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NKG2D signaling shifts the balance of CD8 T cells from single cytokine- to polycytokine-producing effector cells

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Abstract

CD8 T cells play a critical role in immunity against intracellular pathogens and cancer. A primary objective of T cell-based vaccine strategies is the induction of durable and effective immune responses. Achieving this goal involves more than simply boosting the numbers of responding T cells. Of particular interest is the induction of CD8 T cells with polycytokine capability, specifically with the ability of CD8 T cells to co-produce IFNγ, TNFα and IL-2. The presence of these polycytokine-producing CD8 T cells correlates strongly with protection against foreign pathogens and cancer. Therefore, approaches capable of inducing such polyfunctional responses are needed. NKG2D engagement on CD8 T cells has been shown to result in increased effector response. However, the manner in which NKG2D engagement results in improved CD8 T cell effector response is unclear. Here we demonstrate in vitro and in vivo that NKG2D engagement by its natural ligand, Rae-1ε, shifts the balance from single cytokine to polycytokine (IL-2, IFNγ, and TFNα) production. These data define a previously unrecognized process in which NKG2D costimulation on CD8 T cells results in improved effector responses.

Keywords

NKG2D; CD8 T cells; Polycytokine; Effector function

1. Introduction

CD8 T cells play a vital role in protection against intracellular infections and tumors. The essence of their response against recognized antigens is the directed production of cellular mediators, including cytokines, chemokines and cytolytic granules (Price et al., 1999).

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CRediT authorship contribution statement

Jose A. Guevara-Patino and Andrew Zloza: Conceptualization, methodology, writing, Frederick J. Kohlhapp: Data experiment, Writing Original draft preparation. Jeremy A. O'Sullivan1, Tamson V. Moore: Data curation.

Conflict of interest

The authors have no financial conflicts of interest to disclose.

While CD8 T cell responses are often characterized by the detection of single cytokines, studies demonstrate that polycytokine production confers better CD8 T cell-mediated protection (Betts et al., 2006; Zloza et al., 2009; Price et al., 2005).

The number and types of cytokines that are produced by individual CD8 T cells control their ability to mediate effector function as well as develop into memory cells. CD8 T cells secrete IFNγ shortly after cognate antigen recognition. IFNγ induces functions that aid in the generation of an adaptive immune response against pathogens, including proteasomal antigen processing, MHC-I presentation and Th1 lineage commitment (Tewari et al., 2004). IFNγ also regulates expansion, contraction and memory development of CD8 T cells. TNFα mediates multiple intracellular events that affect innate and adaptive responses, including amplification of Th1 responses via induction of IL-12 and IL-18 production, which in turn upregulate IFNγ secretion (Feldmann et al., 1995). IL-2 secreted by T cells promotes self-proliferation and differentiation, and thus aids in the clonal expansion of responding CD8 T cells. CD4 T cells producing only IFNγ have been shown to have reduced formation of memory (Wu et al., 2002; Hayashi et al., 2002; Younes et al., 2003). Furthermore, synergistic function between TNFα and IFNγ have been demonstrated (Bogdan et al., 1990; Liew et al., 1990). Cumulatively, these observations indicate that the generation of CD8 T cells which simultaneously co-produce IFNγ, TNFα and IL-2 (polycytokine-producing CD8 T cells) provides optimal effector function and protection. However, the approaches capable of mediating such poly-functional responses have not yet been fully elucidated.

NKG2D is an activating receptor that has recently generated considerable interest. In T cells, engagement of NKG2D results in augmented TCR activation and function (Diefenbach et al., 2000; Cerwenka et al., 2000; Jamieson et al., 2002; Bauer et al., 1999). Several ligands for NKG2D have been described for humans and mice, including a family of proteins defined in the retinoic acid family (Rae-1ε) (Bauer et al., 1999; Cosman et al., 2001; Diefenbach et al., 2001; Carayannopoulos et al., 2002; Cerwenka et al., 2001). The expression of NKG2D ligands is upregulated in response to cellular stress (i.e. viral infection, U.V. exposure, retinoic acid) (Bauer et al., 1999). Such expression makes NKG2D ligand-expressing cells visible to NK and CD8 T cells for destruction. This activating “danger” signal of NKG2D can also elicit cytokine production (Groh et al., 2001). On NK cells, NKG2D can act as an independent recognition unit; however, when expressed on CD8 T cells, it amplifies signals mediated through the T cell receptor (TCR) and, thus, functions as a costimulatory molecule (Groh et al., 2006; Perez et al., 2019; Roberts et al., 2001; Wu et al., 1999; Zloza et al., 2011).

Herein, we demonstrate that engagement of NKG2D on CD8 T by APCs expressing retinoic acid-inducible Rae-1ε (NKG2D ligand) results in formation of polycytokine-producing CD8 T cells.

2. Materials and methods

2.1. Cell culture

EL4 cells were cultured in RPMI (Mediatech, Inc., Manassas, VA) supplemented with 10 % heat inactivated Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA), 2

mM L-glutamine (Mediatech), and 1 % penicillin/streptomycin (Mediatech). Six-week-old, specific-pathogen-free, C57BL/6 and OT-I T cell receptor transgenic mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed at the animal facility under conventional conditions and animal experimentation was conducted in accordance with the Loyola University Research Administration's Institutional Animal Care and Use Committee (IACUC) guidelines (ACUP # 71510).

2.2. In vitro cell activation

Target EL-4 cells were loaded for 2–4 h with $OVA_{257–264}$ peptide (0–1 μg/ml as indicated; New England Peptide, Gardner, MA) and washed twice to remove unloaded peptide. Targets were co-cultured for 5 days with CD8 T cells isolated by negative-selection from spleen and 30 U/ml IL-2 (R&D Systems, Minneapolis, MN). At day 3, half of the remaining media was replenished, and IL-2 added. At day 5, cells were washed in PBS and stained for surface and intracellular markers or restimulated for 6-hour cytokine detection.

2.3. In vivo peptide vaccination

C57BL/6 mice were injected i.p. with NKG2D blocking antibody (HMG2D; 250 ug/mouse, BioXcell, West Lebanon, NH) or control IgG antibody (LTF2; 250 ug/ml; BioXcell) at day −2 and day 0. At day 0 mice were vaccinated via footpad injection of 10 ug $OVA_{257–264}$ peptide emulsified in TiterMax Classic Adjuvant (Sigma, St. Louis, MO). At day 5, draining inguinal lymph node cells were recovered and restimulated for 6 h for cytokine detection.

2.4. EL4 target cell preparation

The Rae-1ε construct was generated using pcDNA3 (Invitrogen, Carlsbad, CA) which served as the parental backbone. Constructs were transfected using Lipofectamine into EL4 cells not expressing Rae-1ε. Transfected cells were then selected for 2 weeks on 2 mg/ml G418 (Sigma) and single-cell cloned in 96 well plates. Clones with the highest Rae-1ε expression were selected, cultured, and finally Rae-1ε-expressing cells were sorted for by flow cytometry-assisted sorting. The resulting EL4 clone was 100 % Rae-1ε-positive and maintained on G418 with constant monitoring for Rae1-ε expression. Control EL4 cells were transfected with empty pCRAN and similarly maintained.

2.5. Antibodies and flow cytometry

All mouse surface and intracellular antibodies were purchased from EBioscience (San Diego, CA), except CD3 APC-Cy7 (BD Biosciences, San Diego, CA), CD8 Pacific Orange (Invitrogen) and T-bet Pacific Blue (BioLegend, San Diego, CA). OT-I spleen cells were red blood cell depleted using Ack Lysis buffer (BioWhittaker, Walkersville, MD), washed with Phosphate Buffered Saline (PBS, BioWhittaker), incubated with antibodies to extracellular markers for 30 min at 4 °C in the presence of Mouse BD Fc Block (BD Biosciences), washed in PBS and fixed with 2 % formaldehyde, as previously described (Zloza et al., 2022; Bellavance, et al., 2022). For intracellular marker staining, cells were stimulated for 6 h in the presence of GolgiPlug (5 h, BD Biosciences) and stained for extracellular markers. Cells were then fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences), washed with BD Perm/Wash buffer (BD Biosciences) and stained intracellular marker

antibodies for 30 min at 4 °C. Cells were washed twice with BD Perm/Wash buffer and fixed in a 50 % v/v 4 % formaldehyde:BD Perm/Wash buffer solution. Data were acquired using an LSR-II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Flow data analyses consisted of gating on live, non-debris, singlet lymphocytes (identified via area, width and height scatter plots).

Flow cytometry antibodies: Anti-mouse CD3 APC-Cy7 (clone 145–2C11)BiolegendCat# 100330.

RRID:AB_312745.

RRID:AB_1877170Anti-mouse CD62L PerCP-Cy5.5 (clone MEL-14) Biolegend Cat# 104432.

Anti-mouse CD8 FITC (clone 53–6.7)BiolegendCat# 100706.

Anti-mouse/human CD44 BV605 (clone IM7)BiolegendCat#103047.

RRID:AB_2562451.

RRID:AB_2285839.

Anti-mouse IFN-g PE-Cy7 (clone XMG1.2)BiolegendCat# 505826.

RRID:AB_2295770.

Anti-mouse TNF-a eFluor450 (clone MP6-XT22)eBioscienceCat# 48–7321–82.

RRID:AB_1548825.

Anti-mouse IL-2 PE (clone JES6–5H4)BiolegendCat# 503808.

RRID: AB_315302.

BD Cytofix/CytopermBD BiosciencesCat# 554722.

Brefeldin ABiolegendCat# 420601.

MACS BufferMiltenyi BiotecCat# 130–091–221.

2.6. Statistics

Student's t test (two-tailed) was used to calculate the P value. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. NKG2D engagement by the NKG2D ligand, Rae-1ε alters the CD8 T cell cytokine production repertoire and results in increased CD8 T cell polycytokine production

Previous studies have reported that NKG2D co-stimulation of CD8 T cells results in increased production of individual cytokines (Groh et al., 2001; Roberts et al., 2001; Wu et al., 1999; Upshaw et al., 2006). To determine whether such NKG2D co-stimulation results in single or polycytokine production, OT-I splenic CD8 T cells were primed for 5 days with $OVA_{257–264}$-loaded EL4 cells (Fig. 1A) transfected with Rae-1ε (Rae-1ε DNA in pcDNA3) or empty vector (pcDNA3 without Rae-1ε) (described hereafter as EL4/Rae-1ε and EL4/

Empty, respectively) and at day 5 re-stimulated for 6 h with OVA (1 μg/ml). Interestingly, in the absence of NKG2D/Rae-1ε engagement, the main cytokine produced was TNFα (37.4 % at 1 μg $OVA_{257–264}$) with lower IFNγ and IL-2 production (13.4 % and 12.7 %, respectively), while NKG2D/Rae-1ε engagement allowed high and nearly equal production of IFNγ, TNFα and IL-2 (34.5 %, 29.4 %, and 31.6 %, respectively) (Fig. 1B, C).

Based on work showing that potent effectors co-produce multiple cytokines, (Makedonas and Betts, 2006) we examined the CD8 T cell polyfunctionality by IFNγ, TNFα and IL-2 co-production analysis. Through flow cytometric analyses we were able to devise a schematic (Fig. 2A) to yield two forms of polycytokine information. The first polycytokine analysis set examined was percentage of cells able to produce 0, only 1, only 2, or all 3 tested cytokines (without focus on which specific cytokine constituted the *1 cytokine* or *2 cytokines* groups). This first method demonstrated increased cytokine production by activated ($CD44+CD62L^{lo}$) CD8 OT-I T cells with NKG2D/Rae-1ε engagement versus those without (Fig. 2B, C). Additionally, a greater percentage of activated CD8 OT-I T cells in the presence versus absence of NKG2D/Rae-1ε engagement were capable of co-producing all 3 cytokines (15.3 versus 4.6 % at 100 ng/ml, respectively) (Fig. 2D, E).

The second polycytokine analysis set examined the percentage of cells able to produce 1) individual cytokines only (only IFNγ or TNFα or IL-2); 2) each of the 3 possible combinations of 2-cytokine co-production only (IFNγ and TNFα, IFNγ and IL-2, or TNFα and IL-2); and 3) a combination of all 3 cytokines. Importantly, this polycytokine analysis method demonstrated that with NKG2D/Rae-1ε engagement the greatest proportion of responding cells are triple-cytokine co-producers (15.3 % at 100 ng/ml $OVA_{257–264}$), while without NKG2D/Rae-1ε engagement the greatest proportion of cells (17.2 %) produce TNFα only and triple-cytokine producers constitute only 4.7 % of responders (Fig. 3A,B).

To verify that these observed in vitro results apply to an in vivo setting, C57BL/6 mice were vaccinated with $OVA_{257–264}$ peptide/adjuvant at day 0 and administered NKG2D blocking Ab (HMG2D; 250 μg/mouse) or control IgG antibody (250 μg/ml) at days −2 and day 0. Draining inguinal lymph node cells were then restimulated ex vivo at day 5, as described for the in vitro experiments. These data verified our in vitro findings that with NKG2D/Rae-1ε engagement the greatest proportion of responding cells are triple-cytokine co-producers, while without NKG2D/Rae-1ε engagement the greatest proportion of cells produce single cytokines (Fig. 4).

3.2. NKG2D engagement by Rae-1ε results in increased CD8 T cell cytokine production on a per cell basis

Previous reports have demonstrated that polyfunctional T cells have greater per cell cytokine production compared to single-cytokine producing cells (Precopio et al., 2007). In order to determine whether NKG2D/Rae-1ε engagement results in similar higher production of TNFα, IFNγ, and IL-2 on a per cell basis, we determined the mean fluorescence intensity (MFI) of each cytokine from cytokine producing CD8 T cells stimulated with OVA/Empty versus OVA/Rae-1ε in vitro, as described previously, and from C57BL/6 mice vaccinated with $OVA_{257–264}$ peptide ± NKG2D blocking Ab or control IgG antibody in vivo. CD8 T cells restimulated on day 5 with $OVA_{257–264}$ (1 μg/ml) for 6 h. Those CD8 T cells receiving

NKG2D co-stimulation, in vitro or in vivo, demonstrated increased production of each of the cytokines measured (Fig. 5A, B).

4. Discussion

While the presence of NKG2D on CD8 T cells and its costimulatory signaling role have been reported, (Groh et al., 2001; Roberts et al., 2001; Wu et al., 1999; Upshaw et al., 2006) the result of such engagement on polycytokine production has not yet been fully elucidated. Through the use of APCs designed to express Rae-1ε or lack such expression, we demonstrate that CD8 T cell NKG2D engagement by its natural ligand, Rae-1ε, results not only in the previously observed increase in the production of cytokines, (Gonzalez et al., 2006) but moreover results in a previously unappreciated change in the cytokine production repertoire. Specifically, in the absence of Rae-1ε expression on APCs, the majority of responding CD8 T cells produce TNFα, while a lesser proportion produces IFNγ or IL-2. However, NKG2D/Rae-1ε engagement results in a similar proportion of CD8 T cells producing TNFα (as without Rae-1ε engagement), and importantly, such engagement results also in increases in both the proportion of CD8 T cells producing IL-2 and the proportion producing IFNγ. We further show that NKG2D/Rae-1ε engagement augments the proportion of effector CD8 T cells producing two or three cytokines simultaneously. Most importantly, NKG2D/Rae-1ε engagement shifts the balance of cytokine production from single cytokine production to polycytokine (IL-2, IFNγ, and TFNα) production.

On a cellular level, such additive incorporation of IL-2 and IFNγ to cells already producing TNFα may indicate enhanced activation of CD8 T cells by NKG2D/Rae-1ε engagement, as this follows the natural progression of cytokine-production acquisition. Specifically, in CD8 T cells, cytokine production ability has been reported to begin first with TNFα, followed by IFNγ and lastly IL-2 (Sandberg et al., 2001). Hence, the acquisition of IFNγ and IL-2 production ability by CD8 T cells upon NKG2D/Rae-1ε engagement indicates that such engagement may serve as a checkpoint for further progression of cell activation towards polycytokine production.

On a physiologic level, acquisition of the ability to produce IFNγ and IL-2, and more specifically, the acquisition of polycytokine production ability by CD8 T cells affords greater response against recognized antigen. Such improved responses by polyfunctional CD8 T cells have been demonstrated in vaccinia virus infection and among HIV+ long-term non-progressors (Betts et al., 2006; Precopio et al., 2007). Previous studies of cytokine production ability by CD4 T cells against *Mycobacterium tuberculosis*, *Leishmania major* and CMV (Kannanganat et al., 2007; Darrah et al., 2007) and CD8 T cells against vaccinia virus (Precopio et al., 2007) have shown that such cells with polycytokine production ability are superior also in the amount of cytokine which is produced on a per cell basis. Our work here demonstrating that NKG2D/Rae-1ε engagement in vitro or in vivo not only results in a higher proportion of CD8 T cells with polycytokine ability, but also in higher production (MFI) of cytokine production by responding cells, is in agreement with these studies. However, while these CD8 T cell poly-functional studies showed higher expression (MFI) of only IFNγ, and not the other cytokines monitored, our work demonstrates that NKG2D/Rae-1ε engagement results in higher expression of all three cytokines measured

(TNFα, IFNγ, and IL-2). Such findings reveal the improved response conferred by NKG2D/Rae-1ε engagement on CD8 T cells and may explain one method by which polyfunctional CD8 T cells afford better responses.

Despite advances in T cell biology, applicable molecular approaches that prevent unhelped or exhausted states remain largely unknown, hampering efforts for therapeutic applications. Studies show that unhelped CD8 T cells produce decreased IFNγ and IL-2 upon recall stimulation (Shedlock and Shen, 2003; Bourgeois et al., 2002; Janssen et al., 2003). Furthermore, it has been reported that there is a hierarchy of T cell exhaustion with production of IL-2 being compromised first, followed by loss of TNFα and then IFNγ in response to antigen stimulation (Wherry et al., 2003). Our findings thus suggest that deficiencies in cytokine production and autocrine stimulation associated with CD4-unhelped or exhausted CD8 T cell states may be reversed with NKG2D/Rae-1ε engagement.

Collectively, these data define a previously unrecognized manner in which NKG2D/Rae-1ε costimulation on CD8 T cells in vitro and in vivo results in improved effector responses. Moreover, these findings suggest that vaccination methods which co-deliver NKG2D ligands in addition to antigen may produce more effective future protection against the pathogens and may provide key insight into how enduring therapeutic responses might be achieved in patients.

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Data Availability

Data will be made available on request.

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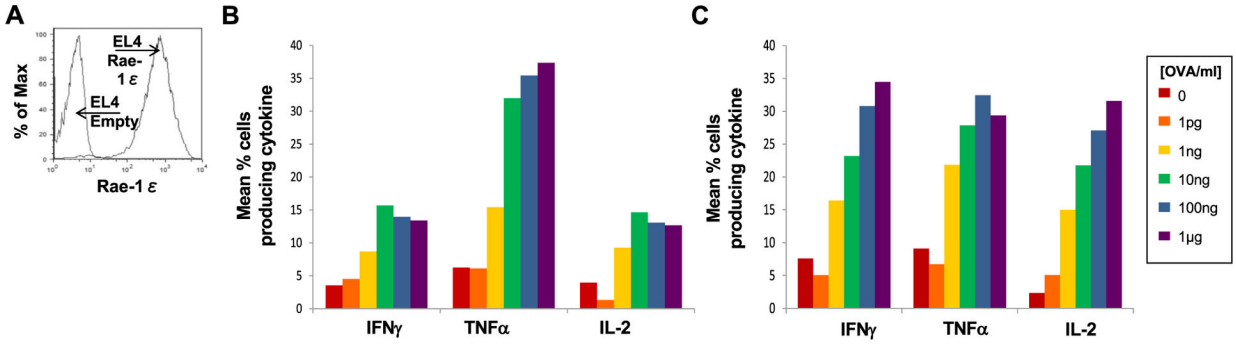

Fig. 1.

NKG2D/Rae-1ε engagement alters the CD8 T cell cytokine production repertoire. (A) EL4 cells were transfected with Rae-1ε or empty vector and subsequently stained for Rae-1ε expression. Data shown are representative of 4 individual experiments with similar results. (B and C) OT-1 CD8 T cells were co-cultured for 5 days with $OVA_{257–264}$-loaded EL4 cells transfected with empty vector (B) or Rae-1ε (C) at $OVA_{257–264}$-loading concentrations (0–1 μg/ml, as per chart color code). Cells were then restimulated for 6 h with $OVA_{257–264}$ peptide (1 μg/ml) in the presence of GolgiPlug and stained subsequently for cytokine production. Gating was performed on live non-debris singlet lymphocytes that were CD3 +CD8CD44 +$CD62^{lo}$. Data are shown as mean percent of all cytokine-producing OT-1 CD8 T cells and separated the one type of cytokine (IL-2, IFNγ, or TNFα) produced. Data shown are representative of 3 individual experiments with similar results evaluated in triplicate.

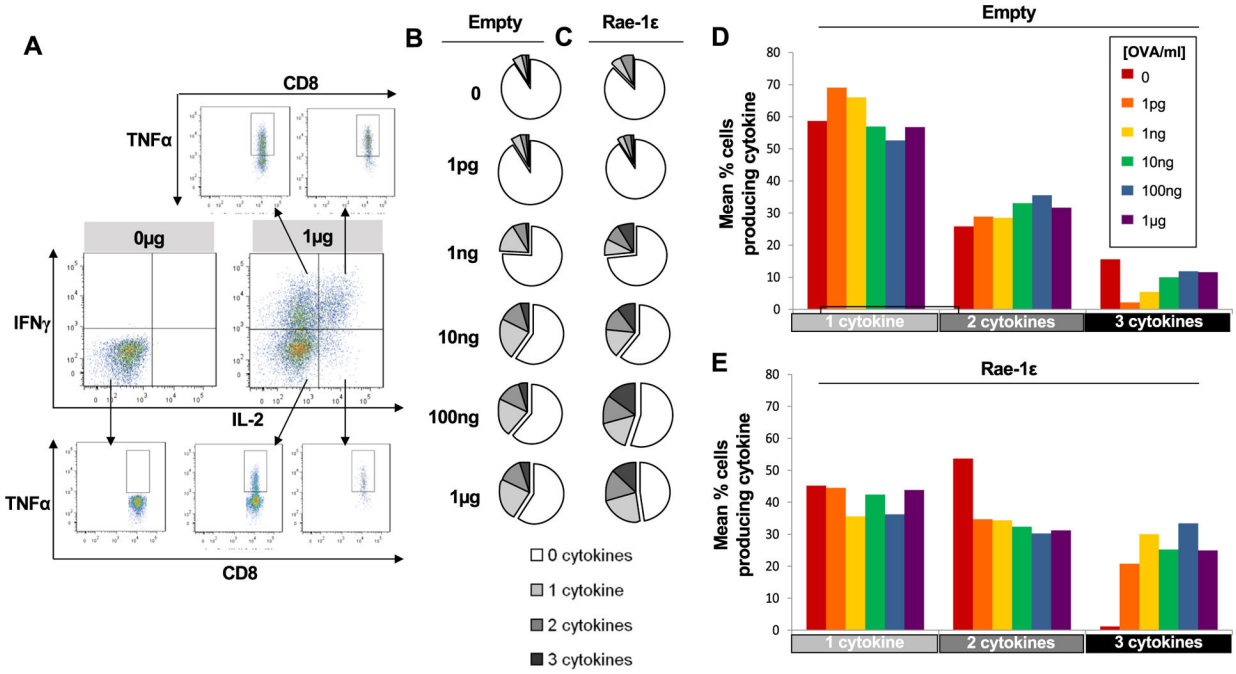

Fig. 2.

NKG2D engagement by Rae-1ε results in an increased proportion of CD8 T cells producing two or three cytokines. (A) Gating strategy for determination of polycytokine production gating on live singlet CD8CD44 +CD62L^{lo} cells. Quadrants were initially formed by plotting IFNγ versus IL-2, and from each of those quadrants subsequent gating was performed for TNFα production. Initial $OVA_{257–264}$ peptide stimulation concentrations (per ml) are shown in the gray shaded box. (B) OT-1 CD8 T cells were co-cultured for 5 days with $OVA_{257–264}$-loaded EL4 cells transfected with Rae-1ε (B and D) or empty (C and E) vector at $OVA_{257–264}$-loading concentrations shown (0–1 μg/ml, as per chart color code). Cells were then restimulated at day 5 for 6 h with $OVA_{257–264}$ peptide (1 μg/ml) in the presence of GolgiPlug and stained subsequently for cytokine production. Gating was performed on live non-debris singlet lymphocytes that were CD3 +CD8CD44 +CD62^{lo}). Data are shown as mean percent of all cytokine-producing OT-1 CD8 T cells and separated by number of cytokines produced: one cytokine (1 cytokine), combination of any two cytokines (2 cytokines), or all 3 cytokines (3 cytokines). Data shown are representative of 3 individual experiments with similar results evaluated in triplicate.

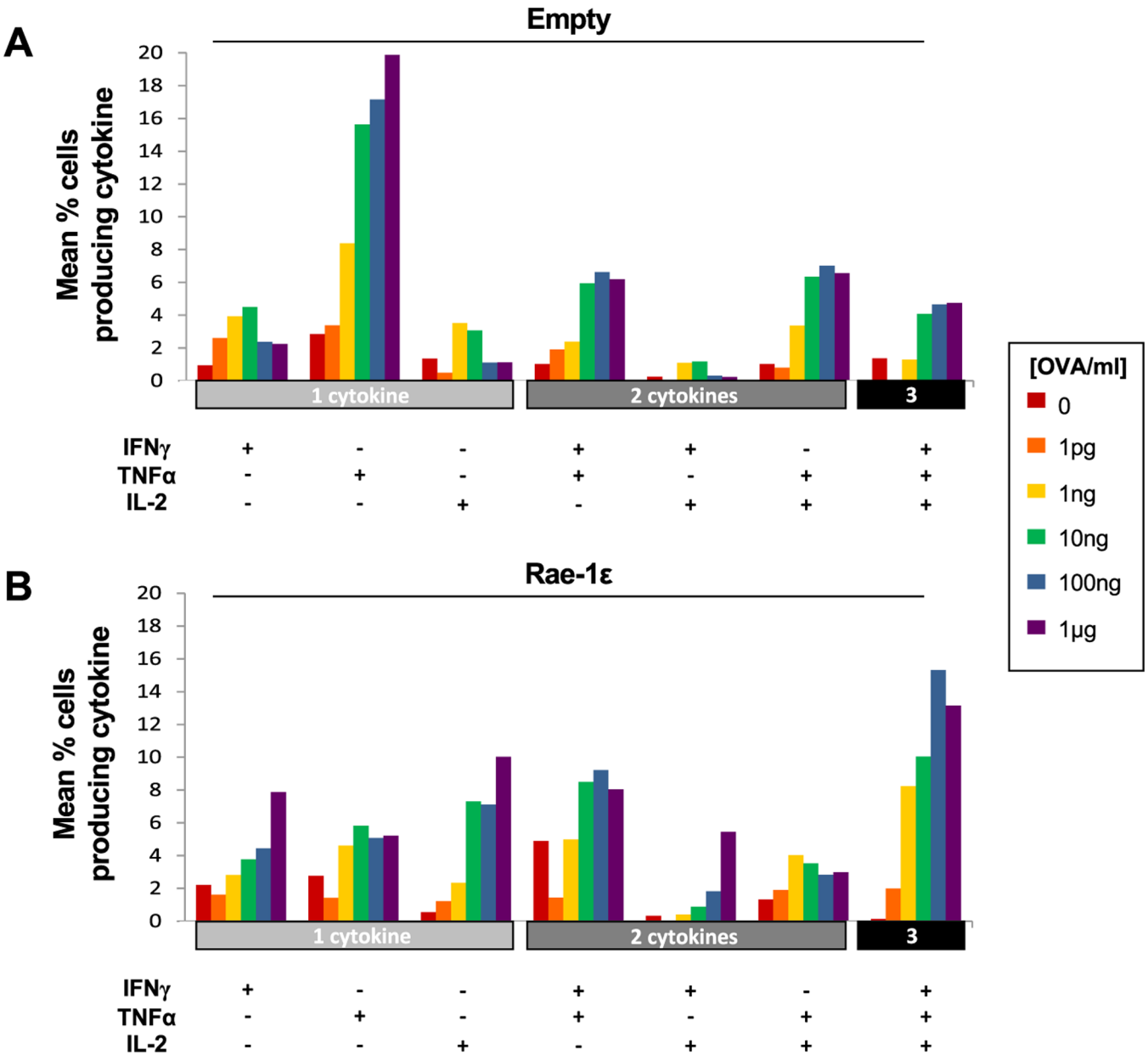

Fig. 3.

NKG2D engagement by Rae-1ε promotes CD8 T cell polycytokine production in vitro. CD8 OT-I T cells were co-cultured for 5 days with EL4 cells transfected with empty (A) or Rae-1ε (B) vector and loaded with $OVA_{257–264}$ (0–1 μg/ml, as per chart color code). Cells were then restimulated at day 5 for 6 h with $OVA_{257–264}$ peptide (1 μg/ml) in the presence of GolgiPlug and stained for polycytokine production. Gating was performed on CD3 +CD8CD44 +$CD62^{lo}$ T cells. Data were expanded to show CD8CD44 +$CD62L^{lo}$ OT-I T cells producing each individual cytokine only (1 cytokine: IL-2, IFNγ, or TNFα only), each of the 3 possible combinations of 2 cytokines (2 cytokines: IL-2 and IFNγ, IL-2 and TNFα, or IFNγ and TNFα), and a combination of all 3 cytokines (IL-2, IFNγ and TNFα; labeled as *3*). The x-axis lists the cytokine or combination produced (production

is denoted by +). Data shown are representative of 3 individual experiments with similar results evaluated in triplicate.

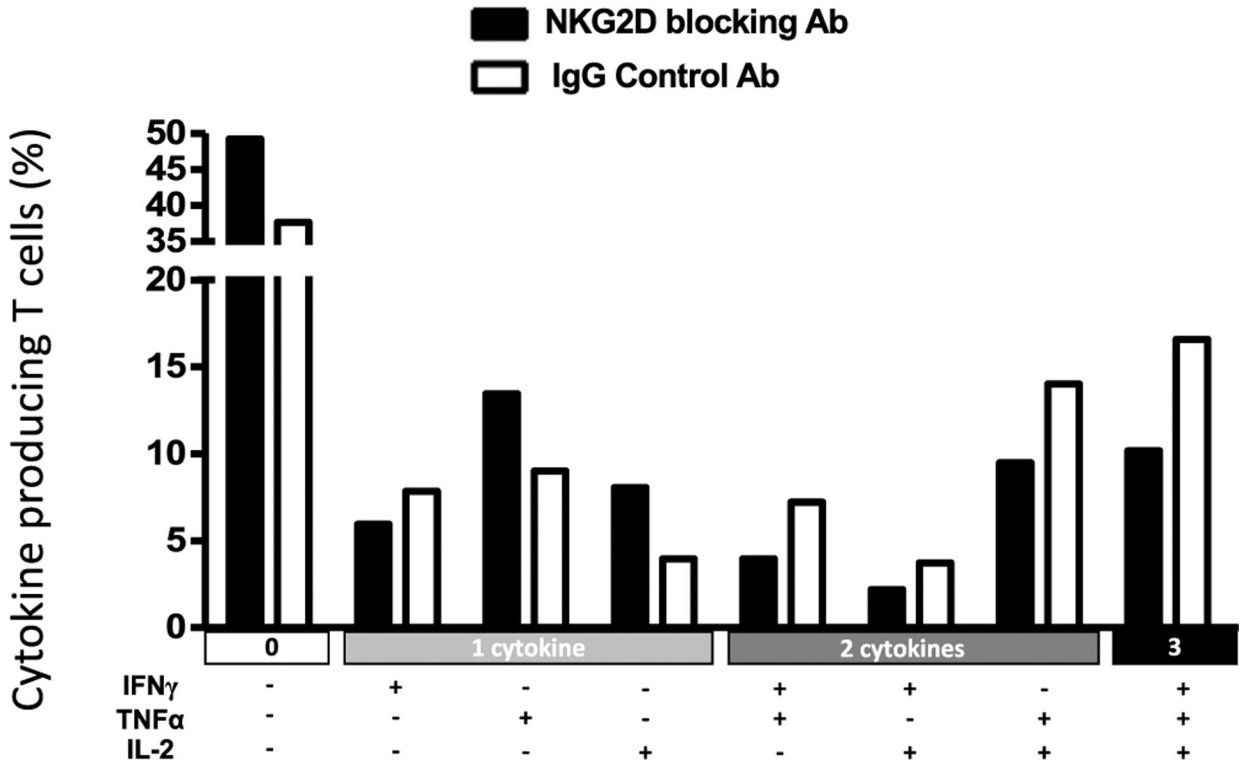

Fig. 4.

NKG2D engagement promotes CD8 T cell polycytokine production in vivo. C57BL/6 mice were vaccinated with OVA 257–264 peptide emulsified in TiterMax classical adjuvant at day 0 and NKG2D blocking Ab (HMG2D; 250 μg/mouse) or control IgG antibody (250 μg/ml) at days −2 and day 0. Draining inguinal lymph node cells were then restimulated at day 5 for 6 h with $OVA_{257–264}$ peptide (1 μg/ml) in the presence of GolgiPlug and stained for polycytokine production. Gating was performed on CD3+CD8CD44+$CD62^{lo}$ T cells. Data were expanded to show CD44+$CD62L^{lo}$ CD8 T cells producing no cytokines (labeled as *0*), each individual cytokine only (1 cytokine: IL-2, IFNγ, or TNFα only), each of the 3 possible combinations of 2 cytokines (2 cytokines: IL-2 and IFNγ, IL-2 and TNFα, or IFNγ and TNFα), and a combination of all 3 cytokines (IL-2, IFNγ and TNFα; labeled as *3*). The x-axis lists the cytokine or combination produced (production is denoted by +). Data shown are cumulative of 2 individual experiments each with 3–5 mice per group.

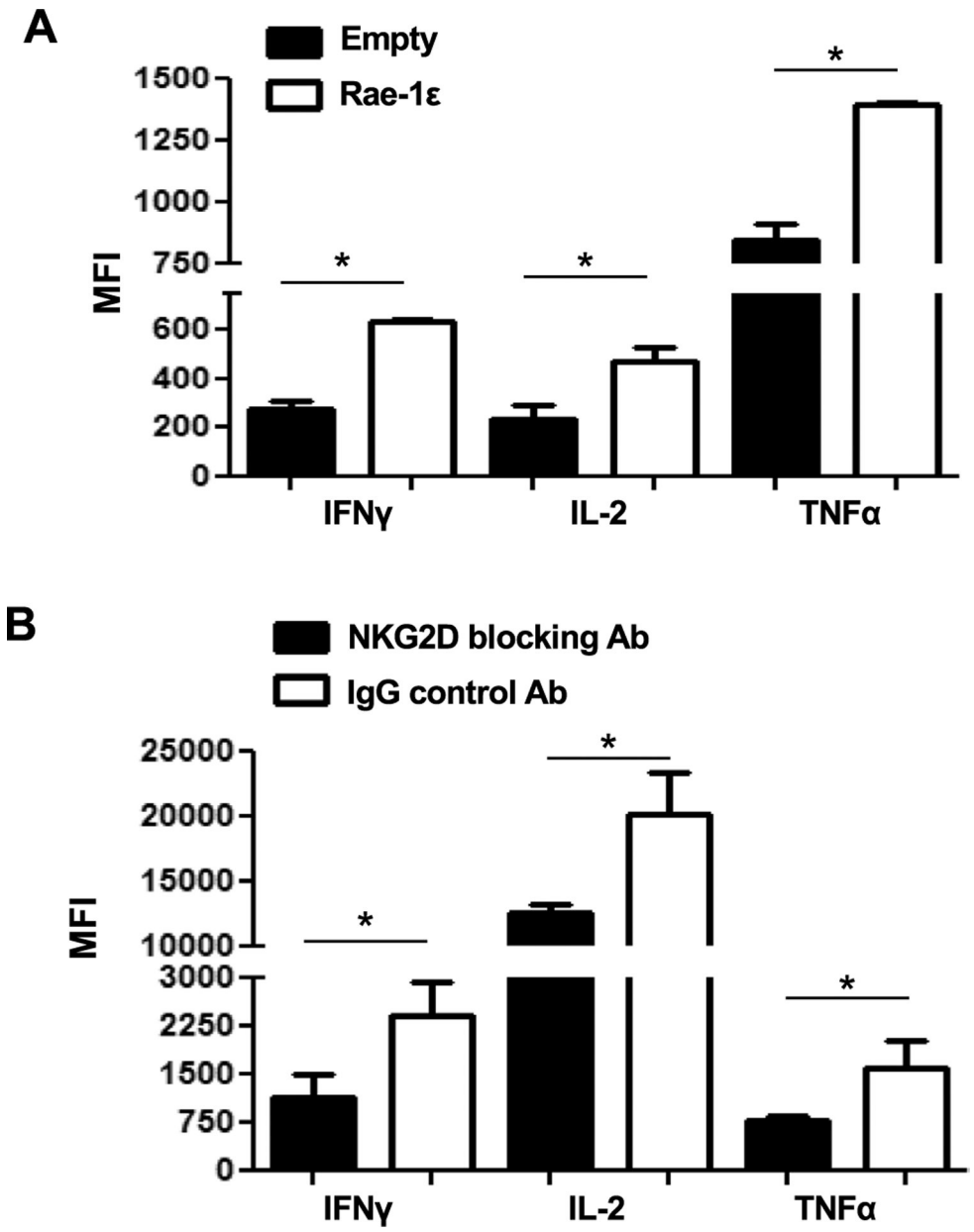

Fig. 5.

NKG2D/Rae-1ε engagement results in increased CD8 T cell cytokine production on a per cell basis. (A) OT-1 CD8 T cells were co-cultured for 5 days with $OVA_{257–264}$-loaded EL4 cells (1 μg/ml) transfected with empty vector (white bar) or Rae-1ε (black bar). At day 5 cells were restimulated for 6 h with $OVA_{257–264}$ peptide (1 μg/ml) in the presence of GolgiPlug and stained subsequently for cytokine production. Gating was performed on live non-debris singlet lymphocytes that were CD3 +CD8 CD44 +$CD62^{lo}$ and mean fluorescence intensity (MFI) was determined. Data shown are representative of 3 individual experiments with similar results. (B) C57BL/6 mice were vaccinated with $OVA_{257–264}$ peptide at day 0 and NKG2D blocking Ab (HMG2D; 250 μg/mouse) or control IgG antibody (250 μg/ml) at days −2 and day 0. Draining inguinal lymph node cells were restimulated at day 5 as

described in A. Data shown are cumulative of 2 individual experimental replicates each with 3–5 mice per group. * Indicates $P < 0.05$.