKG2A+ and NKG2A-	Total	NKG2A+ and NKG2A-	NKG2A+	NKG2A-
Sample F	7 20%	22 63%	6 17%	35	57	57	73
Sample C	12 31%	20 51%	7 18%	39	46	63	62
Sample D	26 65%	10 25%	4 10%	40	44	62	60
Sample N	7 25%	7 25%	14 50%	28	26	61	63
Sample A	26 41%	24 38%	13 21%	63	53	59	59
Totals	78 38%	83 40%	44 21%	205	226	301	317
Average/ sample	16	16	9	41	45	60	63

rowing in these CD94$^+$ cells, often reduced to one or two expansions for some BV families, with a number of BV families lacking detectable representation in the repertoire. On average, 16 oligoclonal expansions were found among all 23 BV families in the CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ IEL subsets (Figure 2C). Nearly all of the repertoire was contained in these oligoclonal expansions, as expected in a population selected for memory/effector functions. Importantly, the large majority of these oligoclonal expansions typically differed between the two CD94 populations, suggesting a link between TCR and NKG2 isotype usage. Using a statistical measure of the dissimilarity of two repertoire distributions, the Hamming distance (HD), which ranges from 0 for identical populations to 100 if there is no overlap, we found that the CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ subsets were nearly as different from one another (HD = 45) than from the reference panel of polyclonal CD4$^+$ PBL (HD = 60 and 63, respectively).

Heterogeneous Expression of NKG2A in a Few CTL Clones

In apparent exception with the general rule established above, in a small number of cases, the β chain length distribution suggested the presence of shared oligoclonal expansions between the two subsets (Figure 2). In PBL sample P, a BV5 oligoclonal expansion of CDR3 length 11 was found in both the CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ subsets. In IEL sample C, the large shared BV2 subset identified by flow cytometry (Figure 2A) consisted of a similar oligoclonal expansion with a β chain CDR3 length = 10 in both the CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ subsets (Figure 2B). A general analysis across all BV families of four IEL samples suggested that up to 21% of the oligoclonally expanded repertoire might be shared between the CD94^{High} NKG2A$^+$ and the CD94^{Low}NKG2A$^-$ cells (Figure 2C). In one case (sample N), up to half the expansions were potentially shared (Figure 2C). These findings suggested that some degree of heterogeneity in NKG2A expression might exist within some clonal expansions.

This was further examined by bulk sequencing of these BV expansions. Sequencing of BV2 of CDR3 length = 10 in the CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ subsets of sample C (Figure 2) revealed identical Vβ chain sequences: STSGSSYEQY. To determine the nature of the α chains associated with this β chain, the BV2$^+$CD94^{High}NKG2A$^+$ and BV2$^+$CD94^{Low}NKG2A$^-$ subsets were FACS-sorted from cell lines derived from fresh CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ IEL. Sequencing and CDR3 length distribution analysis revealed a single AV19 α chain of CDR3 length = 11: ALSEAYVGKLI in both populations, demonstrating a single, massively expanded BV2 IEL clone with heterogeneous surface expression of NKG2A.

NKG2A Commitment of CTL Clones

We reasoned that these instances of intraclonal heterogeneity for NKG2A expression could reflect stochastic commitment by individual members of some clones or, alternatively, could result from variable levels of induction within clonally committed populations. By combining three different approaches, we demonstrated clonal commitment and showed that NKG2A could be induced in the NKG2A-negative members of NKG2A-positive clones. Further, we showed that these NKG2A-negative, NKG2A-committed cells expressed CD94 homodimers.

First, clones with heterogeneous NKG2A expression were investigated. In 3/3 cases studied, the NKG2A negative members of these clones were found to induce NKG2A upon TCR stimulation in vitro, as shown in Figure 3A for the AV19BV2 clone previously identified in Figures 2A and 2B. In this experiment, NKG2A$^-$ AV19BV2 cells were sorted from the CD94^{Low}NKG2A$^-$ IEL sample C, and upon stimulation by anti-CD3, >60% induced surface NKG2A by day 10 in a time-dependent progressive manner. NKG2A mRNA, which was undetectable in the starting NKG2A$^-$ population, was strongly induced as well. Further, NKG2A expression progressively decayed upon removal from the CD3-coated wells and resting, suggesting that sustained TCR stimulation is necessary to maintain the expression of NKG2A. Neither IL-15 nor IL-12 alone could induce NKG2A expression, although IL-15 did induce CD94 in CD94-negative IEL (Jabri et al., 2000, and data not shown). Identical results were obtained with another oligoclonal expansion, BV16, which was also heterogeneous for NKG2A (data not shown). Interestingly, the proportion of cells in the different CD94^{Low}NKG2A$^-$ IEL subsets that could be induced to express NKG2A upon TCR stimulation roughly corresponded to the proportion of cells present in the CD94^{Low}NKG2A$^-$ that were shared with the CD94^{High} NKG2A$^+$ subset. For example, in Figure 3B, the extent of NKG2A induction in the CD94^{Low}NKG2A$^-$ cell lines C and N (20 and 40%, respectively) paralleled the frequency of clones shared with the CD94^{High}NKG2A$^+$ pop-

Figure 2. Clonal Composition of CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ Subsets of IEL and PBL

(A) Freshly isolated IEL and PBL were stained with anti-CD8, anti-CD94, anti-NKG2A, and a panel of anti-TCRβ BV family-specific antibodies to define BV usage in CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ subsets. All predominant BV families representing 5% or more of the cells are shown.

(B) TCR β chain CDR3 length distribution in freshly isolated CD94^{High}NKG2A$^+$ and CD94^{Low}NKG2A$^-$ IEL and PBL. Representative examples illustrate the oligoclonal nature of the TCR repertoires in IEL and PBL. Peaks corresponding to BV12 in IEL and BV13 and BV5 (CDR3 length 10) in PBL were almost exclusively present in the CD94^{Low}NKG2A$^-$ population. Conversely, BV18 in IEL and BV6 in PBL were almost exclusively present in the CD94^{High}NKG2A$^+$population. In contrast, the BV2 peak in IEL and the BV5 (CDR3 length 11) in PBL were present in both the CD94^{Low}NKG2A$^-$ and the CD94^{High}NKG2A$^+$ subsets. BV12 and BV18 IEL peaks correspond to sample D in (A), BV2 in IEL corresponds to sample C, and all PBL peaks correspond to sample P.

(C) Summary of the number of oligoclonal expansions found in all BV families in each sample according to their presence in the CD94^{Low}NKG2A$^-$ or CD94^{High}NKG2A$^+$ subset. The Hamming distances (HD) were calculated to compare the repertoires of the CD94^{Low}NKG2A$^-$, the CD94^{High} NKG2A$^+$, and the reference CD4$^+$ PBL populations for all BV families in all samples studied. HD values range from 0 for identical subsets to 100 for strictly distinct subsets.

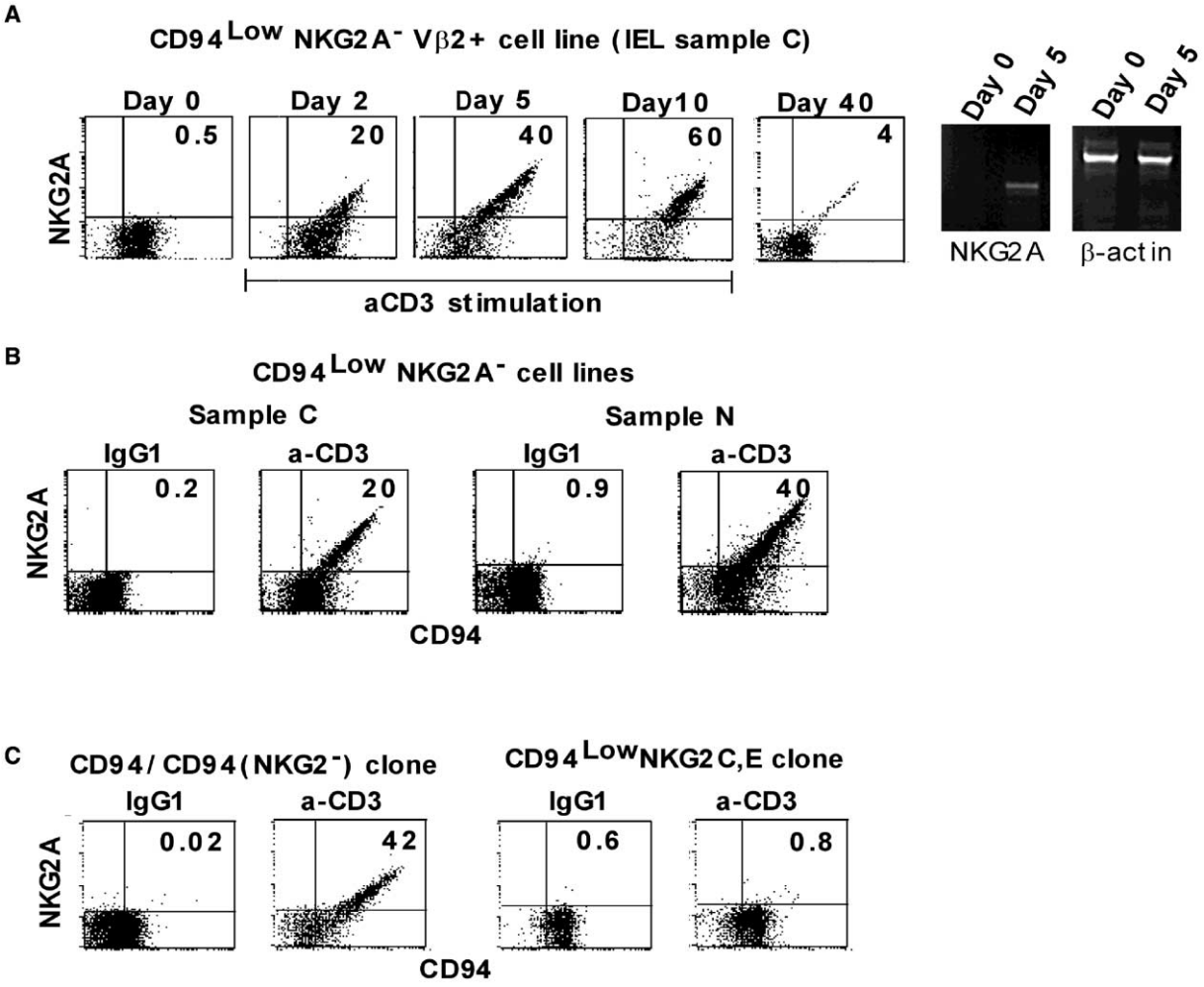


Figure 3. NKG2A Commitment Revealed by TCR Engagement

(A) The CD94^{Low}NKG2A^{-} subset of the Vβ2^{+} IEL oligoclonal expansion in sample C (shown to be heterogeneous for NKG2A expression in Figures 2A and 2B) was purified and stimulated by anti-CD3. Cell surface NKG2A expression progressively increased until d10 when cells were harvested and rested in the absence of anti-CD3. At d40, NKG2A expression was barely detectable. NKG2A transcripts absent at d0 were induced at d5.

(B) Anti-CD3 stimulation was performed to measure the proportion of NKG2A-inducible cells among the CD94^{Low}NKG2A^{-} subsets of samples C and N.

(C) Anti-CD3 stimulated clones expressing CD94 homodimers (CD94^{Low}NKG2^{-}), but not clones expressing activating CD94/NKG2C,E receptors, were induced to express NKG2A.

ulation (18 and 50%, respectively, in Figure 2C). Taken together, these findings supported the notion that members of the same clone shared the same NKG2A commitment, even though in some cases NKG2A expression appeared heterogeneous.

Second, we analyzed the overall population of CD94^{Low}NKG2A^{-} cells that could be induced to express NKG2A upon TCR activation and compared it to the initial CD94^{High}NKG2A^{+} and CD94^{Low}NKG2A^{-} IEL subsets. For this study, CD94^{Low}NKG2A^{-} cells were stimulated with anti-CD3 for 3–4 days, and the NKG2A^{+} (termed NKG2A-induced) cells were sorted for TCR analysis. β chain length distribution and sequencing indicated that, in all cases where clones were shared between CD94^{High}NKG2A^{+} and CD94^{Low}NKG2A^{-} IEL subsets, they were also found in the NKG2A-induced population (Figure 4). For example, the BV4 clone with the CDR3 motif PGGRAYNSPLH and the BV3 clone SPPGLAGNNEQF present in the initial CD94^{High}NKG2A^{+} and CD94^{Low}NKG2A^{-} IEL subsets were also found in the induced NKG2A subset. Remarkably, while these clones represented less than one-third of the initial CD94^{Low} NKG2A^{-} IEL subset, they became dominant in the NKG2A-induced subset. This induction was specific because none of the other 21 clones found in the CD94^{Low} NKG2A^{-} population were induced to express NKG2A, even though their representation was at least 6- to 10-fold greater than the induced clones. In repeated experiments, the TCR repertoire of the NKG2A-induced cells was highly reproducible. Similar results were obtained for 6/6 different BV families (not shown). Under this anti-CD3 stimulation protocol, the stimulation of NKG2A^{-} cell

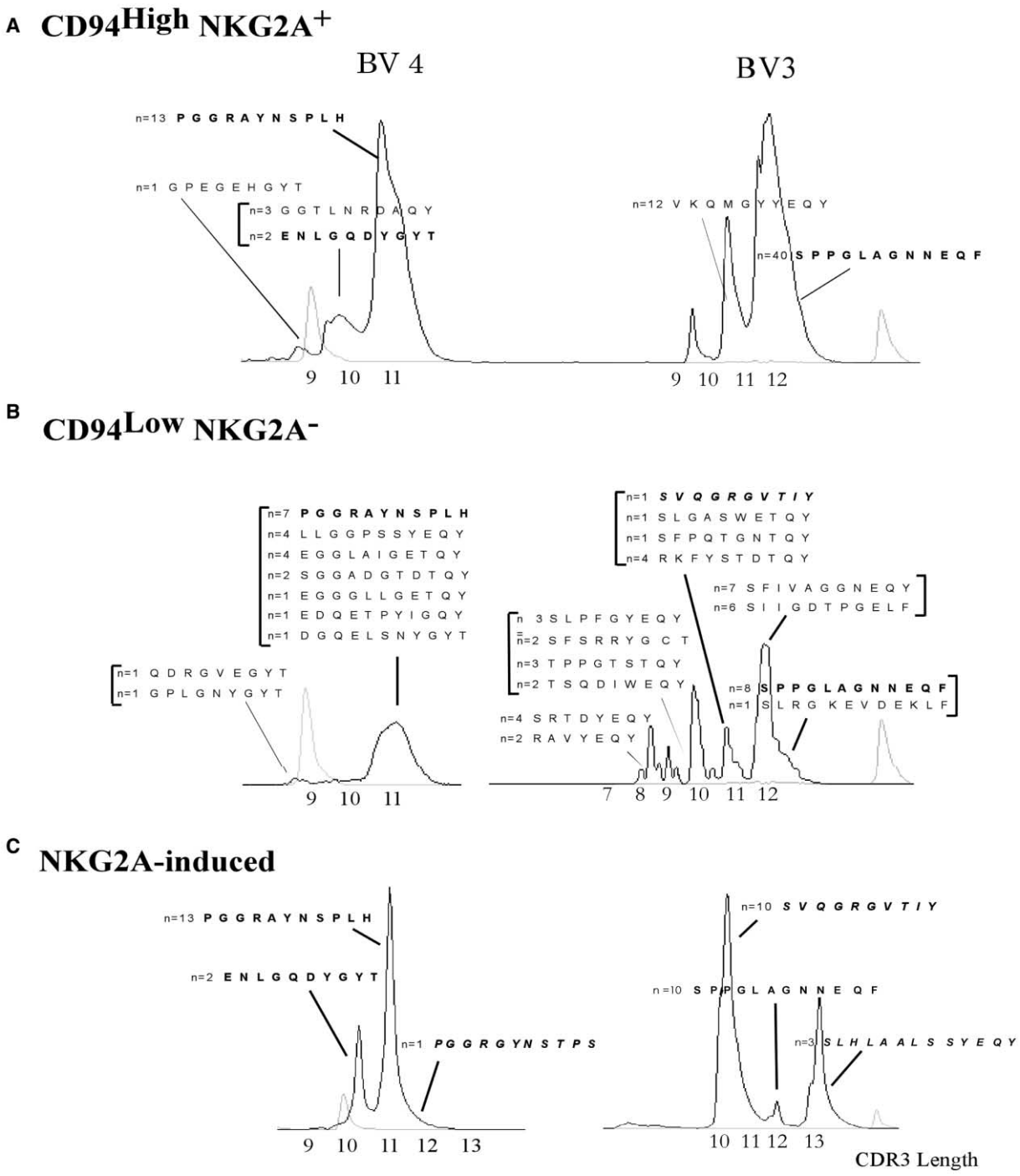


Figure 4. Clonal Patterns of NKG2A Expression and Induction in Precommitted Cells

The BV4 and BV3 repertoires (β chain length distribution and sequencing) of the $CD94^{Low}NKG2A^{-}$ cells induced to express NKG2A ("NKG2A-induced" sorted 3 days after stimulation with anti-CD3) is shown in (C) for comparison with the repertoires of the original, freshly isolated $CD94^{High}NKG2A^{+}$ and $CD94^{Low}NKG2A^{-}$ subsets shown in (A) and (B).

lines such as the one shown in Figure 3B was polyclonal because no significant changes in the Vβ distribution were observed. However, we consistently observed that NKG2A was rapidly and disproportionately induced in a select group of Vβ, ruling out preferential growth of rare, contaminant $NKG2A^{+}$ cells (data not shown). We

also observed rare examples of clones which were only found in the CD94^{Low}NKG2A^{-} subset but could induce NKG2A upon TCR stimulation, such as the BV3 sequence SVQGRGVTIY (Figures 4B and 4C), suggesting that these clones had not been recently activated through their TCR in vivo.

Third, all the CD94^{Low}NKG2A^{-} clones that were shared with the CD94^{High}NKG2A^{+} subset lacked detectable NKG2 C or E/H transcripts, suggesting that they expressed CD94 homodimers. For example, the BV2AV12 and the BV16AV2 CD94^{Low}NKG2A^{-} cells described above that could be NKG2A induced lacked NKG2A, C, and E/H transcripts and instead expressed nonfunctional CD94 receptors (Figure 1D, right panel). In contrast, the NKG2A^{-} cells that remained NKG2A negative upon TCR engagement expressed functional activating CD94/NKG2C or E/H receptors (data not shown). These results suggested that cells expressing CD94 homodimers were precommitted to NKG2A expression. To test this hypothesis, clones expressing CD94 homodimers identified during systematic cloning of CD94^{Low}NKG2A^{-} subsets were tested for their ability to induce NKG2A upon TCR stimulation. These cells represented 15%–20% of the CD94^{Low}NKG2A^{-} subset (Figure 1C), a proportion that correlated well with both the overall percentage of shared clones (see Figure 2C) and the percentage of NKG2A-inducible cells within the CD94^{Low}NKG2A^{-} subset (see Figure 3B). Figure 3C shows a representative example of the 4/4 clones expressing CD94 homodimers that could be induced to express NKG2A. Further, the induction of NKG2A was followed by that of activating NKG2 isoform transcripts, in accordance with our finding that most NKG2A^{+} clones also express activating NKG2 transcripts. In the presence of NKG2A, these activating isoforms should be prevented to costimulate TCR activation. In contrast, NKG2A could not be induced in 40/40 clones expressing functional activating NKG2C, E/H receptors (see one example in Figure 3C). Thus, the component of the CD94^{Low}NKG2A^{-} population which expresses CD94 homodimers (NKG2C, E/H negative) appears to be largely, perhaps entirely, NKG2A committed and poised to express NKG2A on the surface upon TCR stimulation.

Taken together, these results establish that the TCR repertoires of CD94^{High}NKG2A^{+} and CD94^{Low}NKG2A^{-} memory/effector CTL are essentially distinct. The apparent intraclonal heterogeneity with respect to NKG2A expression within some clones corresponds to cells that are NKG2A precommitted but require TCR restimulation for NKG2A reinduction.

CTL Clones Expressing Distinct but Sequence-Related TCR Share the Same NKG2A Commitment

We next wanted to determine whether natural CTL clones expanded by the same antigen in vivo would share the same NKG2A commitment. Multiple studies have described the features that characterize the CDR3 region of antigen-expanded T cells in mice and humans (Bourcier et al., 2001; Kalams et al., 1994; McHeyzer-Williams et al., 1999; Sant'Angelo et al., 1997). These features often allow the identification of T cells driven by the same antigen, even in the absence of knowledge of the antigen (Casanova and Maryanski, 1993; Costello et al., 2001; Yang et al., 1996). We therefore performed plasmid and cell cloning and sequenced multiple β and α chain CDR3 regions found within the same spectratypic bands or oligoclonal expansions, because they would be expected to contain such antigen-driven expansions.

Sequence analysis of CD94^{High}NKG2A^{+} oligoclonal expansions with the same CDR3 length revealed that they were indeed composed of distinct sets of clones with different nucleotide sequences characteristic of antigen drive. Four features of antigen drive are illustrated in Figures 4 and 5:

First, the conserved CDR3 length of expanded clones with the same BV and TRBJ usage (e.g., 4/4 BV9 clones with CDR3 length 9 have the same TRBJ element 2-7*01; 3/4 BV16 clones with CDR3 length 10 have the same TRBJ element 1-2*01).

Second, multiple examples of nongermline-encoded CDR3 sequences were found to be conserved at the amino acid level but degeneratively encoded at the nucleotide level, implying that the TCR were selected at the amino acid level (e.g., in Figure 4: the first four amino acids of the related PGGRGYNSTPSH and PGGRAYN SPLH sequences are nongermline encoded; in Figure 5: for the BV9 sequences, E in position 3 of clone 4, S in position 5 of clone 2, D in position 3, and G-P in position 5-6 of clones 5 and 6 are instances of amino acid conservation; for the BV16 sequences, Q in position 2 of clone 1, V in position 3 of clones 1 and 2, I in position 5 of clones 1 and 3, and D in position 5 of clones 4 and 5 are also instances of amino acid conservation). The pattern of conservation of amino acids corresponding to positions 97, 98, and 100 of β chain, as shown for the BV16 oligoclonal expansion in Figure 5, was also a feature of T cell clonal selection by antigen (Bourcier et al., 2001; McHeyzer-Williams et al., 1999).

Third, to formally establish that the five BV16 clones were driven by the same antigen, we showed that they had homologous α chains as well (Figure 5B). We sorted the BV16-positive T cells from the sample C cell line, cloned them, and determined the $\alpha\beta$TCR sequences of 17 individual T cell clones. Five clonal groups were identified. β chain sequencing retrieved three of the same BV16 sequences as previously found in the fresh population, demonstrating that the clones analyzed were representative of fresh BV16 CD94^{High}NKG2A^{+} population. The two remaining minor clonal groups had the same CDR3 length $= 10$ and shared the degeneratively encoded amino acid motif SxxG, further supporting the presence of antigen drive. Strikingly, the α chains exhibited the same features typical of antigen drive as the β chains. Thus, all five clonal groups shared the same AV12, the same CDR3 length of 9, and a common 4 amino acid motif CVVR (Figure 5B). Furthermore, the third and fourth position in this motif were degeneratively encoded by, respectively, two and four different nucleotide sequences, implying that the homologous α chains had been selected at the protein level by the same peptide. The conclusion that CTL expressing these TCR were not a random set of cells but rather were driven by the same antigen, is based on the following considerations. Assuming that AV family usage is random, the probability that 5/5 BV16 β chains are associated with the same AV is: $(1/46)^5$, where 46 is the number of AV families. This probability should be multiplied by

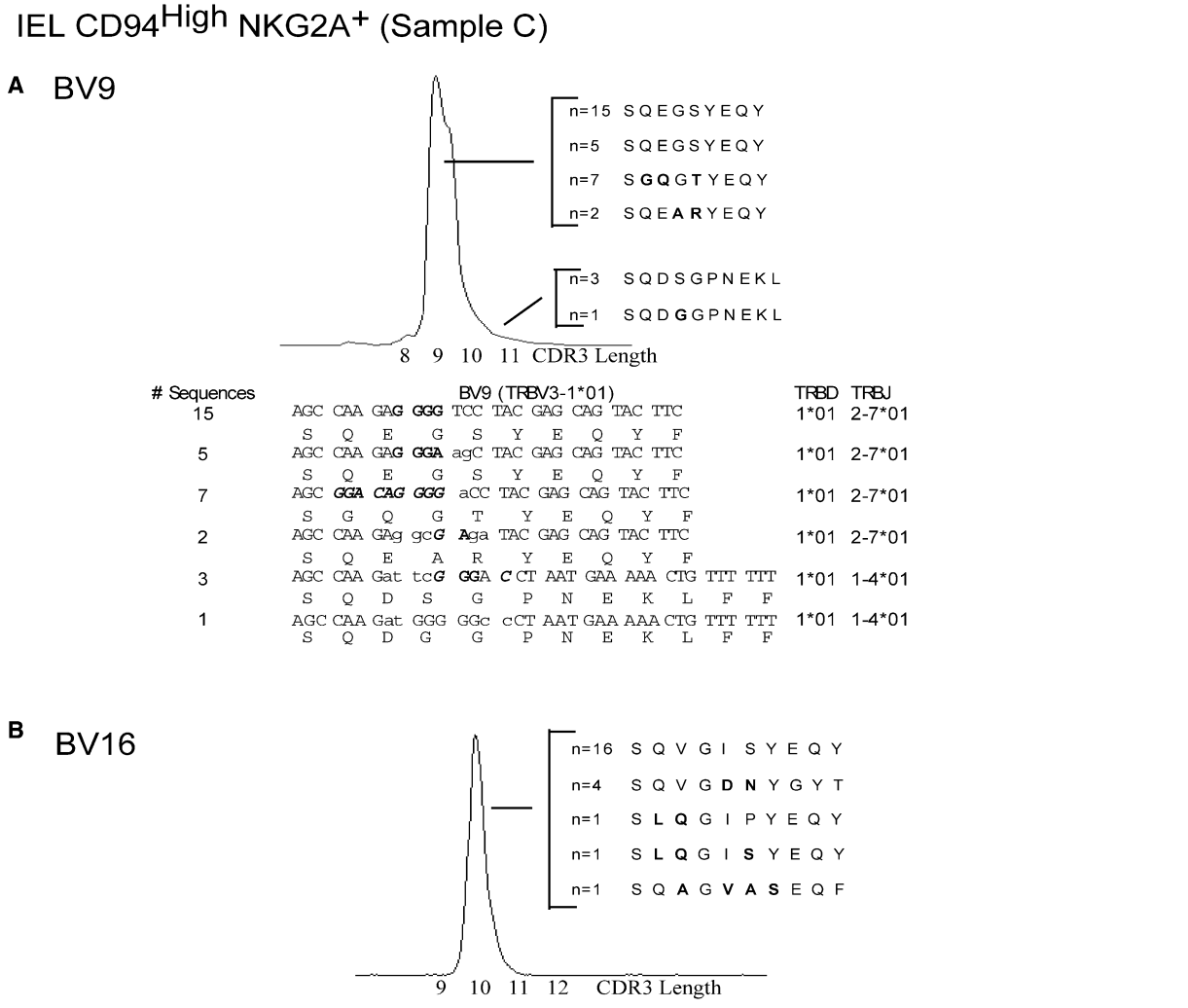


Figure 5. CTL Clones Expressing Sequence-Related TCR Exhibit the Same NKG2A Expression Pattern

(A) Analysis using β chain length distribution and sequencing of two BV9 oligoclonal expansions of CDR3 length = 9 and 11 shows that each oligoclonal expansion is composed of a group of related sequences. Bolded amino acid residues indicate differences from the reference sequence. The lower portion of the figure contains the nucleotide sequence showing differences in the use of somatic and germline elements. Germline origin is indicated in uppercase, with the D element in bolded italics. Somatic origin is shown in lowercase.

(B) β chain sequence analysis of an isolated BV16 oligoclonal expansion of CDR3 length = 10 in freshly purified T cells reveals that the oligoclonal expansion is composed of five related clones. To determine the αβ chain pairing of these clones, the BV16 positive T cells were subcloned and the αβTCR sequences of 17 individual subclones are shown.

the likelihood of selecting five chains of the same CDR 3 length of 9. Assuming 11 to be the median length of the distribution, this is approximately (1/10)[5]. This infinitesimal probability of selecting this combination of structurally similar α chains by chance ($<10^{-13}$) would be further reduced if the probability to express the conserved nongermline-encoded CVVR motif was included. This calculation assumes no pairing bias between AV2 and BV16, which is supported by published studies (Mackensen et al., 1994; Pietra et al., 2001). Thus, the finding that 5/5 different clones with structurally homologous α chains and β chains shared the same NKG2A commitment further strengthens the conclusion already drawn from the extensive TCR β chain repertoire analy-

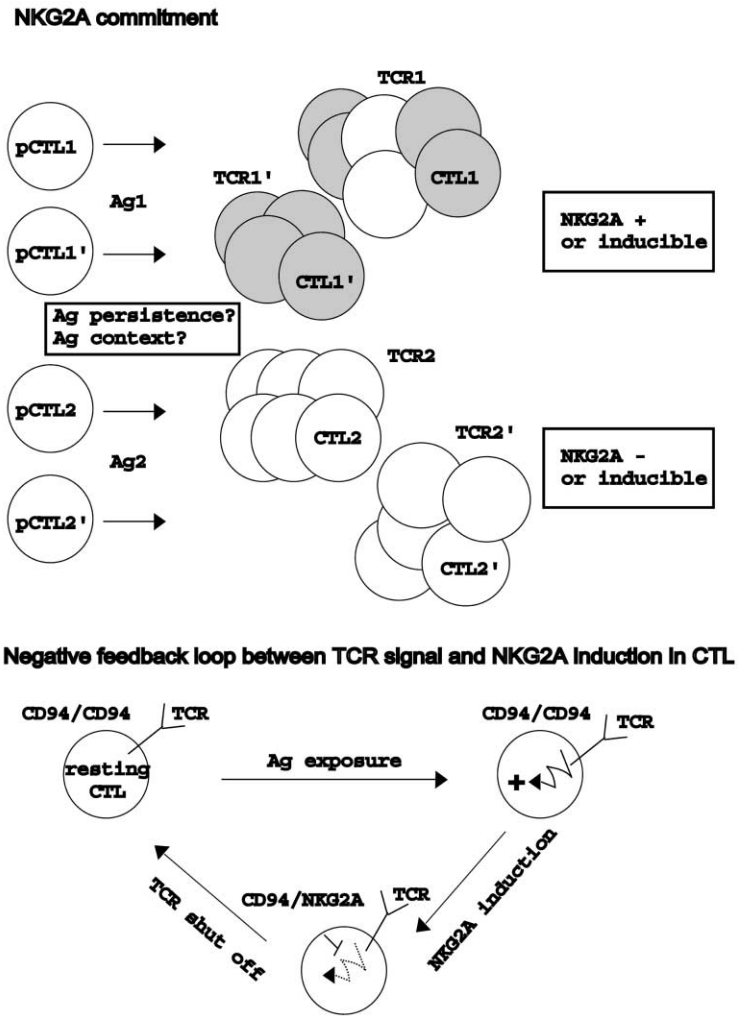


Figure 6. Model for NKG2A Expression and Function in Effector/Memory CTL

(A) Naturally expanded CTL clones expressing related TCR (likely sharing the same antigen specificity) share the same commitment to express NKG2A. Thus, the context in which antigen-specific T cells are primed or expanded during the differentiation process into CD8 effector/memory cells (e.g., the nature of the APC presenting the antigen or the persistent nature of the antigen) determines NKG2A commitment.

(B) NKG2A/TCR dominant-negative circuit loop in effector CTL. Surface expression of NKG2A is dependent on TCR stimulation at the effector stage. Resting effector CTL precommitted to the expression of NKG2A express nonfunctional CD94 homodimers. Upon TCR triggering, they express surface CD94/NKG2A. In turn, CD94/NKG2A exerts a negative feedback signal on TCR activation, if it encounters the appropriate HLA-E ligand. This negative feedback may be an important mechanism to prevent chronic inflammation and autoimmunity in an environment of high antigenic load such as the intestine.

sis in 15 individuals, that TCR specificity for antigen dictates commitment to NKG2A expression. This conclusion implies that NKG2A commitment is a postthymic differentiation event imparted at the clonal level upon in vivo encounter with antigen.

Discussion

By examining memory/effector CTL in humans where a NKG2A-specific mAb is available, we exploited the presence of a clonal marker, the $\alpha\beta$ TCR, and the existence of naturally expanded clones in the blood and intestinal tissue to examine the regulation of NKG2 isotypes at the clonal level and with respect to TCR specificity. We studied $CD94^{High}NKG2A^{+}$ and $CD94^{Low}NKG2A^{-}$ memory/effector subsets freshly purified ex vivo from 15 individuals, as well as corresponding cell lines and clones derived in vitro, combining functional studies and extensive TCR repertoire analysis including determination of TCRα/β pairing.

The results demonstrate that, under natural conditions, commitment to expression of the inhibitory NKG2A isotype is a clonal attribute restricted to a subset of memory/effector CTL expansions. In some cases where a fraction or even the majority of a CTL clone lacked NKG2A, an apparent exception to the clonal rule, TCR engagement promptly reinduced mRNA and protein expression, demonstrating stable commitment at the clonal level. The NKG2A-inducible cells appeared to express CD94 homodimers because they lacked detectable mRNA for NKG2A, C, and E/H isotypes and because CD94 engagement did not modulate TCR activation. In contrast, NKG2A could not be induced in $NKG2A^{-}$ clones expressing activating CD94/NKG2 receptors. We propose a scenario whereby the subset of CTL clones that are committed to NKG2A expression maintain or upregulate NKG2A upon TCR engagement. This in turn exerts a negative feed-back on TCR activation, with subsequent decay in NKG2A expression and return to a state where TCR activation is heightened (Figure 6B). Such regulation, which has not been observed for other inhibitory NKR such as KIR (Vely et al., 2001), is reminiscent of the CTLA-4/B7 regulatory loop (Perkins et al., 1996), but it has specific features adapted to the biology of memory/effector CTL, particularly in tissues such as the intestinal epithelium. Indeed, CD94/NKG2A and HLA-E but not CTLA-4/B7 are significantly expressed in the intestinal environment (Bloom et al.,

1995; Jabri et al., 2000, and B.J., unpublished data). The advantage of such a scenario in a tissue environment of high antigenic load such as the intestine could be to allow some CTL to respond initially maximally to antigens, submitting them later on to a fine-tuning of their TCR signaling threshold in order to downmodulate, for example, persisting immune reactions that might otherwise lead to autoimmunity and chronic inflammation. The flip side of this scenario is that, as recently suggested for the mouse response to Polyoma virus, premature shut-off of the CTL response, perhaps as a viral immune evasion strategy, could facilitate viral persistence and the emergence of virally induced tumors (Moser et al., 2002).

Oligoclonal expansions of memory/effector CTL expressing NKR have previously been detected in blood and tissues (Moretta and Moretta, 1997), but the link between TCR and NKR expression has been difficult to ascertain in vivo. Studies of in vitro-derived human CTL clones found that members of the same clone could be KIR^- or KIR^+ and express different KIR patterns (Uhrberg et al., 2001; Vely et al., 2001), suggesting stochastic expression of these receptors during or after clonal expansion. Our study in the NKG2A system supports and extends the notion that NKR expression by CTL is developmentally a post-TCR phenomenon. However, in marked contrast with the KIR studies, our data demonstrate that NKG2A commitment is not stochastic but is uniformly imparted to the clonal progeny. Most importantly with respect to the regulation of antigen-specific CTL responses, NKG2A commitment appears to be dictated by TCR specificity. First, the TCR repertoire of cells precommitted to the expression of inhibitory NKG2A and those expressing only activating CD94/NKG2 receptors are strictly distinct. Second, our extensive TCR repertoire analysis, which included several α/β chain pairing assignments, permitted the unambiguous identification of multiple examples of clonal expansions expressing related but nonidentical TCR. The role of antigen drive was evident in many cases due to the presence of degeneratively encoded, identical amino acid motifs, or highly conservative amino acid substitutions, whereas it was logically inferred in other cases with more pronounced amino acid changes, by the usage of the same BV, JV element, the same CDR3 length, and the pairing with similar α chains. Thus, the combined results suggest that each of these clones recognizes the same or a similar peptide. In this context, a major finding is that all the identifiable member clones comprising an antigen-driven response consistently shared the same commitment to NKG2A. This finding suggests that only particular antigens and/or particular conditions of antigenic stimulation lead to NKG2A commitment. An alternative explanation is that NKG2A commitment is a temporal marker of CTL clones that recently expanded through the effector phase. However, we do not favor this view for the following reasons. First, fresh $CD94^{High}NKG2A^+$ and $CD94^{Low}NKG2A^-$ CTL had a similar $CD45RO^+$ $CD69^+$ $CCR7^-$ profile and they both exerted redirected cytotoxic activity, the hallmarks of effector CTL (data not shown). Secondly, $NKG2A^-$ CTL clones—with the notable exception of the few NKG2A-committed ones expressing CD94 homodimers—were totally refractory to NKG2A expression upon TCR stimulation and induction of effector functions in vitro. Thus, NKG2A expression appears to be a stable commitment of some memory/effector CTL clones related to their antigen specificity rather than a feature associated with a particular stage of their development. Most interestingly, commitment to NKG2A, when it occurs, seems to be a general feature of all the identifiable CTL involved in the immune response to a particular antigen rather than the attribute of individual clones according to some unique properties, such as high avidity for antigen. One possibility is that NKG2A commitment reflects the persistence of antigen, either in the form of continuous exposure to pathogen or of crossreactivity to a self-peptide. In this context, the presence of NKG2A could prevent clonal exhaustion by terminal differentiation or suppress immunopathological responses. Alternatively, the cytokine environment or the functional features of the activated APC associated with specific contexts such as, for example, viral infection, might induce NKG2A commitment.

Our study examined the natural range of TCR specificities expressed in vivo by natural populations of memory/effector CTL found in human tissues and blood. The findings do not allow the assignment of NKG2A induction to specific immune situations, but they do predict that different immune conditions will result in different patterns of NKG2A induction (Figure 6A). In that respect, a recent report suggested that a majority of mouse CTL specific for the dominant polyoma virus peptide antigen expressed an inhibitory NKG2 isotype, suggesting that at least some viral conditions may be associated with such NKG2A induction (Moser et al., 2002). Together with other recent reports (Speiser et al., 1999; Ugolini and Vivier, 2001), these results support therefore a fundamental role of innate immunity receptors in controlling CTL activation and shaping the memory TCR repertoire. Further studies will be needed to elucidate the conditions leading to NKG2A induction in memory/effector CTL and their relevance to the control of immune responses in healthy and diseased tissues.

Experimental Procedures

mAbs and Cytokines

Fluorochrome-conjugated mAbs against CD3, CD8, TCR$\alpha\beta$, and CD94, unconjugated mAbs against CD3 (UCTH1, IgG1), and CD94 (HP-3D9, IgG1), and isotype controls were purchased from PharMingen (San Diego, CA). Unconjugated anti-human MHC class I ABC B9.12.1 (IgG2a), unconjugated and PE-conjugated anti-CD94 mAb HP-3B1 (IgG2a), PE-conjugated anti-NKG2A Z199 mAb (IgG2b), and FITC- and -PE conjugated mAbs directed against the different TCR BV families were from Coulter-Immunotech (Miami, FL). Recombinant IL-15, IL-7, IL-12, IFNγ, TNF-α, IL-10, TGFβ, and IL-2 were from PharMingen.

Lymphocyte Isolation

IEL were purified from jejunal mucosa obtained from healthy individuals undergoing gastric bypass for morbid obesity as previously described (Taunk et al., 1992). PBL were isolated from whole blood of healthy volunteers by Ficoll density gradient centrifugation (Amersham Pharmacia Biotech) according to standard procedures.

FACS Analysis and Purification of IEL and PBL Subsets

Cells (2×10^5) were incubated with directly conjugated antibodies according to standard protocols. Fluorescence was analyzed on a four-color FACSCALIBUR (Becton Dickinson), with statistical quadrants set to score as negative >99% of control Ig isotype-stained cells.

Freshly isolated IEL and PBL were labeled with anti-CD94, anti-NKG2A, anti-CD8, and anti-TCRαβ mAbs to sort TCRαβ$^+$CD8$^+$ IEL, using a VANTAGE FACS (Becton Dickinson), into CD94^{high}NKG2A$^+$ and CD94^{low}NKG2A$^-$ subsets. Purified subsets were subdivided to extract RNA using Triazol (Life Technologies, Grand Island, NY) and to derive clones and cell lines.

TCR Vβ2$^+$ CD94^{high}NKG2A$^+$ and CD94^{low}NKG2A$^-$ IEL, TCR Vβ9$^+$ CD94^{high}NKG2A$^+$, TCR Vβ16$^+$ CD94^{high}NKG2A$^+$, and CD94^{low}NKG2A$^-$ cells induced to express NKG2A upon TCR stimulation were sorted from the established CD94^{low}NKG2A$^-$ and CD94^{high}NKG2A$^+$ cell lines from sample B. Purified cell populations were used to extract RNA and to derive clones and cell lines.

Cell Lines

Purified lymphocyte populations were either directly stimulated in bulk with 1 μg/ml PHA and a mixture of irradiated heterologous PBLs and EBV-transformed cell lines in RPMI with 100 units/ml IL2 and 10% human AB serum (Sigma Chemicals Co.), as described (Vie et al., 1989), or first cloned by limiting dilution and then stimulated using the same procedure.

Determination of NKG2 Isotype Expression by RT-PCR

Total RNA was extracted from freshly isolated IEL subsets or cell lines according to standard procedures and reverse transcribed using the Promega AMV reverse transcriptase (Madison, WI). First strand cDNA (5 μl) was used as a template for PCR with specific 5′ and 3′ primers chosen in distinct exons. NKG2A/B: 5′ACACTGCAG AGATGGATAACC, 3′AAAATGAGCCCGACACAAATG; NKG2C: 5′TGC AGAGATGAGTAAAC, 3′AACGCAAATGCTTTACT; NKG2E: 5′ACA CAGCTGCAGAGATGAA, 3′GTGTTGATTTCTTGAGC; NKG2H: 5′ATG AGTAAACAAAGAGGAACCTT, 3′AAATAACACAATTCATTTTAAGGC. RT-PCR consisted of 35 cycles at 95°C for 1 min, 55°C (for NKG2A/B and NKG2C) or 58°C (for NKG2E and H) for 1 min, and 72°C for 1 min. Amplified PCR products were analyzed on a 1.5% agarose gel.

CDR3 Length Distribution and Sequence Repertoire Analysis

RNA extraction, generation of oligo(dT) primed cDNA, and CDR3 length distribution analysis were performed as described (Costello et al., 2001). The CDR3 region was defined as the nucleotides 3′ to the conserved CAS motif found in all BV families and 5′ to the FGXG motif. Repertoire analysis of the α chain was performed similarly. For sequencing, the appropriate PCR products were subcloned using a Topo TA Cloning Kit (Invitrogen, Carlsbad, CA), and approximately 50 clones were processed as described (Costello et al., 2001). The TCR nomenclature proposed by Arden et al. (1995) has been supplanted by that of Rowen et al. (1996); see http://imgt.cnusc.fr:8104 (Glusman et al., 2001). However, we retained the old BV nomenclature for correlation with the anti-BV mAb specificities. The degree of clonality was estimated by the Hamming distance (HD) and by the number and size of oligoclonal expansions as described (Costello et al., 2001). An oligoclonal expansion was defined as being 5% greater than the expected area for the given BV family at the 95th percentile cutoff in a normal reference profile. Statistical significance was calculated using the Mann-Whitney U Wilcoxon rank test in SPSS 6.1 (SPSS, Chicago, IL).

Antibody-Mediated Stimulation Assays

For induction of NKG2A, CD94^{low}NKG2A$^-$ freshly purified IEL, cell lines, or clones were cultured in 96-or 24-well plates (Costar, Cambridge, MA) coated with 10 μg/ml (unless otherwise specified) anti-CD3 or irrelevant IgG1 MOPC-21 control antibody.

NKG2A

For Fc-dependent redirected cytotoxicity, CD94^{low}NKG2A$^-$ and CD94^{high}NKG2A$^+$ cell lines and clones were incubated for 4 hr with ^{51}Cr-labeled FcγR$^+$ P815 cells at various E:T ratios in the presence of soluble anti-CD94 (1 μg/ml), and/or anti-CD3 at 20 or 40 ng/ml. Control mouse mAbs were MOPC-21 (IgG1) and MOPC-173 mAbs (IgG2a) and/or B9.12.1 (IgG2a) anti-human HLA-A/B/C. ^{51}Cr release was measured with a γ counter. Maximum release was determined by addition of 2% cetrimide and spontaneous release ranged from 5 to 20% of the maximum. % specific cytotoxicity was calculated as 100×(cpm experimental−cpm spontaneous)/(cpm maximum−cpm spontaneous).

Acknowledgments

The authors thank H. Vie for his advice in deriving human CTL clones, M. Bonneville for discussions, and A. Bendelac and L. Ferradini for discussions and critical reading of the manuscript. This work is supported by National Institutes of Health grants RO1 DK 58727-01A1 and DK 42166-11, ACE U19 AI46132, and grants FIS 00/0181 and SAF2001/0696.

Received: May 31, 2002
Revised: August 7, 2002

References

Arden, B., Clark, S.P., Kabelitz, D., and Mak, T.W. (1995). Human T-cell receptor variable gene segment families. Immunogenetics *42*, 455–500.

Arstila, T.P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. (1999). A direct estimate of the human αβ T cell receptor diversity. Science *286*, 958–961.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science *285*, 727–729.

Bellon, T., Heredia, A.B., Llano, M., Minguela, A., Rodriguez, A., Lopez-Botet, M., and Aparicio, P. (1999). Triggering of effector functions on a CD8$^+$ T cell clone upon the aggregation of an activatory CD94/kp39 heterodimer. J. Immunol. *162*, 3996–4002.

Bloom, S., Simmons, D., and Jewell, D.P. (1995). Adhesion molecules intercellular adhesion molecule-1 (ICAM-1), ICAM-3 and B7 are not expressed by epithelium in normal or inflamed colon. Clin. Exp. Immunol. *101*, 157–163.

Blumberg, R.S., Yockey, C.E., Gross, G.G., Ebert, E.C., and Balk, S.P. (1993). Human intestinal intraepithelial lymphocytes are derived from a limited number of T cell clones that utilize multiple Vβ T cell receptor genes. J. Immunol. *150*, 5144–5153.

Bourcier, K.D., Lim, D.G., Ding, Y.H., Smith, K.J., Wucherpfennig, K., and Hafler, D.A. (2001). Conserved CDR3 regions in T-cell receptor (TCR) CD8+ T cells that recognize the Tax11–19/HLA-A*0201 complex in a subject infected with human T-cell leukemia virus type 1: relationship of T-cell fine specificity and major histocompatibility complex/peptide/TCR crystal structure. J. Virol. *75*, 9836–9843.

Braud, V.M., Allan, D.S., O'Callaghan, C.A., Soderstrom, K., D'Andrea, A., Ogg, G.S., Lazetic, S., Young, N.T., Bell, J.I., Phillips, J.H., et al. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature *391*, 795–799.

Casanova, J.L., and Maryanski, J.L. (1993). Antigen-selected T-cell receptor diversity and self-nonself homology. Immunol. Today *14*, 391–394.

Colonna, M., Moretta, A., Vely, F., and Vivier, E. (2000). A high-resolution view of NK-cell receptors: structure and function. Immunol. Today *21*, 428–431.

Cosman, D., Mullberg, J., Sutherland, C.L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N.J. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity *14*, 123–133.

Costello, P.J., Winchester, R.J., Curran, S.A., Peterson, K.S., Kane, D.J., Bresnihan, B., and FitzGerald, O.M. (2001). Psoriatic arthritis joint fluids are characterized by CD8 and CD4 T cell clonal expansions appear antigen driven. J. Immunol. *166*, 2878–2886.

Glusman, G., Rowen, L., Lee, I., Boysen, C., Roach, J.C., Smit, A.F., Wang, K., Koop, B.F., and Hood, L. (2001). Comparative genomics of the human and mouse T cell receptor loci. Immunity *15*, 337–349.

Groh, V., Rhinehart, R., Randolph-Habecker, J., Topp, M.S., Ridell, S.R., and Spies, T. (2001). Costimulation of CD8αβ T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat. Immunol. *2*, 255–260.

Jabri, B., de Serre, N.P., Cellier, C., Evans, K., Gache, C., Carvalho, C., Mougenot, J.F., Allez, M., Jian, R., Desreumaux, P., et al. (2000). Selective expansion of intraepithelial lymphocytes expressing the HLA-E-specific natural killer receptor CD94 in celiac disease. Gastroenterology *118*, 867–879.

Kalams, S.A., Johnson, R.P., Trocha, A.K., Dynan, M.J., Ngo, H.S., D'Aquila, R.T., Kurnick, J.T., and Walker, B.D. (1994). Longitudinal analysis of T cell receptor (TCR) gene usage by human immunodeficiency virus 1 envelope-specific cytotoxic T lymphocyte clones reveals a limited TCR repertoire. J. Exp. Med. *179*, 1261–1271.

Lanier, L.L., and Phillips, J.H. (1996). Inhibitory MHC class I receptors on NK cells and T cells. Immunol. Today *17*, 86–91.

Lee, N., Llano, M., Carretero, M., Ishitani, A., Navarro, F., Lopez-Botet, M., and Geraghty, D.E. (1998). HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc. Natl. Acad. Sci. USA *95*, 5199–5204.

Long, E.O., Burshtyn, D.N., Clark, W.P., Peruzzi, M., Rajagopalan, S., Rojo, S., Wagtmann, N., and Winter, C.C. (1997). Killer cell inhibitory receptors: diversity, specificity, and function. Immunol. Rev. *155*, 135–144.

Lopez-Botet, M., Llano, M., Navarro, F., and Bellon, T. (2000). NK cell recognition of non-classical HLA class I molecules. Semin. Immunol. *12*, 109–119.

Mackensen, A., Carcelain, G., Viel, S., Raynal, M.C., Michalaki, H., Triebel, F., Bosq, J., and Hercend, T. (1994). Direct evidence to support the immunosurveillance concept in a human regressive melanoma. J. Clin. Invest. *93*, 1397–1402.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu. Rev. Immunol. *12*, 991–1045.

McHeyzer-Williams, L.J., Panus, J.F., Mikszta, J.A., and McHeyzer-Williams, M.G. (1999). Evolution of antigen-specific T cell receptors in vivo: preimmune and antigen-driven selection of preferred complementarity-determining region 3 (CDR3) motifs. J. Exp. Med. *189*, 1823–1838.

Medzhitov, R., and Janeway, C.A., Jr. (2000). How does the immune system distinguish self from nonself?, Semin. Immunol. *12*, 185–8; discussion 257–344.

Moretta, A., and Moretta, L. (1997). HLA class I specific inhibitory receptors. Curr. Opin. Immunol. *9*, 694–701.

Moser, J.M., Gibbs, J., Jensen, P.E., and Lukacher, A.E. (2002). CD94-NKG2A receptors regulate antiviral CD8+ T cell responses. Nat. Immunol. *3*, 189–195.

Namekawa, T., Snyder, M.R., Yen, J.H., Goehring, B.E., Leibson, P.J., Weyand, C.M., and Goronzy, J.J. (2000). Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis. J. Immunol. *165*, 1138–1145.

Perez-Villar, J.J., Melero, I., Rodriguez, A., Carretero, M., Aramburu, J., Sivori, S., Orengo, A.M., Moretta, A., and Lopez-Botet, M. (1995). Functional ambivalence of the Kp43 (CD94) NK cell-associated surface antigen. J. Immunol. *154*, 5779–5788.

Perkins, D., Wang, Z., Donovan, C., He, H., Mark, D., Guan, G., Wang, Y., Walunas, T., Bluestone, J., Listman, J., et al. (1996). Regulation of CTLA-4 expression during T cell activation. J. Immunol. *156*, 4154–4159.

Pietra, G., Romagnani, C., Falco, M., Vitale, M., Castriconi, R., Pende, D., Millo, E., Anfossi, S., Biassoni, R., Moretta, L., and Mingari, M.C. (2001). The analysis of the natural killer-like activity of human cytolytic T lymphocytes revealed HLA-E as a novel target for TCR α/β-mediated recognition. Eur. J. Immunol. *31*, 3687–3693.

Raulet, D.H., Vance, R.E., and McMahon, C.W. (2001). Regulation of the natural killer cell receptor repertoire. Annu. Rev. Immunol. *19*, 291–330.

Roberts, A.I., Lee, L., Schwarz, E., Groh, V., Spies, T., Ebert, E.C., and Jabri, B. (2001). NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. J. Immunol. *167*, 5527–5530.

Rowen, L., Koop, B.F., and Hood, L. (1996). The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. Science *272*, 1755–1762.

Sant'Angelo, D.B., Waterbury, P.G., Cohen, B.E., Martin, W.D., Van Kaer, L., Hayday, A.C., and Janeway, C.A., Jr. (1997). The imprint of intrathymic self-peptides on the mature T cell receptor repertoire. Immunity *7*, 517–524.

Schatzle, J.D., Sheu, S., Stepp, S.E., Mathew, P.A., Bennett, M., and Kumar, V. (1999). Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. Proc. Natl. Acad. Sci. USA *96*, 3870–3875.

Smith, H.R., Chuang, H.H., Wang, L.L., Salcedo, M., Heusel, J.W., and Yokoyama, W.M. (2000). Nonstochastic coexpression of activation receptors on murine natural killer cells. J. Exp. Med. *191*, 1341–1354.

Speiser, D.E., Pittet, M.J., Valmori, D., Dunbar, R., Rimoldi, D., Lienard, D., MacDonald, H.R., Cerottini, J.C., Cerundolo, V., and Romero, P. (1999). In vivo expression of natural killer cell inhibitory receptors by human melanoma-specific cytolytic T lymphocytes. J. Exp. Med. *190*, 775–782.

Steinman, R.M., Turley, S., Mellman, I., and Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. J. Exp. Med. *191*, 411–416.

Taunk, J., Roberts, A.I., and Ebert, E.C. (1992). Spontaneous cytotoxicity of human intraepithelial lymphocytes against epithelial cell tumors. Gastroenterology *102*, 69–75.

Ugolini, S., and Vivier, E. (2001). Multifaceted roles of MHC class I and MHC class I-like molecules in T cell activation. Nat. Immunol. *2*, 198–200.

Uhrberg, M., Valiante, N.M., Young, N.T., Lanier, L.L., Phillips, J.H., and Parham, P. (2001). The repertoire of killer cell Ig-like receptor and CD94:NKG2A receptors in T cells: clones sharing identical $\alpha\beta$ TCR rearrangement express highly diverse killer cell Ig-like receptor patterns. J. Immunol. *166*, 3923–3932.

Van Kerckhove, C., Russell, G.J., Deusch, K., Reich, K., Bhan, A.K., DerSimonian, H., and Brenner, M.B. (1992). Oligoclonality of human intestinal intraepithelial T cells. J. Exp. Med. *175*, 57–63.

Vely, F., Peyrat, M., Couedel, C., Morcet, J., Halary, F., Davodeau, F., Romagne, F., Scotet, E., Saulquin, X., Houssaint, E., et al. (2001). Regulation of inhibitory and activating killer-cell Ig-like receptor expression occurs in T cells after termination of TCR rearrangements. J. Immunol. *166*, 2487–2494.

Vie, H., Chevalier, S., Garand, R., Moisan, J., Praloran, V., Devilder, M., Moreau, J., and Soulillou, J. (1989). Clonal expansion of lymphocytes bearing the γ-δ-T-cell-receptor in a patient with large granular lymphocyte disorder. Blood *74*, 285–290.

Yang, Y., Charlton, B., Shimada, A., Dal Canto, R., and Fathman, C.G. (1996). Monoclonal T cells identified in early NOD islet infiltrates. Immunity *4*, 189–194.

Zajac, A.J., Vance, R.E., Held, W., Sourdive, D.J., Altman, J.D., Raulet, D.H., and Ahmed, R. (1999). Impaired anti-viral T cell responses due to expression of the Ly49A inhibitory receptor. J. Immunol. *163*, 5526–5534.