nature medicine

NKG2D signaling on CD8+ T cells represses T-bet and rescues CD4-unhelped CD8+ T cell memory recall but not effector responses

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CD4-unhelped CD8+ T cells are functionally defective T cells primed in the absence of CD4+ T cell help. Given the co-stimulatory role of natural-killer group 2, member D protein (NKG2D) on CD8+ T cells, we investigated its ability to rescue these immunologically impotent cells. We demonstrate that augmented co-stimulation through NKG2D during priming paradoxically rescues memory, but not effector, CD8+ T cell responses. NKG2D-mediated rescue is characterized by reversal of elevated transcription factor T-box expressed in T cells (T-bet) expression and recovery of interleukin-2 and interferon-γ production and cytolytic responses. Rescue is abrogated in CD8+ T cells lacking NKG2D. Augmented co-stimulation through NKG2D confers a high rate of survival to mice lacking CD4+ T cells in a CD4-dependent influenza model and rescues HIV-specific CD8+ T cell responses from CD4-deficient HIV-positive donors. These findings demonstrate that augmented co-stimulation through NKG2D is effective in rescuing CD4-unhelped CD8+ T cells from their pathophysiological fate and may provide therapeutic benefits.

Memory CD8+ T cells confer efficient and long-lasting immunity against secondary pathogen exposure[1,2]. Events during primary exposure (priming) affect the quality of the initial effector and subsequent memory cytotoxic T lymphocyte (CTL) responses. Unless environmental cues (for example, CD4+ T cell help or inflammation) are present, T cell receptor (TCR) signaling does not result in effective activation of CD8+ T cells[3–7]. Furthermore, in the absence of CD4+ T cell help, the resultant CD4-unhelped CD8+ T cells do not differentiate into sustainable memory cells[4].

NKG2D is expressed on natural killer (NK) cells and on activated CD8+ T cells and binds to the ligands retinoic acid early inducible protein 1ε (Rae-1ε), MULT-1 and H60 in mice[8–13], and MICA/B and ULBP in humans[14,15]. NKG2D engagement on CD8+ T cells contributes to TCR signaling, co-stimulation and amplification of T cell signals and recognition of stress-induced proteins[16–18]. In addition to this canonical function, NKG2D also has a role in CD8+ T cell–mediated autoimmune pathophysiology[19]. Therefore, strategies augmenting NKG2D engagement on CD8+ T cells and harnessing its new functions identified here may result in the rescue of CD4-unhelped CD8+ T cell responses.

RESULTS

NKG2D co-stimulation rescues CD8+ T cell memory expansion

CD8+ T cells that do not receive CD4+ T cell help during priming undergo impaired memory-recall responses[6,20]. Because NKG2D engagement on CD8+ T cells contributes to co-stimulation[16,17], we hypothesized that increased NKG2D engagement by Rae-1ε on antigen-presenting cells (APCs) during priming would rescue memory-recall CD4-unhelped CD8+ T cell expansion. We used skin gene gun vaccination[21] (*in vivo* biolistic transfection) to co-deliver DNA plasmids encoding chicken ovalbumin (OVA) and Rae-1ε (OVA + Rae-1ε), OVA and empty vector (OVA + empty), or both empty vectors (empty + empty) to skin APCs. Using a Rae-1ε–GFP fusion vector[22], we verified that skin DNA delivery resulted in elevated expression of Rae-1ε protein on draining lymph node APCs (**Supplementary Fig. 1**).

Next, we assessed the effects of OVA + Rae-1ε vaccination (the NKG2D co-stimulation regimen) on CD8+ T cell memory-recall responses. C57BL/6 mice received gene gun vaccinations three times (days 0, 5 and 10) with or without antibody-mediated CD4 depletion (days −2, 0, 5 and 10), were rested for 4 weeks during memory formation and then received one memory boost vaccination

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Received 10 November 2011; accepted 20 January 2012; published online 26 February 2012; doi:10.1038/nm.2683

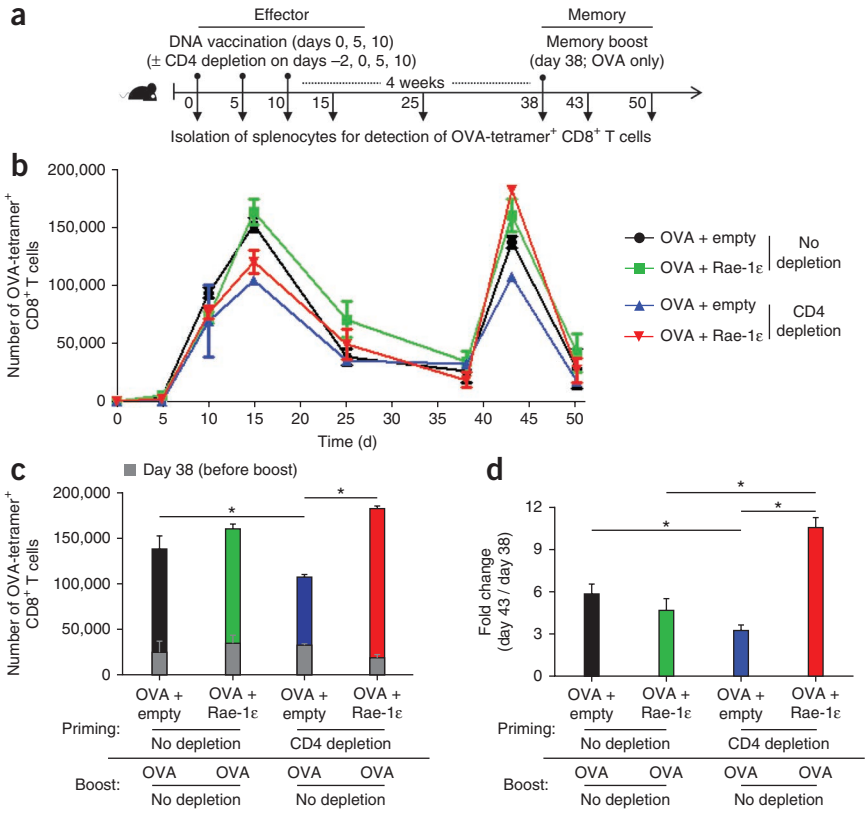

Figure 1 NKG2D engagement by Rae-1ε rescues CD4-unhelped CD8+ T cell memory-recall expansion. (**a**) Experimental design for vaccination and CD4+ cell depletion. (**b**) Expansion kinetics of OVA-tetramer+ CD8+ T cells (± s.e.m.) calculated per spleen. (**c**) Mean number of OVA-tetramer+ CD8+ T cells (+ s.e.m.) per spleen on days 38 and 43. (**d**) Data from **c** shown as a fold change (+ s.e.m.). All data are cumulative from three to five mice (analyzed individually) per group from one experiment of three experiments conducted with similar results. $*P < 0.05$.

(OVA only without Rae-1ε and without CD4 depletion) on day 38 (**Fig. 1a**). OVA-specific CD8+ T cell numbers were determined by OVA-tetramer staining of splenocytes (**Fig. 1b** and **Supplementary Fig. 2**) and confirmed in the draining inguinal lymph node (**Supplementary Fig. 3**). We observed similar post-contraction numbers (day 38, before boost) of OVA-specific CD8+ T cells in all groups (**Fig. 1b**,**c**). Remarkably, the NKG2D co-stimulation regimen at priming resulted in complete rescue of CD4-unhelped OVA-specific CD8+ T cell expansion at the memory-recall phase (**Fig. 1c**,**d** and **Supplementary Fig. 3b**).

NKG2D co-stimulation rescues CD8+ T cell memory function

Given the ability of the NKG2D co-stimulation regimen to augment memory-recall expansion, we hypothesized that such engagement during priming may rescue memory cytolytic molecule and cytokine production by CD4-unhelped CD8+ T cells. Notably, upon memory boost with OVA only, CD4-unhelped CD8+ T cells that received the NKG2D co-stimulation regimen during priming showed complete rescue of granzyme B, interleukin-2 (IL-2) and interferon-γ (IFN-γ) production (**Fig. 2a**). The effect of the memory boost vaccination (that is, a single vaccination) on naive cells on day 38 was negligible (**Supplementary Fig. 4**).

To further investigate NKG2D-mediated rescue of CD4-unhelped CD8+ T cell memory-recall responses, we examined antigen-specific target lysis ability (**Fig. 2b**). CD4 depletion at priming significantly weakened CD8+ T cell memory-recall lytic activity (CTL lysis) (**Fig. 2c**,**d**). Notably, administration of either Rae-1ε (to C57BL/6 mice) or H60 (NKG2D ligand in B6BCF1 mice) during priming resulted in complete rescue of CD4-unhelped CD8+ T cell memory CTL lysis (**Fig. 2d**). As CD4+ T cells were present during the boost vaccination, we next analyzed memory-recall responses in their absence. CD4 depletion during the memory boost resulted in decreased memory-recall CTL lysis in all groups (**Supplementary Fig. 5**). Although we observed the greatest decrease in the group primed without the NKG2D co-stimulation regimen and depleted of CD4+ T cells both during priming and memory boost, we nevertheless observed rescue of CTL lysis with the NKG2D co-stimulation regimen (**Supplementary Fig. 5**).

NKG2D is necessary on CD8+ T cells for memory rescue

To demonstrate that the observed rescue of memory-recall responses is dependent on NKG2D and, specifically, on CD8+ T cell NKG2D engagement, we conducted a series of experiments. First, we blocked NKG2D with antibody during priming and observed decreased memory CTL lysis (**Fig. 3a**). Vaccination of NKG2D-blocked, CD4-depleted mice with OVA + Rae-1ε did not rescue CD4-unhelped CD8+ T cell lysis (**Fig. 3a**).

Second, given that NK and NKT cells express NKG2D, we determined their role in NKG2D-mediated CD8+ T cell rescue. We depleted C57BL/6 mice of NK1.1+ cells[23] and transferred Thy1.1-marked OT-I CD8+ T cells into them. This design allowed induction of detectable OVA-specific responses with a single vaccination, thereby abrogating the need for further NK1.1 depletions and thus avoiding depletion of activated NK1.1-expressing OT-I CD8+ T cells. In this experiment, priming of CD8+ T cells occurred after NK1.1 depletion antibody had cleared (data not shown) and before NK and NKT cells had returned (**Fig. 3b**). Memory-recall OVA-specific CD8+ T cell lysis was not negatively affected by NK1.1 depletion during priming, and CD4-unhelped CD8+ T cell lysis was rescued by the NKG2D engagement regimen (**Fig. 3c**).

Lastly, to demonstrate that NKG2D-mediated rescue is the result of NKG2D engagement on CD8+ T cells, we vaccinated CD45.1+ C57BL/6 wild-type (WT) mice that received adoptively transferred CD45.1− CD8+ T cells from NKG2D-deficient (*Klrk1*$^{-/-}$)[24] (**Fig. 3d**) or CD45.1− WT mice. Compared to transferred WT CD8+ T cells, transferred NKG2D-deficient CD8+ T cells showed decreased memory-recall expansion (**Fig. 3e**). OVA + Rae-1ε vaccination at priming did not rescue memory-recall expansion (**Fig. 3e**) of CD4-unhelped NKG2D-deficient transferred CD8+ T cells but did rescue expansion of CD4-unhelped WT transferred CD8+ T cells (**Fig. 3e**) and endogenous CD8+ T cells in both hosts (data not shown).

NKG2D-mediated rescue is not independent of IL-15

Roles for IL-15 in NKG2D upregulation[25] and signaling[26] and rescue of CD4-unhelped CD8+ T cells[27] have been reported. Therefore, we

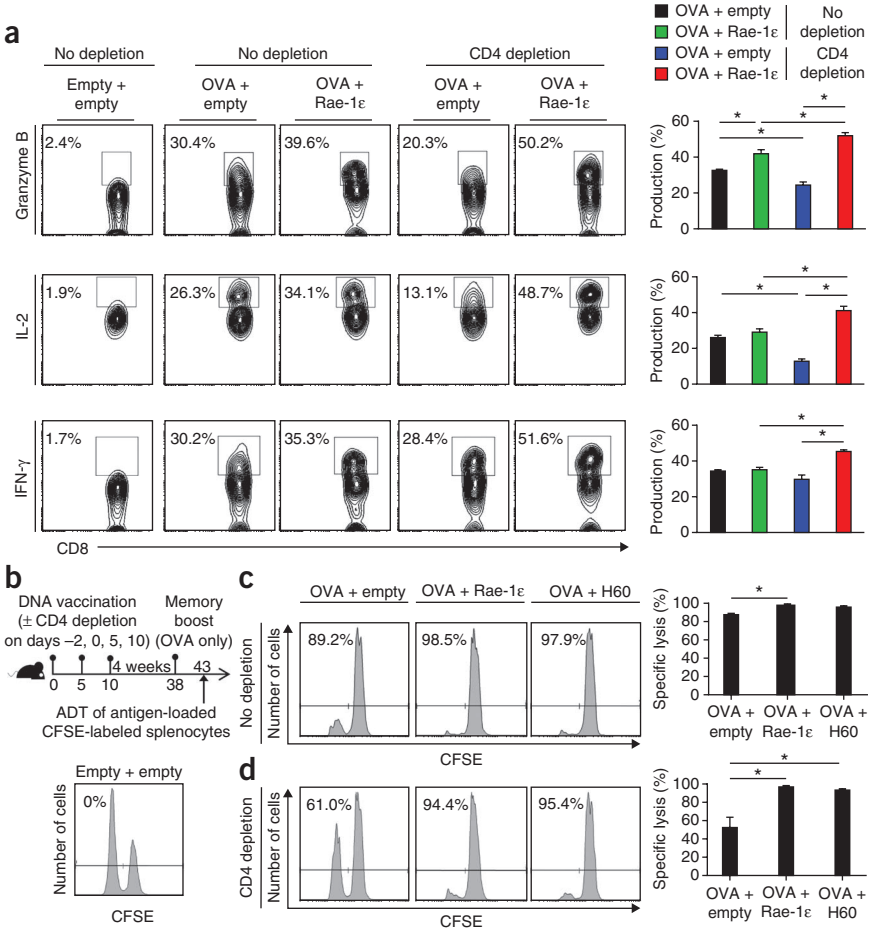

Figure 2 NKG2D-mediated rescue of CD4-unhelped CD8$^+$ T cell memory-recall responses involves cytokine and lytic molecule production and lytic activity. (**a**) Splenic OVA-tetramer$^+$ CD8$^+$ T cell production of granzyme B, IL-2 and IFN-γ on day 43 for mice treated as described in **Figure 1a**. FACS plots show percentage of CD8$^+$ T cells producing the labeled cytokine. They are from one representative mouse per group of one experiment. The graphs at right are cumulative percentage cytokine production from one experiment of three experiments conducted with similar results. (**b**) Experimental design and *in vivo* specific lysis (%) of $OVA_{257–264}$-loaded (CFSE^{lo}) and irrelevant peptide–loaded ($hgp100_{25–33}$, CFSE^{hi}) targets from a control vaccinated (empty + empty) mouse. ADT, adoptive transfer. (**c**,**d**) Specific lysis (%) from mice vaccinated with OVA + empty, OVA + Rae-1ε and OVA + H60 as described in **b** without (**c**) and with (**d**) CD4 depletion. Percentages in the FACS plots represent specific lysis from one representative mouse from one experiment. Bar graph data at right are cumulative from three to five mice analyzed individually per group from one experiment of at least three experiments conducted with similar results. *$P < 0.05$. All error bars represent s.e.m.

investigated IL-15 presentation (via dendritic cell IL-15Rα expression) and found it to be equal under our four vaccination conditions (**Supplementary Fig. 6a**).

Memory phase expansion (**Supplementary Fig. 6b**) and CTL lysis (**Supplementary Fig. 6c**) of OVA-specific CD8$^+$ T cells were greatly reduced in IL-15–deficient (*Il15*$^{-/-}$) mice in comparison to WT mice. The NKG2D co-stimulation regimen was partially able to rescue CD4-unhelped CD8$^+$ T cell responses in the absence of IL-15 to the level observed without CD4 depletion but was unable to rescue to the level observed in WT mice (**Supplementary Fig. 6b**,**c**). Thus, NKG2D-mediated rescue of CD8$^+$ T cells is not independent of IL-15.

NKG2D co-stimulation does not rescue effector responses

We next investigated CD8$^+$ T cell responses at the effector phase. We observed a decreased expansion of CD4-unhelped OVA-specific CD8$^+$ T cells at the effector phase (day 15), which, unexpectedly, was not augmented by the NKG2D co-stimulation regimen (**Fig. 1b** and **Supplementary Fig. 7**).

We observed an increase in CD8$^+$ T cell effector phase granzyme B production (**Supplementary Fig. 8a**) and target lysis (**Supplementary Fig. 8b**) in nondepleted mice receiving the NKG2D co-stimulation regimen as opposed to mice depleted of CD4$^+$ cells. However, lack of CD4 help resulted in unrescued minimal target lysis in mice receiving OVA + empty, OVA + Rae-1ε and OVA + H60 vaccination (**Supplementary Fig. 8c**,**d**).

NKG2D engagement results in JNK2-mediated T-bet suppression

CD4-unhelped CD8$^+$ T cells have greater T-bet protein expression than their CD4-helped counterparts[28], and T-bet expression during the effector phase distinguishes short-lived effector cells (SLECs; T-bet^{hi}) from memory precursor effector cells (MPECs; T-bet^{lo})[28–31]. To determine whether effector phase T-bet expression correlates with NKG2D-mediated rescue of CD4-unhelped CD8$^+$ T cell memory-recall responses, we determined T-bet expression in OVA-specific CD8$^+$ T cells. In the context of CD4 help, OVA-specific CD8$^+$ T cells consistently had low T-bet expression throughout the effector and memory phases (**Fig. 4a–c**). Conversely, CD4-unhelped OVA-specific CD8$^+$ T cells primed without the NKG2D co-stimulation regimen had significantly increased expression of T-bet (**Fig. 4a–c**). Notably, T-bet expression levels in CD4-unhelped CD8$^+$ T cells were reduced (45%) with the NKG2D co-stimulation regimen, were similar to T-bet levels of CD4-helped CD8$^+$ T cells throughout the effector phase and were lower during the memory phase (**Fig. 4a–c**). Corroborating these findings, T-bet levels were higher in NKG2D-deficient versus WT OVA-specific CD8$^+$ T cells transferred into a WT host, especially under CD4-depletion conditions (**Fig. 4d**), and were higher when compared to those in endogenous CD8$^+$ T cells (**Supplementary Fig. 9a**). Likewise, T-bet expression in OVA-specific CD8$^+$ T cells was elevated in IL-15–deficient versus WT hosts, especially under CD4-depletion conditions (**Fig. 4e**), and only partially repressed by the NKG2D co-stimulation regimen (**Fig. 4e** and **Supplementary Fig. 9b**).

Studies have shown that co-stimulation of CD8$^+$ T cells results in activation of the c-Jun N-terminal kinase 2 (JNK2) pathway[32] and that JNK2-knockout mice have increased levels of T-bet expression[33]. In our studies, *in vitro*–activated NKG2D-deficient CD8$^+$ T cells expressed higher T-bet (**Fig. 5a**) and lower phosphorylated JNK2 (pJNK2) levels (**Fig. 5b**) compared to WT counterparts. Additionally, WT CD8$^+$ T cells similarly activated in the presence of NKG2D-blocking antibody showed lower pJNK2 levels (**Fig. 5b**). On the basis of these observations

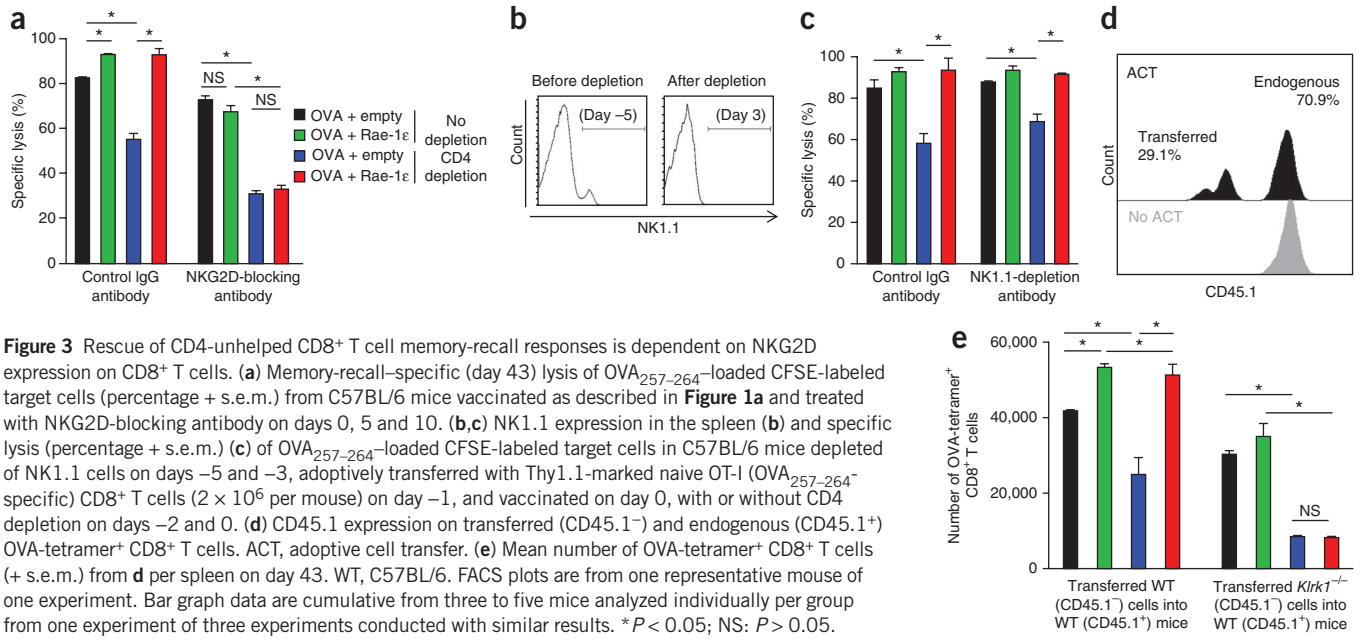

Figure 3 Rescue of CD4-unhelped CD8$^+$ T cell memory-recall responses is dependent on NKG2D expression on CD8$^+$ T cells. (**a**) Memory-recall–specific (day 43) lysis of $OVA_{257–264}$–loaded CFSE-labeled target cells (percentage + s.e.m.) from C57BL/6 mice vaccinated as described in **Figure 1a** and treated with NKG2D-blocking antibody on days 0, 5 and 10. (**b**,**c**) NK1.1 expression in the spleen (**b**) and specific lysis (percentage + s.e.m.) (**c**) of $OVA_{257–264}$–loaded CFSE-labeled target cells in C57BL/6 mice depleted of NK1.1 cells on days –5 and –3, adoptively transferred with Thy1.1-marked naive OT-I ($OVA_{257–264}$-specific) CD8$^+$ T cells (2×10^6 per mouse) on day –1, and vaccinated on day 0, with or without CD4 depletion on days –2 and 0. (**d**) CD45.1 expression on transferred (CD45.1$^-$) and endogenous (CD45.1$^+$) OVA-tetramer$^+$ CD8$^+$ T cells. ACT, adoptive cell transfer. (**e**) Mean number of OVA-tetramer$^+$ CD8$^+$ T cells (+ s.e.m.) from **d** per spleen on day 43. WT, C57BL/6. FACS plots are from one representative mouse of one experiment. Bar graph data are cumulative from three to five mice analyzed individually per group from one experiment of three experiments conducted with similar results. $^*P < 0.05$; NS: $P > 0.05$.

and evidence that NKG2D modulates JNK signaling[34,35], we hypothesized that NKG2D signaling represses T-bet via JNK2. JNK2 pathway inhibition of *in vitro* OT-I CD8$^+$ T cell activation with $OVA_{257–264}$ peptide–loaded EL4 mouse lymphoma cells expressing Rae-1ε resulted in a significant increase in T-bet to levels resembling OT-I CD8$^+$ T cells activated without NKG2D co-stimulation (**Fig. 5c**).

NKG2D confers protection in CD4-dependent infections

To address the condition in which CD4$^+$ T cell counts *in vivo* remain continuously low or absent, we characterized the ability of NKG2D to rescue CD8$^+$ T cell responses under continuous CD4$^+$ cell depletion via CD4-specific antibody administered every 5 d (days –2 to 43) (**Fig. 6a**). Under these conditions, CD8$^+$ T cell memory expansion was significantly reduced (**Fig. 6b**) and T-bet expression further increased (**Fig. 6c**) compared to CD4 depletion only during priming (**Figs. 1** and **4**). However, CD8$^+$ T cells primed with the NKG2D co-stimulation regimen and continuously depleted of CD4$^+$ T cells showed rescued total memory-recall responses (**Fig. 6c**) as well as memory-recall responses on a per-cell basis in an *ex vivo* CTL assay where OVA-tetramer$^+$ CD8$^+$ T cell numbers were equalized from the vaccination conditions (**Supplementary Fig. 10**).

To assess functional responses in an *in vivo* pathogenic model, we used a lethal influenza-PR/8 infection model, in which the virus expresses OVA and clearance via CD8$^+$ cells is CD4 dependent (**Fig. 6d**). Absence of the NKG2D co-stimulation regimen and presence of continuous CD4 depletion in C57BL/6 mice resulted in

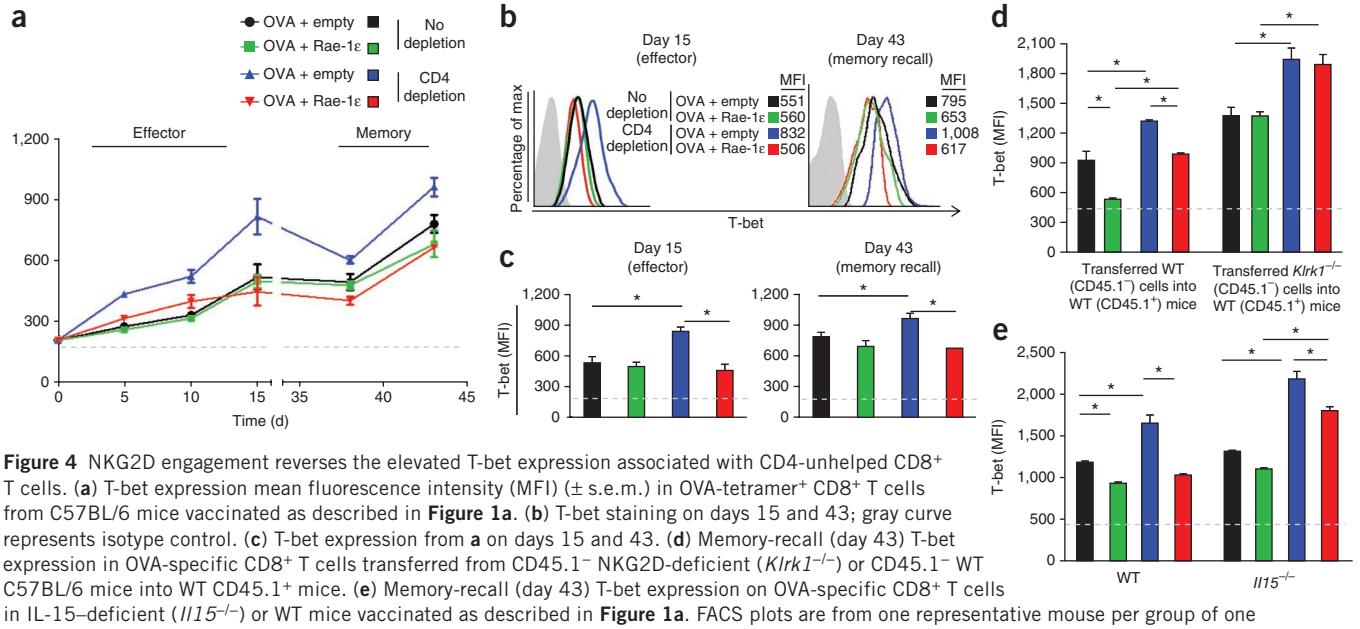

Figure 4 NKG2D engagement reverses the elevated T-bet expression associated with CD4-unhelped CD8$^+$ T cells. (**a**) T-bet expression mean fluorescence intensity (MFI) (± s.e.m.) in OVA-tetramer$^+$ CD8$^+$ T cells from C57BL/6 mice vaccinated as described in **Figure 1a**. (**b**) T-bet staining on days 15 and 43; gray curve represents isotype control. (**c**) T-bet expression from **a** on days 15 and 43. (**d**) Memory-recall (day 43) T-bet expression in OVA-specific CD8$^+$ T cells transferred from CD45.1$^-$ NKG2D-deficient (*Klrk1*$^{-/-}$) or CD45.1$^-$ WT C57BL/6 mice into WT CD45.1$^+$ mice. (**e**) Memory-recall (day 43) T-bet expression on OVA-specific CD8$^+$ T cells in IL-15–deficient (*Il15*$^{-/-}$) or WT mice vaccinated as described in **Figure 1a**. FACS plots are from one representative mouse per group of one experiment. All other data are cumulative from three to five mice analyzed individually per group from one experiment of three experiments conducted with similar results. Dashed lines represent MFI of background flow cytometric staining for T-bet. $^*P < 0.05$; NS: $P > 0.05$.

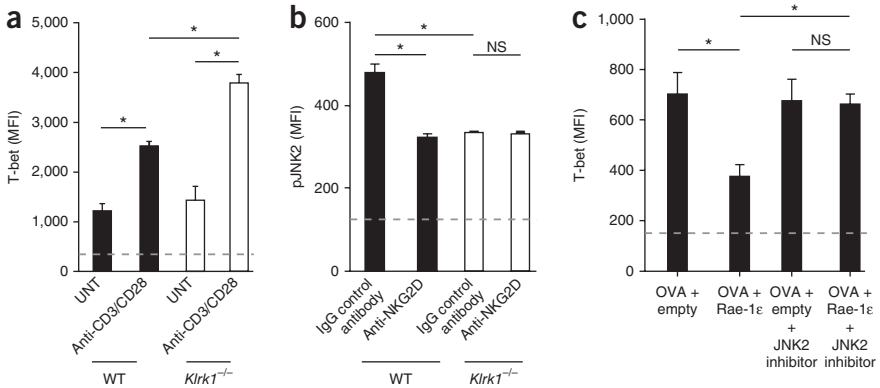

Figure 5 Suppression of T-bet by NKG2D–Rae-1ε engagement is mediated through JNK2. (**a**) CD8$^+$ T cell T-bet MFI (+ s.e.m.) from *Klrk1*$^{-/-}$ or WT C57BL/6 splenocytes stimulated *in vitro* with antibodies to CD3 and CD28 (1 μg ml^{-1} each) for 72 h or left untreated (UNT). (**b**) pJNK2 MFI (+ s.e.m.) from CD8$^+$ T cells stimulated as in **a** with or without NKG2D-blocking antibody (anti-NKG2D). (**c**) T-bet MFI (+ s.e.m.) of OT-I CD8$^+$ T cells co-cultured with $OVA_{257–264}$-loaded EL4 cells transfected with either Rae-1ε or empty vector and treated with or without a JNK2 inhibitor (JNK inhibitor IX; 25 nM). Bar graph data are cumulative from cells of three to five mice analyzed individually per group from one experiment of three experiments conducted with similar results. Dashed lines represent MFI of background flow cytometric staining for the respective markers. *$P < 0.05$; NS: $P > 0.05$.

decreased CD8$^+$ T cell memory-recall responses (**Fig. 6e**) and low survival (~20%) (**Fig. 6f**) after influenza-PR/8 infection. In contrast, the NKG2D co-stimulation regimen resulted in augmented CD8$^+$ T cell memory-recall responses and survival (**Fig. 6e**,**f**). Further, CD8$^+$ T cells from mice lacking NKG2D expression were unable to respond to influenza-PR/8 challenge *in vivo* despite receiving OVA + Rae-1ε vaccination during priming (**Fig. 6e**).

To further assess the role of NKG2D co-stimulation in a pathogenic model where chronic CD4 deficiency has a major role in disease progression, we determined the response of HIV-specific CD8$^+$ T cells from chronic HIV-positive donors, whose lack of CD4$^+$ T cell help leads to CD4-unhelped CD8$^+$ T cells[36], and compared these with CD8$^+$ T cells from long-term nonprogressor (LTNP) HIV-positive donors whose maintained CD4$^+$ T cell help leads to potent anti-HIV

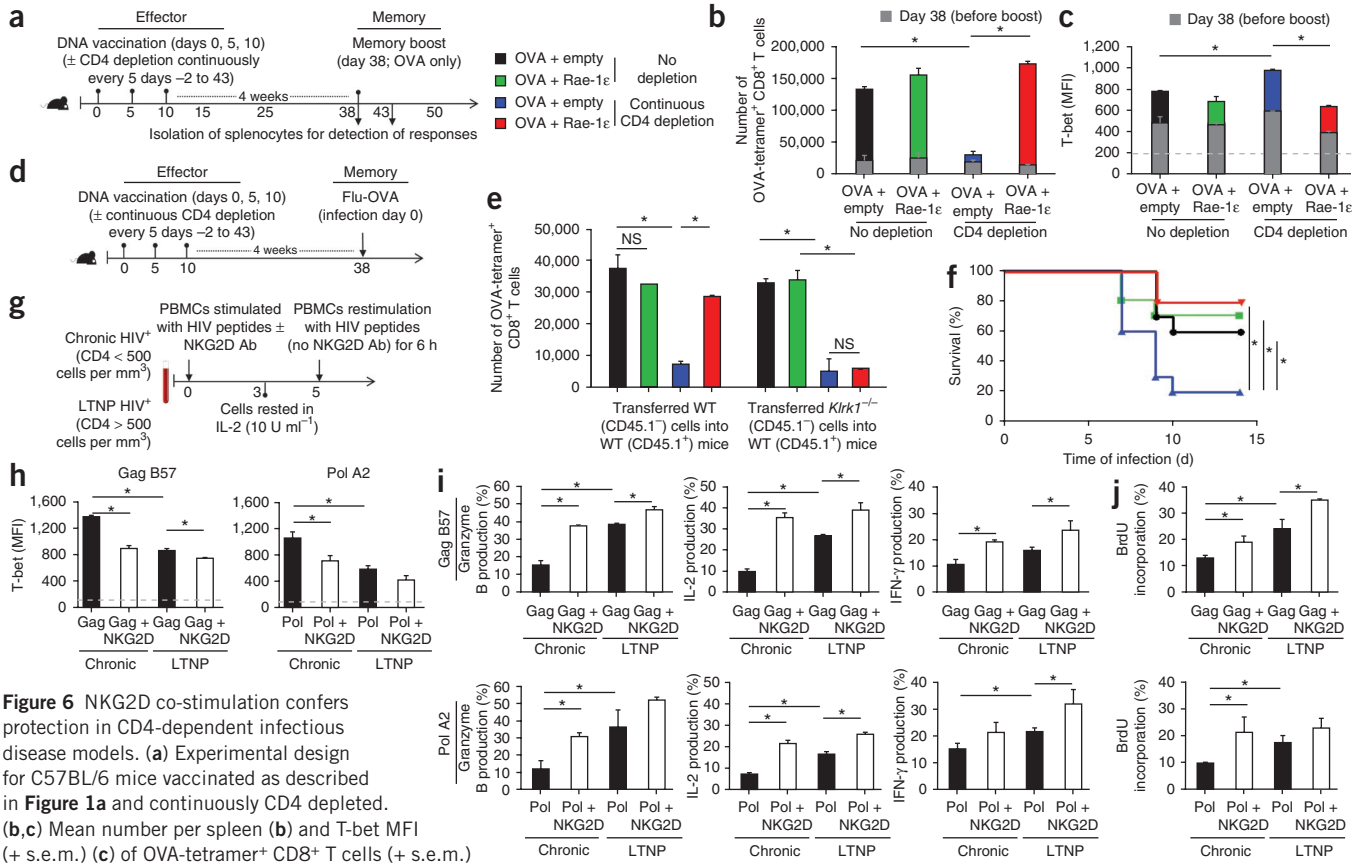

Figure 6 NKG2D co-stimulation confers protection in CD4-dependent infectious disease models. (**a**) Experimental design for C57BL/6 mice vaccinated as described in **Figure 1a** and continuously CD4 depleted. (**b**,**c**) Mean number per spleen (**b**) and T-bet MFI (+ s.e.m.) (**c**) of OVA-tetramer$^+$ CD8$^+$ T cells (+ s.e.m.) on days 38 and 43 from mice in **a**. (**d**) Experimental design of influenza-PR/8 intranasal infection. (**e**) Number of OVA-tetramer$^+$ CD8$^+$ T cells from experiment in which WT or *Klrk1*$^{-/-}$ CD8$^+$ T cells were adoptively transferred into WT hosts and vaccinated and infected as described in **d**. Bar graph data in **b**, **c** and **e** are cumulative from three to five mice analyzed individually per group from one experiment of three experiments conducted with similar results. (**f**) Survival curve for one experiment described in **d** and repeated twice with similar results. (**g**) Experimental design for stimulation of HIV-positive chronic or LTNP donor cells with pooled HIV peptides. (**h**–**j**) HIV-tetramer$^+$ CD8$^+$ T cell MFI (+ s.e.m.) of T-bet (**h**), mean percentage producing granzyme B, IL-2 or IFN-γ (+ s.e.m.) (**i**), and mean percentage incorporating BrdU (+ s.e.m.) (**j**) from at least three donors per group. Gag B57, Gag HLA-B57 tetramer-PE (KAFSPEVIPMF); Pol A2, HIV Pol HLA-A2 tetramer-PE (ILKEPVHGV). Dashed lines in **c** and **h** represent MFI of background flow cytometric staining for the respective markers. *$P < 0.05$; NS: $P > 0.05$.

$CD8^+$ T cell responses[37] (**Supplementary Table 1**). To replicate the conditions used in our *in vivo* mouse studies, we stimulated donor peripheral blood mononuclear cells (PBMCs) with pooled HIV peptides in the presence or absence of exogenous NKG2D agonist antibody, rested them for 2 d and re-stimulated them with pooled HIV peptides (without NKG2D agonist antibody) (**Fig. 6g**). As in the mouse models, NKG2D co-stimulation of human $CD8^+$ T cells from chronic HIV donors lowered T-bet expression (**Fig. 6h**) and rescued their recall ability to produce granzyme B, IL-2 and IFN-γ (**Fig. 6i**) and proliferate (**Fig. 6j**). Further, the phenotype and functionality of NKG2D-rescued recall $CD8^+$ T cell responses from chronically infected HIV-positive donors resembled those associated with $CD8^+$ T cell responses from HIV-positive LTNPs (**Fig. 6h–j**).

DISCUSSION

We demonstrate that augmented NKG2D engagement on $CD8^+$ T cells rescues CD4-unhelped $CD8^+$ T cell memory-recall responses. Contrary to our expectations, the NKG2D co-stimulation regimen did not rescue CD4-unhelped effector responses. This paradoxical finding raises the question of how memory-recall responses can be rescued in the absence of effector responses. We discovered that, through augmentation of JNK2 phosphorylation, increased NKG2D co-stimulation results in CD4-unhelped $CD8^+$ T effector cells with reduced expression of T-bet, a transcription factor that at low expression drives formation of effector cells with increased memory potential[28–31]. This leads us to propose that the effector-phase responses and memory potential of $CD8^+$ T cells may be dictated by separate signals and, furthermore, that NKG2D co-stimulation provides $CD8^+$ T cells with memory potential programming for conversion to potent memory $CD8^+$ T cells even in the absence of primary effector responses.

Similar to the impaired effector cytolytic responses of CD4-unhelped $CD8^+$ T cells in our study, previous studies[5,38] have shown comparable (that is, nearly absent) CD4-helpless effector responses on day 14. However, some studies have shown that CD4-unhelped $CD8^+$ T cell effector responses are typically less affected and that memory-recall responses are greatly impaired[4,6,7,39,40]. This difference may be attributed to the level of inflammation-mediated response present during the effector phase. In the studies conducted using infection models, CD4-unhelped $CD8^+$ T cell responses may have arisen from infection-associated inflammation signals. In our study, skin gene gun delivery causes limited inflammation[41] and thus aids in deconstructing effector response mechanisms.

Despite the absence of effector responses from CD4-unhelped $CD8^+$ T cells vaccinated without the NKG2D co-stimulation regimen, we observed memory responses, albeit defective ones. These preserved memory responses are attributable to the return of $CD4^+$ T cell help during memory-phase boost vaccination. Recent studies support the notion that CD4-unhelped responses can be rescued in the memory phase[5,42]. Notably, even with continuous CD4 depletion throughout both the effector and memory phases, the NKG2D co-stimulation regimen still rescued $CD8^+$ T cell memory responses.

Studies have shown that IL-15 can induce NKG2D expression on $CD8^+$ T cells[25] and that IL-15 is necessary for NKG2D signaling[26]. In our study, NKG2D-mediated rescue in CD4-unhelped $CD8^+$ T cells was partially dependent on IL-15. These data may provide the mechanism by which IL-15 has been shown to aid in CD4-unhelped memory response rescue[27]. IL-2, a cytokine augmented in our studies with increased NKG2D engagement, has been reported to compensate for the lack of IL-15 (ref. 43). These results may warrant future investigation in determining whether the partial rescue observed in IL-15–deficient mice may be mediated by NKG2D signaling through alternative molecular pathways[44] or whether other common γ-chain cytokines compensate for the lack of IL-15 in this situation.

Even under deleterious conditions in an *in vivo* pathogenic model of lethal influenza-PR/8 infection during continuous immunocompromising CD4 depletion, augmented NKG2D co-stimulation provided mice with rescued memory-recall $CD8^+$ T cells and a high rate of survival. These findings raise the possibility of including an NKG2D co-stimulation regimen in human vaccination protocols. Specifically in the context of influenza vaccines, which in a nonadjuvant form do not drive strong $CD4^+$ T cell responses[45], these data suggest a potential enhancement, via the NKG2D co-stimulation regimen, of the effectiveness of $CD8^+$ T cell responses. Additionally, such an NKG2D co-stimulation regimen may avoid vaccine side effects attributable to robust $CD4^+$ T cell activation in the presence of strong adjuvants[46].

Finally, we demonstrate that the NKG2D co-stimulation regimen observations equally pertain to a human disease system in which $CD4^+$ T cells are progressively depleted by HIV, resulting in $CD8^+$ T cells that are characteristically CD4 unhelped[47]. Whereas increased T-bet expression by Nef peptide–stimulated, IFN-γ–producing $CD8^+$ T cells from HIV elite controllers (a subset of LTNPs) has been reported[48], our study demonstrates that NKG2D co-stimulation reduced T-bet expression of Gag tetramer– and Pol tetramer–specific $CD8^+$ T cells from chronically infected HIV donors and rescued their ability to acquire cytolytic potential. Furthermore, such NKG2D co-stimulation rescued *ex vivo* $CD8^+$ T cell responses from chronically infected HIV-positive donors to resemble the phenotype and functionality associated with HIV-positive LTNP $CD8^+$ T cell responses. Augmented NKG2D co-stimulation may have a role in improving $CD8^+$T cell responses against HIV itself and against AIDS-associated pathogens.

This work demonstrates that NKG2D co-stimulatory signaling during priming may have therapeutic value, specifically in the development of optimal memory-recall responses and the reversal of the impotent state of CD4-unhelped $CD8^+$ T cells. These findings may be useful in T cell–based vaccine design and adoptive T cell therapy, where potent effector and memory formation are vital for successful eradication of acute and recurrent disease, especially in situations where $CD4^+$ T cells are depleted as a result of the disease (for example, HIV/AIDS), exhausted or suppressed as a sequela of the given pathology (for example, chronic infection or cancer) or therapeutically depleted to remove immune suppression (for example, cancer). In such situations, delivery of NKG2D ligand may be a feasible substitute for $CD4^+$ T cell help.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

For their kind gifts, we thank: A. Houghton (pCRAN vector and OVA DNA; Memorial Sloan-Kettering Cancer Center); V. Kumar and L. Chlewicki (non–Rae-1ε–expressing EL-4 cells; The University of Chicago); L. Lanier (genes encoding Rae-1ε, Rae-1ε–GFP, and H60; University of California, San Francisco); A. Tenorio (HIV-positive donor identification; Rush University Medical Center); W. Yokoyama (NKG2D-deficient mouse spleens; Washington University School of Medicine in St. Louis), and D. Raulet (NKG2D-decifient mice, University of California, Berkeley). We thank B. Jabri, A. Bendelac, M.I. Nishimura and M.J. Turk for constructive discussions and manuscript edits. We are grateful to the flow cytometry facilities at The University of Chicago and at Loyola University Chicago for their invaluable support and to the Frank W. Fitch Monoclonal Antibody Facility of The University of Chicago Cancer Center (funded by

US National Cancer Institute Cancer Center Support Grant 5P30CA014599-35) for CD4 depletion antibody production. HMG2D-specific antibody was used with permission from H. Yagita (Juntendo University School of Medicine). This work was supported in part by the American Cancer Society (ACSLIB112496-RSG) to J.A.G.-P.; American Cancer Society-Illinois Division (Young Investigator Award Grant 07-20) to J.A.G.-P.; Croatian Ministry of Science, Education and Sports (062-0621261-1271), as well as the Croatian-Israeli Grant to B.P.; the US National Institutes of Health R21CA127037 to J.A.G.-P., PO1AI082971 to L.A.-H., K22AI077714 to P.G.T.; T32 Immunology Training Grant, The University of Chicago, 5T32AI007090 to A.Z., F.J.K. and J.A.O.; and the Cancer Research Foundation (Young Investigator Award) to J.A.G.-P.

AUTHOR CONTRIBUTIONS

A.Z., F.J.K. and J.A.G.-P. designed the study and wrote the manuscript; J.A.G.-P. supervised the project. G.E.L. generated the DNA constructs and performed WT CTL experiments. A.Z. performed the cytokine, kinetics, T-bet/JNK2, HIV and remainder of CTL experiments. J.M.S. designed the cytokine staining and JNK2 experiments. T.V.M., V.V., J.W.W. and A.Z. performed the influenza experiments. F.J.K., A.T.L., J.A.O., M.C.J., E.C.B., A.Z. and J.A.G.-P. performed DNA vaccinations and flow cytometric experiments. L.A.-H. collaborated on the HIV experiment; A.L.M., P.G.T. and A.I.S. collaborated on the influenza experiment. B.P. and B.Z. developed the NKG2D-deficient mice and collaborated on associated experiments. All authors edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice, donors and cells. Six-week-old, specific pathogen–free, male C57BL/6 (B6), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) and B6.SJL-PtprcaPepcb/BoyJ (CD45.1$^+$) mice (Jackson Laboratories); C57BL/6NTac-IL15tm1Imx N5 (IL-15–deficient; *Il15*$^{-/-}$) and C57BL/6 control mice (Taconic); Thy1.1-marked OT-I and B6BCF1 (C57BL/6 × Balb/c F1 hybrid) mice (bred in house); and NKG2D-deficient (*Klrk1*$^{-/-}$) mice (D. Raulet) were housed under conventional conditions. We conducted experiments in accordance with The University of Chicago Institutional Animal Care and Use Committee (IACUC) and the Loyola University Chicago IACUC guidelines. We obtained peripheral blood from chronic HIV-positive donors (documented as HIV positive for at least 5 years without extended antiretroviral therapy, with CD4$^+$ T cell count <500 cells per mm^3 and uncontrolled plasma HIV-1 RNA levels) and LTNP donors (documented as HIV positive for at least 5 years without extended antiretroviral therapy, with CD4$^+$ T cell count >500 cells per mm^3 and low or undetectable plasma HIV-1 RNA levels). We conducted human cell research in accordance with guidelines on human research and the approval of the Institutional Review Board of Rush University Medical Center. We obtained donor informed consent in accordance with the Declaration of Helsinki. All cells were cultured in RPMI supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 2 mM L-glutamine (Mediatech) and 1% penicillin-streptomycin (Mediatech).

***In vitro* culture.** Mouse EL-4 target cells were loaded (for 2 h) with $OVA_{257–264}$ peptide (1 μg ml^{-1}) and co-cultured for 72 h with CD8$^+$ T cells (isolated by negative selection from OT-I mouse spleens) and 30 U ml^{-1} IL-2 (R&D Systems) with or without JNK inhibitor IX (*N*-[3-cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl]-1-naphthamide; EMD Chemicals; 25 ng ml^{-1}). We primed human HIV-positive donor PBMCs for 3 d with pooled HIV peptides spanning the Gag or Pol regions[49] (NIH AIDS Research and Reference Reagent Program) at 2 μg per peptide per ml with or without NKG2D agonistic antibody (1D11, 2 μg ml^{-1}) (BioLegend), rested them for 2 d in medium with minimal IL-2 (10 U ml^{-1}) and re-stimulated them as before with HIV peptides (no NKG2D agonistic antibody) for 6 h.

DNA vaccination. We performed vaccinations (4 μg plasmid DNA per mouse per vaccination) using a Helios gene gun (Bio-Rad) as previously described[21,50]. DNA vaccines (OVA + Rae-1ε, OVA + empty, OVA + H60, empty + empty) (empty bullets were made with everything the same except without OVA, Rae and H60) were generated using the pCRAN multiple cloning site variant of pcDNA3 (Invitrogen). DNA was produced in large quantities and purified by GeneArt. We generated bullets containing DNA as previously described[21].

Influenza infection. Mice were anesthetized with ketamine and xylazine[51], weighed and given influenza A virus (PR/8; strain A/Puerto Rico/8/1934 H1N1 modified to express OVA) via intranasal administration (80,000 half-maximal embryo infectious dose). We killed mice on day 3 of infection for spleen recovery or weighed them throughout the infection and killed them when they reached <70% preinfection weight.

Cytotoxic T lymphocyte lysis assays. C57BL/6 splenocyte target cells were peptide-loaded (with 1 μg ml^{-1} of SIINFEKL ($OVA_{257–264}$) or irrelevant peptide (KVPRNQDWL, $hgp100_{25–33}$)), CFSE-labeled (0.5 and 8 μM, respectively) and adoptively transferred (1:1 ratio, 2 × 10^7 cells total) via retro-orbital injection. Eighteen hours later, transferred spleen cells were analyzed by flow cytometry for CFSE loss and specific lysis was calculated[52].

Flow cytometry. We purchased all flow cytometry antibodies from Ebioscience, except antibodies to mouse CD3 (BD Biosciences), T-bet (BioLegend) and pJNK2 (Santa Cruz Biotechnology). OVA MHC-I tetramer–PE (SIINFEKL), HIV Gag HLA-B57 tetramer–PE (KAFSPEVIPMF) and HIV Pol HLA-A2 tetramer–PE (ILKEPVHGV) were purchased from Beckman Coulter. These OVA and HIV tetramers are tetramers that are fluorescently labeled (with PE) and have an MHC-I that holds the respective peptide. We performed flow cytometric antibody staining and analysis as previously described[49,53–55]. Cell staining data were acquired using an LSR-II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). We gated on live lymphocytes using LIVE/DEAD staining (R&D Systems), forward scatter area (FSC-A) versus side scatter area (SSC-A), followed by forward scatter width (FSC-W) versus side scatter width (SSC-W), FSC-A versus forward scatter height (FSC-H) and SSC-A versus side scatter height (SSC-H) plots. Cell counts were calculated using PKH26 beads, as previously described[56,57].

Antibody depletions, blocking and stimulation. We delivered depleting or blocking monoclonal antibodies (CD4-specific antibody (GK1.5; BioXcell and The University of Chicago Frank W. Fitch Monoclonal Antibody Facility) NK1.1-specific antibody (PK136, BioXcell) and NKG2D-specific antibody (HMG2D, BioXcell)) via intraperitoneal injection at 500 μg per mouse per depletion or blocking. *In vitro* NKG2D co-stimulation of human PBMCs was performed using NKG2D -specific agonistic antibody (1D11, BioLegend).

Adoptive CD8$^+$ T cell transfer. We magnetically isolated cells from mouse spleens via negative selection using the MACS CD8α$^+$ T cell negative isolation kit (Miltenyi Biotec) or a positive isolation kit (naïve (CD44$^-$) CD8$^+$ T cells: R&D Systems, CDllc$^+$ cells: Miltenyi Biotec). Cells were delivered via retro-orbital injections in 100 μl serum-free PBS.

EL4 target cell preparation. We generated Rae-1ε and H60 constructs using the pcDNA3 vector (Invitrogen) and transfected them using lipofectamine into EL4 cells (V. Kumar and L. Chlewicki) not expressing Rae-1ε, as described previously[50]. Control EL4 cells were transfected with empty pCRAN.

Statistical analyses. We used the log-rank test for comparison of survival curves. For the remainder of statistical analyses we used Student's *t* test (two-tailed). A *P* value of < 0.05 was considered statistically significant.

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