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# Costimulation through NKG2D Enhances Murine CD8<sup>+</sup> CTL Function: Similarities and Differences between NKG2D and CD28 Costimulation<sup>1</sup>

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Multiple studies have demonstrated that the NK cell activating receptor NKG2D can function as a costimulatory receptor for both mouse and human CD8<sup>+</sup> T cells. However, it has recently been suggested that stimulation through NKG2D is insufficient for costimulation of CD8<sup>+</sup> T cells. To aid in the delineation of NKG2D function in CTL responses, we investigated whether stimulation of NKG2D by the natural ligand RAE1 $\epsilon$  was able to costimulate effector functions of a murine CTL line generated from DUC18 TCR transgenic mice. We found that NKG2D was able to costimulate DUC CTL responses and did so in a manner similar to CD28 costimulation. The T cells exhibited increased proliferation, IFN- $\gamma$  release, and cytotoxicity when presented antigenic peptide by P815 cells expressing RAE1 $\epsilon$  or B7-1 compared with untransfected P815. In addition, both RAE1 $\epsilon$  and B7-1 enhanced Ag-independent IFN- $\gamma$  secretion in response to IL-12 and IL-18 by DUC CTL. However, only costimulation through CD28 allowed for DUC CTL survival upon secondary stimulation, whereas ligation of NKG2D, but not CD28, induced DUC CTL to form an immune synapse with target cells in the absence of TCR stimulation. Understanding the outcomes of these differences may allow for a better understanding of T cell costimulation in general. *The Journal of Immunology*, 2005, 175: 2825–2833.

Naive CD8<sup>+</sup> T cells require engagement of the TCR by MHC molecules presenting antigenic peptide along with secondary costimulatory signals, such as CD28 ligation by B7 family member molecules, to be fully activated and to differentiate into CTL. In contrast, costimulation is not required for CTL to exert effector functions, but can enhance CTL activity. There is a panoply of costimulatory molecules present on both naive and activated T cells that are able to enhance T cell activation or effector function (reviewed in Refs. 1 and 2). The expression of the ligands for the majority of these costimulatory molecules is largely restricted to professional APC, allowing for a role in naive CD8<sup>+</sup> T cell activation, but limiting the potential for these molecules to enhance CTL responses that are directed at virally infected cells or tumor cells. However, the ligands for one potential costimulatory molecule expressed on CTL, NKG2D, are expressed on potential CTL targets.

NKG2D was first characterized as an activating receptor on NK cells (3, 4), but has now been shown to be expressed on a variety of immune cells. NKG2D is constitutively expressed on all human and mouse NK cells (5), but the rest of the cellular expression profile of this receptor differs between mice and humans. In the

mouse, NKG2D is expressed on activated, but not naive, CD8<sup>+</sup> T cells, and subsets of  $\gamma\delta$  T cells and NKT cells (5, 6). In the human, NKG2D is expressed on all CD8<sup>+</sup> T cells, naive and activated, all  $\gamma\delta$  T cells, and possibly on some activated CD4<sup>+</sup> T cells (3, 7, 8). A number of NKG2D ligands have been described, all of which are distantly related to MHC class I molecules in sequence. These ligands, which have all been shown to be up-regulated either in virally infected cells, tumor cells, or otherwise stressed cells, include the RAE1 (RAE1 $\alpha$ - $\epsilon$ ) proteins, H60, and MULT1 in the mouse (5, 9, 10), and MICA/B and the RAET1 family member molecules in the human (3, 11–15). In both mice and humans, expression of NKG2D ligands by target cells potentially induces NK cell cytotoxicity (3, 5, 9, 16, 17). However, the function of NKG2D on CD8<sup>+</sup> T cells is not as clear.

Multiple studies have demonstrated that NKG2D can function as a costimulatory receptor for both mouse and human CD8<sup>+</sup> T cells (3, 5, 9, 16, 17). However, it has recently been suggested that stimulation through NKG2D alone is insufficient for costimulation of CD8<sup>+</sup> T cells, but rather synergizes with other costimulatory molecules (18). Additional studies suggest that under certain circumstances NKG2D may be able to function as a CTL stimulatory receptor, acting independently of TCR stimulation to facilitate tumor killing (8, 19). To aid in the delineation of NKG2D function in CTL responses, we investigated whether stimulation of NKG2D by natural ligands was able to costimulate effector functions of a murine CTL line generated from DUC18 TCR transgenic mice (20). We found that NKG2D was indeed able to costimulate DUC CTL; however, its costimulatory effects differed somewhat from those of CD28.

## Materials and Methods

### Mice

All mice were housed under specific pathogen-free conditions in the Washington University animal facilities in accordance with institutional guidelines. DUC18 TCR transgenic male mice (BALB/c) (20) 7–9 wk old were

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used for experiments. DUC18 TCR transgenic mice were crossed to DAP-10<sup>-/-</sup> (C57BL/6) (21), and sex-matched DUC DAP-10<sup>+/+</sup> and DAP-10<sup>-/-</sup> littermates were used for experiments.

### Cytokines and peptide

Recombinant human IL-2 (rhIL-2)<sup>3</sup> was purchased from Biogen Idec, and recombinant murine IL-12 (rmIL-12) was purchased from BioSource International. The previously identified epitope tumor-expressing mutated ERK-1 (tERK-1) (QYIHSANVL) was synthesized and purified, as previously described (20, 22).

### In vitro activation of DUC T cells

Following RBC lysis, DUC18 splenocytes ( $5 \times 10^6$ /ml) were cultured in six-well plates (5–6 ml/well) in IMDM plus 10% FCS and 55  $\mu$ M 2-ME with 1  $\mu$ M tERK. Three days later, the cells were split 1:2. The following day (day 4), the cells were harvested by purification with Ficoll-Paque Plus (Amersham Biosciences) and placed in fresh medium with rhIL-2 (40 U/ml). When used, rmIL-12 (20 ng/ml) (BioSource International) was also added at this time. The cells were then cultured for another 24 h before harvesting for experiments. The live cells harvested were >95% CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>.

### Passage of DUC T cell line

Each round of stimulation, DUC T cells ( $5 \times 10^5$  cells/well) and mitomycin C-treated P815/B7-1 cells ( $5 \times 10^5$ /well) were cultured together in IMDM plus 10% FCS in a 24-well plate along with  $10^{-9}$  M tERK and 40 U/ml IL-12 for 5 days.

### P815 cell lines

All cell lines were cultured in IMDM plus 10% FCS. P815 is a mastocytoma cell line isolated from a DBA/2 mouse and expresses the H-2<sup>d</sup> haplotype, as do the DUC CTL. RAE1 $\epsilon$  was introduced into P815 to make the P815/RAE1 $\epsilon$  cell line by retroviral transduction. Briefly, RAE1 $\epsilon$  cDNA, the cloning of which has been described elsewhere (23), was placed into pMXIRES-enhanced GFP (24), courtesy of T. Kitamura (University of Tokyo, Tokyo, Japan). This vector was transfected into Plat-E cells (25) using Fugene (Roche), and viral supernatant was harvested 48 h later. P815 cells were then cultured with the viral supernatant and 10  $\mu$ g/ml sequ-brene (Sigma-Aldrich) for 24 h, followed by another 24 h in fresh medium. Enhanced GFP-expressing P815 cells were then collected using a FACS-Vantage SE (BD Biosciences). P815/B7-1 cells (26) were kindly provided by T. Gajewski (University of Chicago, Chicago, IL).

### Generation of anti-RAE1 polyclonal Abs

Oligonucleotides 5'-CATGCCATGGGCAGCCTAGATGATGCACACTCT-3' and 5'-GAAGATCTTCAAGAAGTAGAGTGGCTGGT-3' were used to amplify RAE1 $\epsilon$  cDNA encoding the ectodomain sequence from LDDAHS to PPPHST. To generate the required restriction sites, the final protein included the amino acids MGS appended to the N terminus. The resultant PCR product was ligated into the *Nco*I and *Bam*HI sites of the pET15b expression vector (Novagen). Bacteria (BL21(DE3)-codon plus from Stratagene) transformed with the resultant plasmid were used to produce RAE1 $\epsilon$  inclusion bodies. These were refolded, as previously described (27). Adult laying hens were immunized five times with 100  $\mu$ g of refolded protein in Freund's adjuvant, after which eggs were harvested and processed to yield essentially pure IgY, all according to the standard procedures of Aves Labs. This IgY was shown to react specifically with RAE1 $\gamma$ ,  $\delta$ , and  $\epsilon$  proteins by flow cytometry (data not shown).

### Generation of biotinylated proteins

**Cloning and production of biotinylated rNKG2D ligands.** cDNA for H60, RAE1 $\epsilon$ , and MULT1 were obtained, as described previously (10, 23). Using PCR and standard techniques, the extracellular domains from H60 (MSDGTDSL to QGLSVT), RAE1 $\epsilon$  (MGSLLDDAH to LPPSHST), and MULT1 (MGIEETAS to GSFST) were amplified such that onto each C terminus was appended the amino acid sequence GLNDIFEAQKIEWH. The latter sequence includes a minimal peptide substrate for biotin protein ligase (28). Amino acids in boldface were added to the N termini of the ligand domains to accommodate appropriate restriction sites and an initiator methionine. Resultant PCR products were ligated into the *Nco*I and *Xho*I sites of pET28a (Novagen) yielding constructs that encoded the bold-faced amino acids, followed by the ligand ectodomains, a biotin ligase

substrate, and, lastly, a hexahistidine tag. These bacterial expression vectors were transformed into BL21(DE3)-codon plus bacteria (Stratagene) and grown in Luria-Bertani medium containing 34  $\mu$ g/ml chloramphenicol and 50  $\mu$ g/ml kanamycin until an  $A_{280.1 \text{ nm}}$  of 0.8 was achieved. Expression was then induced using 1 mM isopropylthiogalactoside (Sigma-Aldrich) for 3–4 h. Bacterial pellets were processed and refolded essentially as described previously (27). Following further purification on Superdex 75 size exclusion columns (Amersham Biosciences) in 20 mM HEPES/150 mM NaCl/1 mM EDTA, pH 7.5, proteins were biotinylated using BirA ligase (Avidity), according to the manufacturer's directions. Biotinylated proteins were concentrated and repurified using size exclusion chromatography, as above. They were used for experimentation, as described below.

### NKG2D, RAE1 $\epsilon$ , and B7-1 expression analysis

NKG2D expression by the DUC CTL was determined by staining with either a PE-labeled NKG2D-specific Ab (eBioscience), a nonlabeled NKG2D-specific Ab (clone C7 (29)), followed by a PE-labeled anti-hamster Ab (Jackson ImmunoResearch Laboratories), or RAE1 $\epsilon$  tetramers made by mixing biotinylated RAE1 $\epsilon$  with streptavidin-PE (eBioscience). RAE1 $\epsilon$  and B7-1 expression was measured by staining cells with the pan anti-RAE1 or B7-1 Ab (BD Biosciences), followed by a Cy-5-labeled anti-chicken (Jackson ImmunoResearch Laboratories) or a PE-labeled anti-hamster Ab (BD Biosciences), respectively.

### Proliferation assays

DUC CTL were labeled with 10  $\mu$ M CFSE and put into a 24-well plate ( $5 \times 10^5$ /well) with target cells ( $5 \times 10^5$ /well) with or without  $10^{-8}$  M tERK for 48 h. The cells were then harvested and stained with a PE-labeled V $\beta$ 8.3-specific Ab and propidium iodide, and the cells were analyzed using a FACScan (BD Biosciences).

### Cytotoxicity assays

DUC CTL cytotoxicity was measured using a standard chromium release assay. <sup>51</sup>Cr-labeled target cells were plated in 96-well round-bottom plates (10,000 cells/well) with DUC CTL starting at an E:T cell ratio of 30:1. The plates were spun briefly and cultured for 4 h at 37°C. The supernatants were then collected and read on a MicroBeta counter (PerkinElmer), and the percent specific lysis was determined as: ((sample counts per minute – spontaneous counts per minute)/(maximum counts per minute – spontaneous counts per minute))  $\times$  100.

### Intracellular cytokine staining

DUC CTL that had been cultured with IL-12 ( $10^5$ /well) and target cells ( $10^5$ /well) were plated in a 96-well V-bottom plate, spun briefly, and cultured at 37°C for 4 h. The cells were then stained with a FITC-labeled CD8-specific Ab, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% saponin in PBS, and stained with a PE-labeled IFN- $\gamma$ -specific Ab (BD Biosciences). The cells were then analyzed using a FACScan (BD Biosciences).

### IFN- $\gamma$ ELISA

DUC CTL that had been cultured with IL-12 were cultured for 24 h with target cells with or without 10 ng/ml rmIL-18 (BioSource International). Supernatants from these cultures and recombinant murine IFN- $\gamma$  (provided by R. Schreiber, Washington University, St. Louis, MO) as a standard were incubated overnight in 96-well Immulon 2 HB plates (Thermo Labsystems) that were precoated with 1.5  $\mu$ g/ml anti-mouse IFN- $\gamma$  (clone H22; provided by R. Schreiber). The plates were washed with PBS/0.5% Tween 20, and goat anti-mouse IFN- $\gamma$  serum (provided by R. Schreiber) diluted in IMDM plus 10% FCS was added for 1 h. The plates were washed again, and bovine anti-goat IgG-HRP (Santa Cruz Biotechnology) diluted in IMDM plus 10% FCS was added (1  $\mu$ g/ml) for 0.5 h. The plates were washed again, and ABTS in citrate buffer was added as substrate (Sigma-Aldrich). The absorbance was then measured at 405 nm. A standard curve was generated and used to determine the amount of IFN- $\gamma$  present in each supernatant.

### Lipid bilayer preparation

GPI-anchored ICAM-1 labeled with Cy5-N-hydroxysuccinimide (Amersham Biosciences) was incorporated into liposomes, as described previously (30). Biotinylated liposomes were made by mixing egg phosphatidylcholine with biotinylated phosphatidylcholine at a 100:1 ratio. Planar bilayers were made by mixing the ICAM-1-containing liposomes and the biotinylated liposomes 1:1 on clean glass coverslips in a parallel plate flow cell (Bioptechs). Streptavidin Alexa Fluor 488 or 555 conjugate (Molecular

<sup>3</sup> Abbreviations used in this paper: rhIL, recombinant human IL; C-SMAC, central supramolecular activation cluster; HBS, HEPES buffered saline; IS, immune synapse; rmIL, recombinant murine IL; tERK, tumor-expressing mutated ERK-1.

Probes/Invitrogen Life Technologies) was flowed (1  $\mu\text{g}/\text{ml}$ ) over the bilayer, followed by a wash with 5 ml of HEPES-buffered saline (HBS). When used, biotinylated anti-CD3 $\epsilon$  (2C11, 1  $\mu\text{g}/\text{ml}$ ; BD Biosciences), RAE1 $\epsilon$ , MULTI1, or H60 (1  $\mu\text{g}/\text{ml}$ ) was flowed over the bilayer, followed by a wash with HBS. Cells were then injected into the warmed (37°C) flow cells in HBS plus 1% human serum albumin (Alpha Therapeutic). In some experiments, the T cells were fixed by flowing over 49% paraformaldehyde and stained with an anti-TCR $\beta$ -FITC (BD Biosciences). Cells were imaged using a Zeiss LSM510 confocal system. All images of cells on bilayers were taken with the pinhole open.

#### Formation and imaging of T cell-P815 conjugates

DUC CTL ( $10^6$ ) were mixed with target cells ( $10^6$ ) in 100  $\mu\text{l}$  of IMDM without FCS in 1.5-ml tubes. The cells were spun briefly and incubated 5 min at 37°C. The cells were then fixed in 4% paraformaldehyde in PBS, treated with 0.1 M glycine in PBS, and washed once with PBS before being placed on poly(L-lysine) (Sigma-Aldrich)-coated slides. The cells were stained with rat anti-mouse LFA-1 (H155.78 (31)), followed by Cy3-labeled goat anti-rat (Jackson ImmunoResearch Laboratories) and either biotinylated anti-mouse CD3 $\epsilon$  Ab (2C11; BD Biosciences), chicken anti-mouse RAE1, or hamster anti-mouse B7-1 (BD Pharmingen), followed by streptavidin Alexa Fluor 488, Cy5-labeled rabbit anti-chicken (Jackson ImmunoResearch Laboratories), or Cy5-labeled goat anti-hamster (Jackson ImmunoResearch Laboratories), respectively. Confocal images were taken using a Zeiss LSM510 confocal system.

## Results

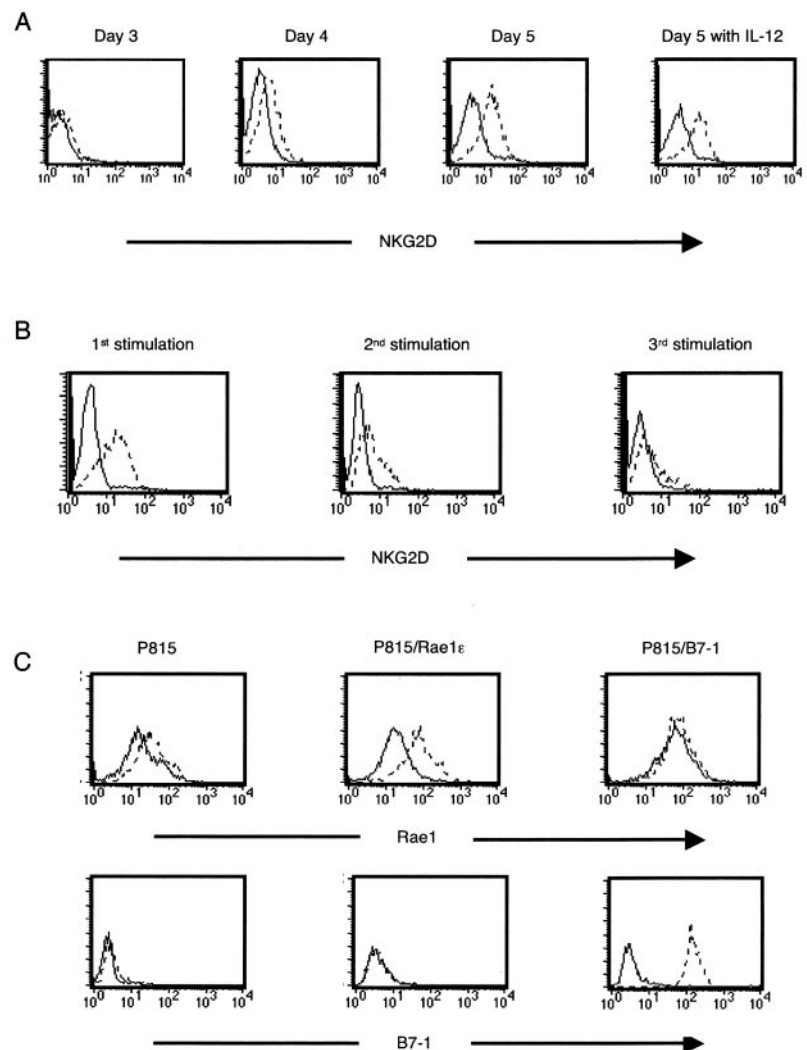
### DUC T cells express NKG2D upon activation

To characterize the function of NKG2D in CD8 $^+$  T cell responses, we studied CD8 $^+$  T cells from the DUC18 TCR transgenic mouse (20).

This mouse produces CD8 $^+$  H-2k $^d$ -restricted T cells specific for a peptide (named tERK) encoded by a mutated ERK2 protein expressed by the syngeneic CMS5 fibrosarcoma. We first confirmed that NKG2D surface expression is up-regulated in DUC T cells following activation. DUC T cells were stained with an NKG2D-specific Ab following *in vitro* activation with the tERK peptide and autologous splenocytes and analyzed using flow cytometry (Fig. 1A). NKG2D expression was easily detected on the surface of DUC T cells on day 4 and continued to increase on day 5. Because IL-12 is important in the differentiation of CD8 $^+$  T cells into CTL (32–36), we also tested whether culturing activated DUC T cells with IL-12 for the last 24 h of culture affected NKG2D surface expression. This exogenous IL-12 had no detectable effect on NKG2D surface expression. This result does not, however, rule out a possible role for endogenous IL-12 in NKG2D expression on the DUC CTL. Because NKG2D is thought to be expressed on memory, as well as activated, CD8 $^+$  T cells (17), we expected NKG2D surface expression to remain stable over multiple rounds of *in vitro* stimulation. We found, however, that NKG2D surface expression decreased with each subsequent round of stimulation (Fig. 1B). Therefore, all of our studies were performed with DUC CTL generated by a single stimulation.

### The NKG2D ligand RAE1 $\epsilon$ enhances CTL effector functions

To test the effect of NKG2D stimulation on DUC CTL function, we expressed the NKG2D ligand RAE1 $\epsilon$  in P815 cells, an MHC-compatible target for DUC18 T cells (Fig. 1C). We used P815 cells



**FIGURE 1.** NKG2D expression by DUC T cells is up-regulated upon primary stimulation, but is down-regulated after multiple rounds of *in vitro* stimulation. *A*, DUC T cells were harvested 3, 4, and 5 days following activation with or without IL-12. The cells were stained with an anti-NKG2D Ab, followed by a secondary anti-hamster Ab (dotted line) or secondary Ab alone (solid line). *B*, DUC T cells were harvested after one, two, and three rounds of *in vitro* stimulation and stained with a PE-labeled NKG2D-specific Ab (dotted line) or control Ab (solid line). *C*, Expression of RAE1 and B7-1 by target cells. P815, P815/RAE1 $\epsilon$ , and P815/B7-1 cells were stained with a pan anti-RAE1 Ab, followed by a secondary anti-chicken Ab, and anti-B7-1 (dotted lines) or control Abs (solid lines). Results shown are representative of multiple independent experiments.

transfected with B7-1 to compare any effect seen with NKG2D ligation to CD28 ligation. The nonmanipulated P815 cell line does not naturally express any NKG2D ligands, as measured by NKG2D tetramer staining (data not shown), or CD28 ligands (26). In addition, the three cell lines expressed comparable levels of ICAM-1 (data not shown). We began by determining whether RAE1 $\epsilon$  or B7-1 expression could enhance the formation of conjugates with DUC CTL. Because the affinity of NKG2D for RAE1 $\epsilon$  (28 nM  $K_D$  (23)) is much greater than that of CD28 for B7-1 (20  $\mu$ M  $K_D$  (37)), we expected RAE1 $\epsilon$  to enhance the number of DUC CTL that formed conjugates with P815 cells to a greater extent than B7-1. However, both B7-1 and RAE1 $\epsilon$  were able to augment conjugate formation between DUC CTL and P815 cells both in the absence and presence of antigenic peptide (Fig. 2, A and B). These results suggest that these costimulatory molecules may enhance conjugate formation not simply by increasing adhesion between the two cells, but rather via initiating a signaling pathway.

We next tested whether NKG2D stimulation could enhance multiple DUC CTL effector functions. Expression of RAE1 $\epsilon$ , as with B7-1, on P815 cells increased DUC CTL proliferation (Fig. 2C), cytotoxicity (Fig. 2D), and IFN- $\gamma$  secretion (Fig. 2E) in response to antigenic peptide. And like B7-1, RAE1 $\epsilon$  was unable to induce any of these effector responses in the absence of TCR stimulation by antigenic peptide. In addition, both RAE1 $\epsilon$  and B7-1 enhanced IFN- $\gamma$  secretion by DUC CTL in response to the cytokine combination of IL-12 and IL-18 in the absence of Ag. Although it had been demon-

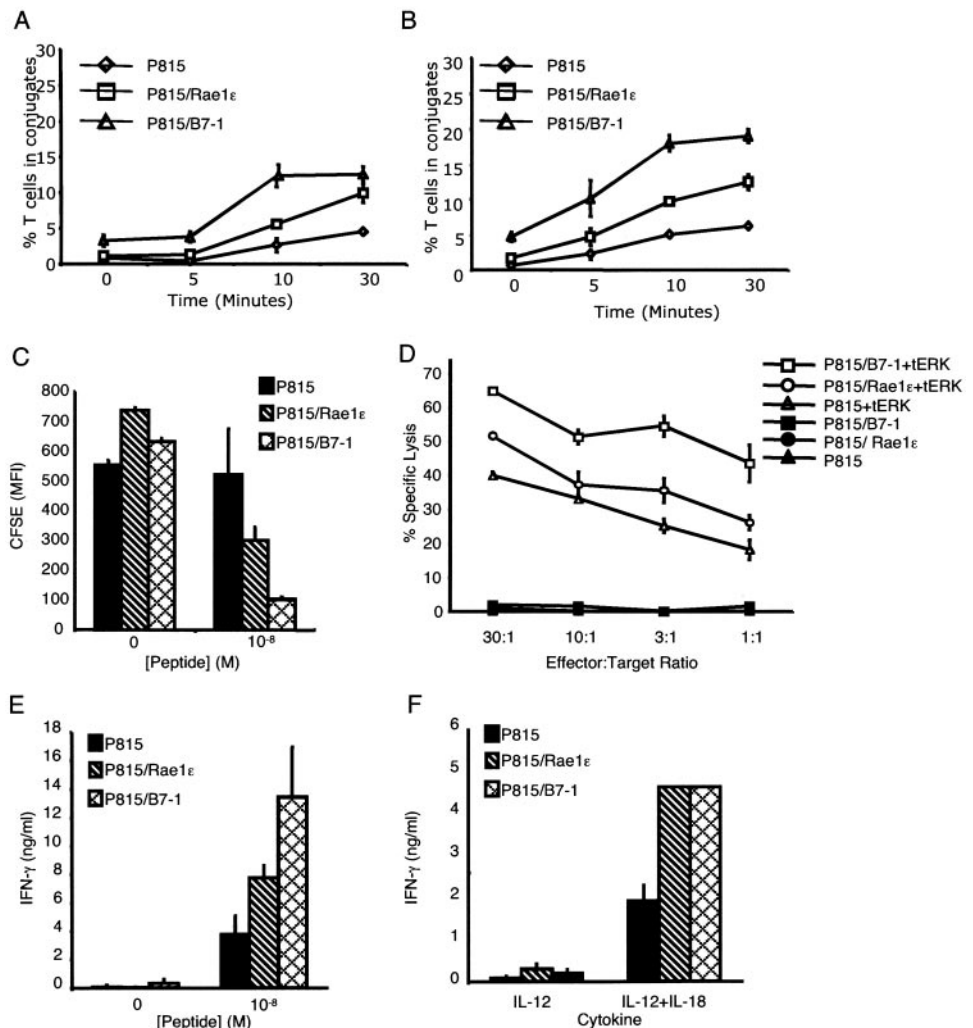
strated previously that both activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells secrete IFN- $\gamma$  in the absence of TCR stimulation in response to the combination of IL-12 and IL-18 (38–41), whether costimulatory molecules could enhance this cytokine secretion had not previously been investigated. Taken together, these results demonstrate that stimulation of NKG2D, as with CD28, can enhance many CTL effector functions, both Ag dependent and independent.

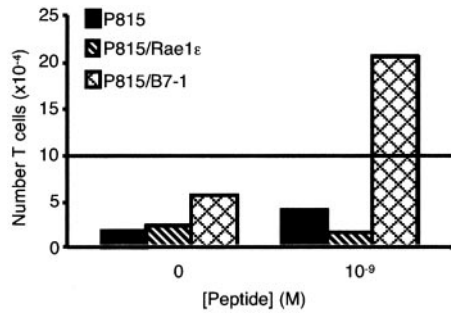
We also investigated whether RAE1 $\epsilon$  could enhance the survival of DUC CTL. To do this, we cultured DUC CTL with P815, P815/B7-1, or P815/RAE1 $\epsilon$  cells with or without tERK peptide (Fig. 3). After 48 h, we determined the number of live cells present in each of the cultures. Far fewer T cells than were plated were recovered from any of the cultures that did not contain peptide as well as cultures containing peptide and P815 or P815/RAE1 $\epsilon$  cells. In contrast, approximately twice the number of T cells plated were recovered from the cultures containing P815/B7-1 cells and peptide. These results, taken together with the proliferation data (Fig. 2C), suggest that whereas CD28 costimulation by B7-1 was able to enhance both DUC CTL proliferation and survival following Ag stimulation, NKG2D stimulation by RAE1 $\epsilon$  was only capable of enhancing T cell proliferation, but not survival.

#### *NKG2D stimulation induces DUC CTL to form immune synapses (IS) in the absence of Ag*

IS have been shown to form between T cells and APC or target cells that the T cells recognize. IS are characterized by a distinct

**FIGURE 2.** Both RAE1 $\epsilon$  and B7-1 are able to costimulate DUC CTL effector responses. The number of DUC CTL in conjugates with target cells was measured over time in the absence of Ag (A) or in the presence of  $10^{-6}$  M tERK (B). C, Proliferation of CFSE-labeled DUC CTL cultured in the presence of target cells pulsed with 0 or  $10^{-8}$  M tERK for 2 days was measured as a decrease in CFSE fluorescence intensity. D, Killing of target cells pulsed with 0 or  $10^{-6}$  M tERK was measured in a standard chromium release assay. E, IFN- $\gamma$  production by IL-12-stimulated DUC CTL ( $0.5 \times 10^6$ ) cultured in the presence of target cells ( $0.5 \times 10^6$ ) pulsed with 0 or  $10^{-6}$  M tERK for 16 h was measured by ELISA. F, IFN- $\gamma$  production by IL-12-stimulated DUC CTL cultured in the presence of target cells and with or without IL-18 (20 ng/ml) for 16 h was measured by ELISA. Results shown are representative of multiple independent experiments.



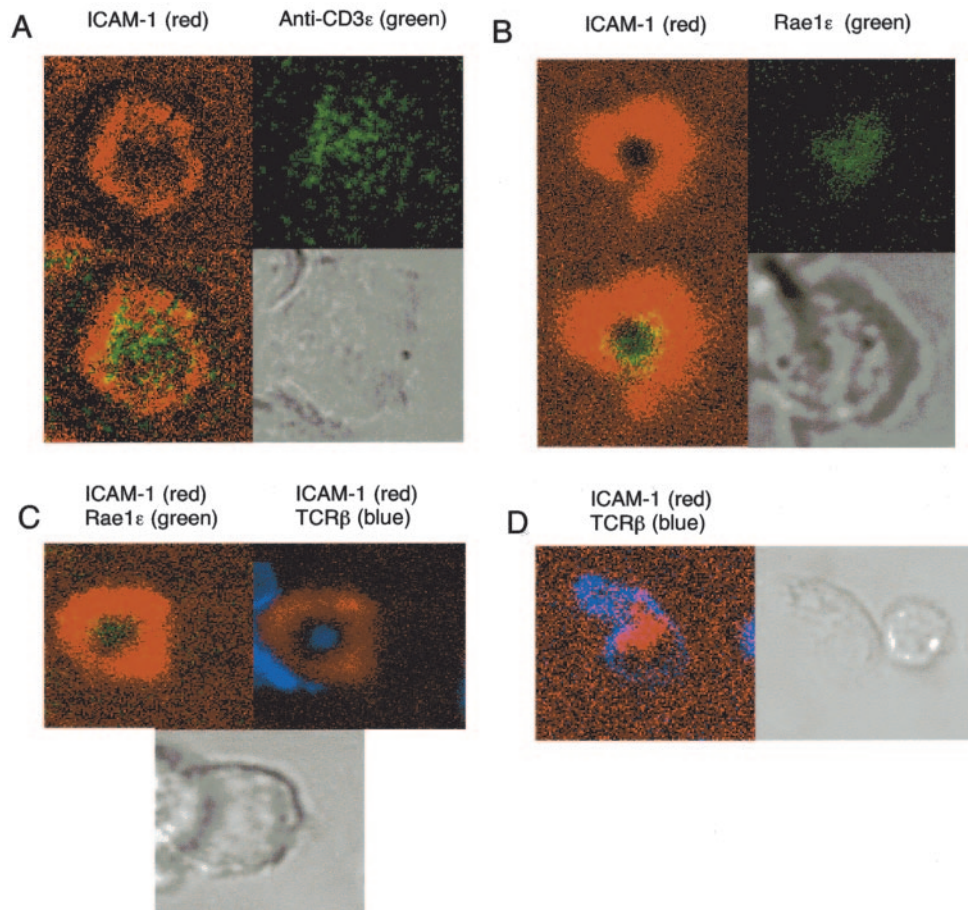


**FIGURE 3.** B7-1, but not RAE1 $\epsilon$ , is able to enhance the survival of DUC CTL upon Ag stimulation. A total of 10<sup>5</sup> DUC CTL (marked by a line) was cultured with target cells in the presence of 0 or 10<sup>-9</sup> M tERK. Two days later, the number of live DUC T cells was determined by trypan blue exclusion and staining with Abs against V $\beta$ 8.3 and CD8 and propidium iodide. Results shown are representative of three independent experiments.

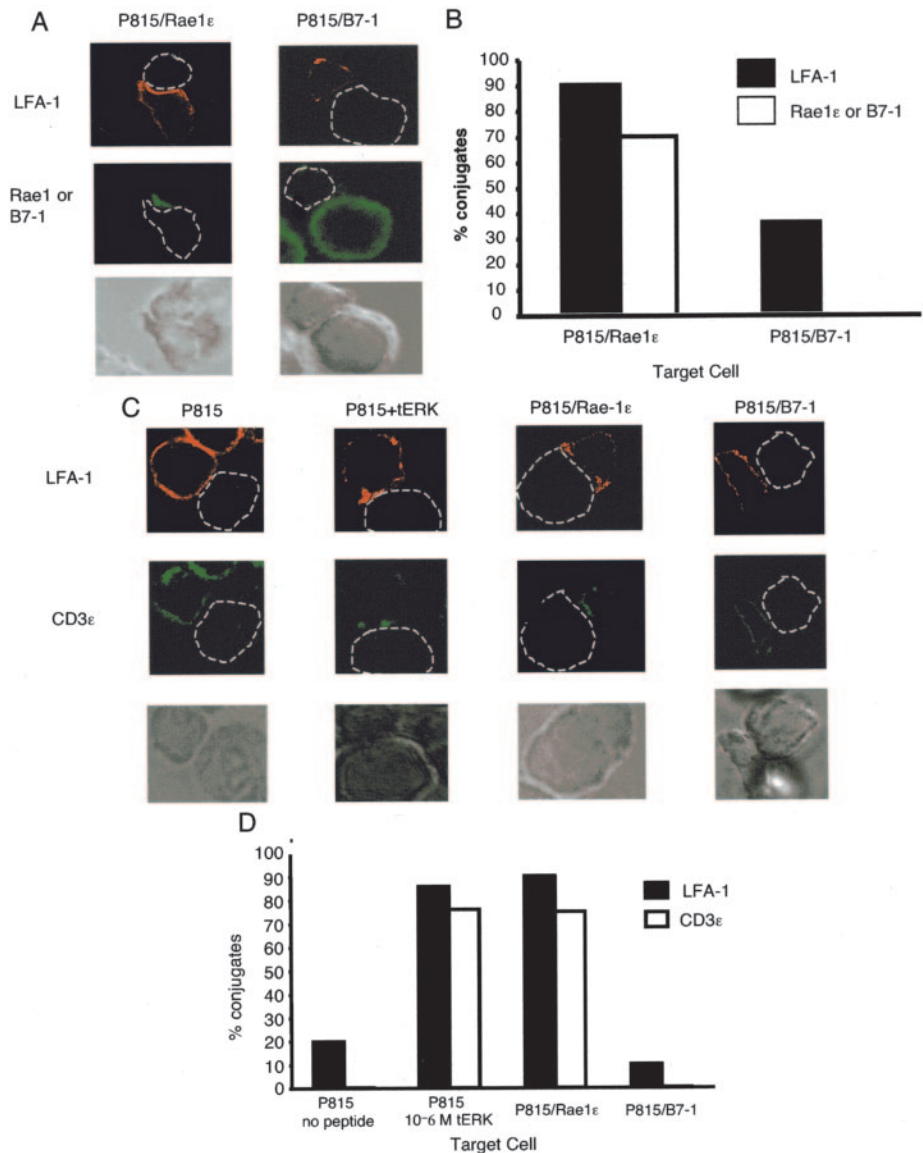
rearrangement of cell surface proteins in the contact surface between T cells and APC or target cells, resulting in a stable cluster of molecules in the center of the contact (the central supramolecular activation cluster (C-SMAC)) surrounded by a ring of adhesion molecules (the peripheral-SMAC) (30, 42, 43). This structure is thought to be important in regulating T cell responses (30, 42, 44–46), and TCR stimulation has been thought to be required for IS formation. Because NKG2D has such a high affinity for its ligands, we tested whether NKG2D stimulation could induce IS formation. Lipid bilayers were generated, as previously described (30, 44), using ICAM-1 and biotinylated lipid. We used fluorescent

streptavidin to couple various biotinylated molecules to the bilayer. As a positive control, bilayers were generated with ICAM-1 and biotinylated anti-CD3 $\epsilon$ . Once plated on these bilayers, DUC CTL quickly stopped and formed IS, seen as a peripheral ring of ICAM-1 surrounding a central cluster of anti-CD3 Abs (Fig. 4A). The T cells formed a similar structure when placed onto lipid bilayers containing ICAM-1 and RAE1 $\epsilon$ , MULT1, or H60, with a peripheral ring of ICAM-1 surrounding a central cluster of the NKG2D ligand (Fig. 4B). ICAM-1 rings (P-SMAC) did not form when the CTL were plated on bilayers containing only ICAM-1; instead, T cells continued to crawl (Fig. 4D). To establish whether the C-SMAC contained TCR in the IS formed with NKG2D ligands, the T cells were fixed and stained with an anti-TCR $\beta$  Ab. Surprisingly, synapses formed in the presence of NKG2D ligands also appeared to recruit TCR to the C-SMAC (Fig. 4C). These data suggest that NKG2D ligands are able to induce IS formation with recruitment of NKG2D and TCR to the C-SMAC.

Reorganization of receptors and ligands in planar bilayers can be more robust than in cell-cell systems (46), so we wanted to confirm the NKG2D-induced IS formation using cell conjugates. Conjugates were formed between DUC CTL and the three P815 cell lines. Conjugates were then fixed and stained with LFA-1 and RAE1 or B7-1-specific Abs (Fig. 5). In the majority of DUC-P815/RAE1 $\epsilon$  conjugates, LFA-1 and RAE1 $\epsilon$  were both clustered at the cell-cell interface (Fig. 5A, left panel, and B). In contrast, conjugates formed between DUC CTL and P815 or P815/B7-1 had little, if any, LFA-1 clustered at the cell-cell interface, and none had clustering of B7-1 (Fig. 5A, right panel, and B). Staining with Abs to CD3 $\epsilon$  confirmed that there was also clustering of TCR in the synapses formed with P815/RAE1 $\epsilon$  (Fig. 5C, third panel). This



**FIGURE 4.** DUC CTL form IS on lipid bilayers containing ICAM-1 and NKG2D ligands. Lipid bilayers containing A, ICAM-1 (red) and anti-CD3 $\epsilon$  Abs (green); B and C, ICAM-1 (red) and RAE1 $\epsilon$  (green); or D, ICAM-1 alone were formed on glass coverslips in flow cells, as described in *Materials and Methods*. With the flow cells at 37°C, DUC CTL, were flowed onto the bilayers. C and D, The T cells were fixed and stained with an anti-TCR $\beta$  Ab. Representative cells are shown from multiple independent experiments. Similar results were obtained with the NKG2D ligands MULT-1 and H60.



**FIGURE 5.** RAE1 $\epsilon$ , but not B7-1, induces IS formation in the absence of Ag. **A**, Conjugates were formed between DUC CTL and P815/RAE1 $\epsilon$  or P815/B7-1. The conjugates were fixed and stained with Abs against LFA-1 (red) and RAE1 or B7-1 (green). The experiments shown are representative of multiple independent experiments. **B**, Quantitation of molecular segregation in conjugates formed between DUC CTL and P815/RAE1 $\epsilon$  or P815/B7-1. Twenty conjugates formed with each target cell were scored for clustering of LFA-1 and RAE1 $\epsilon$  or B7-1 at the cell-cell interface. **C**, Conjugates were formed between DUC CTL and P815 without peptide, P815 with 10<sup>-6</sup> M peptide, P815/RAE1 $\epsilon$ , or P815/B7-1. The conjugates were fixed and stained with Abs against LFA-1 (red) and CD3 $\epsilon$  (green). The experiments shown are representative of multiple independent experiments. **D**, Quantitation of IS formation between DUC CTL and P815, P815/RAE1 $\epsilon$ , or P815/B7-1. Twenty conjugates formed with each target cell were scored for clustering of LFA-1 and CD3 $\epsilon$  at the cell-cell interface.

TCR clustering was similar to clustering induced by antigenic peptide (Fig. 5C, *second panel*). As expected, there was no TCR clustering in synapses formed with the P815 or P815/B7-1 cells in the absence of peptide (Fig. 5C, *first and fourth panels*). These data demonstrate that NKG2D, but not CD28, stimulation can induce DUC CTL to form IS with target cells in the absence of TCR stimulation. These results suggest that NKG2D is capable of sending a productive signal to DUC CTL in the absence of a formal signal through the TCR, although no effector functions that we measured were induced by NKG2D stimulation alone (Fig. 2).

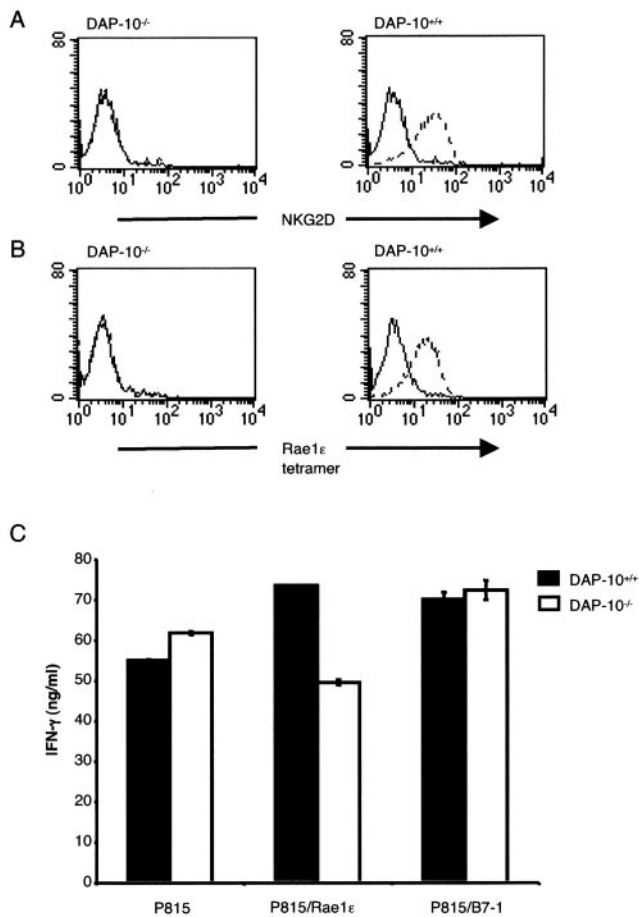
#### *RAE1 $\epsilon$ enhancement of DUC CTL effector function is mediated through NKG2D*

To confirm that the enhancement of DUC CTL activity by RAE1 $\epsilon$  was through NKG2D, we made use of DAP-10<sup>-/-</sup> mice (21). Because NKG2D requires DAP-10 to reach the plasma membrane in T cells, DAP-10<sup>-/-</sup> CTL express very little NKG2D on their cell surface. We crossed the DUC transgenic mice to the DAP-10<sup>-/-</sup> mice and generated DAP-10<sup>-/-</sup> DUC CTL. We confirmed by staining with an NKG2D-specific Ab that these cells expressed very little NKG2D on the cell surface (Fig. 6A). In addition, we verified with RAE1 $\epsilon$  tetramers that there was no other receptor

able to bind to RAE1 $\epsilon$  on the T cells (Fig. 6B). Unlike what was observed with wild-type DUC CTL generated from a DAP-10<sup>+/+</sup> littermate, RAE1 $\epsilon$  was not able to increase IFN- $\gamma$  production in response to antigenic peptide by DAP-10<sup>-/-</sup> DUC CTL (Fig. 6C). These results confirmed that the enhancement of DUC CTL effector function that we had observed by RAE1 $\epsilon$  was indeed through NKG2D-mediated signaling.

## Discussion

Using primary murine T cells, we demonstrated that ligation of NKG2D by the natural ligand RAE1 $\epsilon$  can enhance CTL effector function. The proliferative, cytotoxic, and IFN- $\gamma$  responses of DUC CTL in response to Ag were all increased in the presence of RAE1 $\epsilon$ . In addition, NKG2D stimulation enhanced the Ag-independent IFN- $\gamma$  response of DUC CTL induced by the combination of IL-12 and IL-18. It is possible that these enhanced responses seen in the presence of RAE1 $\epsilon$  may be simply the result of increased adhesion due to the high affinity of NKG2D-RAE1 $\epsilon$  interaction. However, because of the ability of NKG2D ligation to induce IS formation (discussed below), it is likely that NKG2D is



**FIGURE 6.** RAE1 $\epsilon$  costimulation of DUC CTL effector function is mediated through NKG2D. *A* and *B*, Five days following *in vitro* activation with the tERK peptide, DAP-10<sup>+/+</sup> and DAP-10<sup>-/-</sup> DUC CTL were stained with an Ab specific for NKG2D or with a RAE1 $\epsilon$  tetramer. *C*, IFN- $\gamma$  production by DAP-10<sup>+/+</sup> and DAP-10<sup>-/-</sup> DUC CTL ( $10^6$ ) in response to targets pulsed ( $10^6$ ) with  $10^{-6}$  M tERK was measured by ELISA. These results are representative of two independent experiments.

delivering a costimulatory signal to the T cells. The NKG2D-mediated enhancement that we observed is consistent with other reports suggesting that this molecule can costimulate both mouse and human CD8<sup>+</sup> T cell responses (3, 5, 9, 16, 17, 47). It contradicts, however, the results of Ehrlich et al. (18), which suggest that NKG2D can only costimulate murine CD8<sup>+</sup> T cells in combination with additional cofactors, such as CD28. Although it is possible that not all CTL respond to NKG2D in a similar manner, we obtained similar results with OT-1 CTL (our unpublished data). It may be that T cells need to be in the correct activation state to become responsive to stimulation through NKG2D. The T cells used in the previous study were cultured for 7–8 days and were given rmIL-7 and a high concentration of rhIL-2 (200 U/ml). A lower amount of rhIL-2 (40 U/ml) was used in our studies, and rmIL-12 (20 ng/ml) was added for IFN- $\gamma$  studies to skew the cells to a T cytotoxic 1 phenotype (48). In addition, to maximize NKG2D expression, we tested our cells on day 5. These differences in cell culture conditions may potentially explain why the previous study was unable to demonstrate NKG2D costimulation while we were.

In many ways, costimulation of DUC CTL by NKG2D was similar to that by the prototypical costimulatory molecule CD28. Both NKG2D and CD28 stimulation increased the proliferative, cytotoxic, and cytokine response of the T cells in response to Ag,

as well as Ag-independent IFN- $\gamma$  secretion in response to IL-12 and IL-18. There were notable differences between the costimulatory capacity of these molecules, however. First, in the majority of our assays, B7-1 had a greater costimulatory effect than RAE1 $\epsilon$ . This may be because B7-1 is genuinely a superior costimulatory molecule for DUC CTL. However, because we have no way to compare the expression level of the two molecules in our cell lines, we cannot rule out the possibility that RAE1 $\epsilon$  is expressed at a lower level in our cell line than B7-1 is, giving the misleading impression that RAE1 $\epsilon$  is an inferior costimulatory molecule.

A significant difference between NKG2D and CD28 was that only CD28 allowed for recovery of a significant number of DUC CTL following secondary stimulation, suggesting that CD28, but not NKG2D, enhanced DUC CTL survival. However, we cannot rule out the possibility that the larger cell recovery with CD28 ligation was due to enhanced proliferation of a small number of cells rather than an increase in survival. This alternative explanation is unlikely, however, given the described role of CD28 in up-regulation of Bcl-x<sub>L</sub> expression and T cell survival. It is more likely that the difference in cell recovery reflects a difference in T cell survival. Expression of RAE1 $\epsilon$  on the P815 cells lent no survival advantage to DUC CTL over untransfected P815. This finding conflicts with data suggesting that one of the human NKG2D ligands, Letal, enhances CD8<sup>+</sup> T cell survival (49). This difference could, however, reflect a difference between mouse and human NKG2D function. Or it is also possible that while the one NKG2D ligand that we tested, RAE1 $\epsilon$ , was unable to enhance murine CTL survival, one of the other NKG2D ligands would be capable of doing so.

The finding that the survival of DUC CTL was not enhanced by NKG2D costimulation may give some insight into how costimulation by CD28 is capable of enhancing T cell survival. The cytoplasmic tails of both CD28 and DAP-10, the adaptor molecule required for NKG2D signaling in T cells, contain a YNMN motif. Through this motif, both CD28 and DAP10 can bind the p85 subunit of PI3K as well as the adaptor protein Grb2 (4, 50). Studies aimed at determining which portion of CD28 is required for the induction of Bcl-x<sub>L</sub> and the increased survival of T cells have been equivocal. The YNMN motif has been implicated in some studies (51, 52), but has been shown to be dispensable in others (53). ICOS, another T cell costimulatory molecule of the CD28 family, contains a YMFN motif that binds to the p85 subunit of PI3K, but not to Grb2 (54). Costimulation through ICOS, like NKG2D, while increasing PI3K activity, does not enhance T cell survival (55). These data were taken to imply that the CD28-mediated survival signal must be delivered via Grb2 binding to the YNMN motif. However, while DAP10 is able to bind Grb2 as well as p85, costimulation with NKG2D was unable to enhance DUC CTL survival. All together, these data suggest that the YNMN motif may not be sufficient for delivery of the survival signal mediated by CD28.

Another striking difference between NKG2D and CD28 costimulation was that ligation of NKG2D, but not CD28, induced DUC CTL to form IS in the absence of TCR stimulation. This disparity may be due to the large difference of affinity that the two receptors have for their ligands ( $\sim 28$  nM  $K_D$  for RAE1 $\epsilon$ /NKG2D (23) vs 20  $\mu$ M  $K_D$  for B7/CD28 (37)). It will be of interest to see whether one of the lower affinity NKG2D ligands, such as RAE1 $\alpha$  ( $K_D$  690 nM (56)), is also able to induce IS formation, and whether signaling elements in DAP10 are required. Despite IS formation, NKG2D stimulation alone did not drive any of the effector functions that we tested. Such Ag-independent synapse formation in the absence of any measured effector function also occurs with  $\gamma\delta$  T cells in response to ligation of NKG2D (57). The purpose of this



IS formation in the absence of effector function is not clear. It is interesting to speculate that interactions of NKG2D with its ligands may target CTL to distressed cells, such as virally infected cells or tumor cells, in vivo. The NKG2D-driven IS formation may then function to lower the activation threshold of T cells. This could have implications for the pathogenesis of autoimmunity.

In conclusion, our data demonstrate that NKG2D can costimulate a murine CTL response. Although the effects of NKG2D costimulation overlapped those of CD28 costimulation, the effects were not identical. Understanding these differences, as well as costimulation pathways involved in CTL effector function in general, may be beneficial clinically. Delineating the function of NKG2D is of particular interest because this receptor may be involved in autoimmune disease and may be able to aid in CD8<sup>+</sup> T cell-dependent tumor-specific responses (7, 16, 58, 59).

## Disclosures

The authors have no financial conflict of interest.

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