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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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Abstract

CD4-unhelped CD8+ T cells are functionally-defective cells primed in the absence of CD4+ T cell help and present a critical problem. Based on the co-stimulatory and non-canonical roles of 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, responses. NKG2D-mediated rescue is

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AUTHOR CONTRIBUTIONS

A.Z., F.J.K., and J.A.G. designed the study and wrote the manuscript; J.A.G. 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. performed DNA vaccinations and flow cytometric experiments. L.A. 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.

characterized by reversal of elevated T-bet expression and recovery of IL-2 and IFN-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.

INTRODUCTION

Memory $CD8^+$ T cells confer efficient and long-lasting immunity against secondary pathogen exposure[1, 2]. Events during primary exposure (priming) impact the quality of the initial effector and subsequent memory cytotoxic T lymphocyte (CTL) responses. Unless environmental cues (*e.g.*, $CD4^+$ 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 help, these resultant "CD4-unhelped" $CD8^+$ T cells do not differentiate into sustainable memory cells[4].

NKG2D on natural killer (NK) cells and on activatedef $CD8^+$ T cells binds to retinoic acid early inducible-1 gene ε (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-associated ligands[16–18]. Besides this canonical function, NKG2D is implicated in $CD8^+$ T cell-mediated autoimmune pathophysiology[19]. Therefore, strategies augmenting NKG2D engagement on $CD8^+$ T cells and harnessing its non-canonical functions may result in the rescue of CD4-unhelped $CD8^+$ T cell responses.

RESULTS

NKG2D co-stimulation regimen rescues CD4-unhelped $CD8^+$ T cell memory recall expansion

$CD8^+$ T cells that do not receive $CD4^+$ T cell help during priming undergo impaired memory recall expansion[6, 20]. Because NKG2D engagement on $CD8^+$ T cells contributes to co-stimulation[16, 17], we hypothesized that increased NKG2D engagement by Rae-1ε from antigen presenting cells (APCs) during priming would rescue memory recall CD4-unhelped $CD8^+$ T cell expansion. We employed skin gene gun vaccination[21] (*in vivo* biolistic transfection) to co-deliver DNA plasmids encoding chicken ovalbumin (OVA) and NKG2D ligand Rae-1ε (OVA/Rae-1ε), OVA and empty vector (OVA/Empty), or empty vectors (Empty/Empty) to 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) ± CD4 depletion (days −2, 0, 5, and 10), were rested for 4 weeks during memory formation, and then received one memory boost vaccination (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 in the spleen (Fig. 1b and Supplementary Fig. 2) and confirmed in the draining inguinal lymph node (Supplementary Fig. 3). Similar post-contraction levels (day 38, before boost) of OVA-specific $CD8^+$ T cells were observed 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 cells at the memory recall phase (Fig. 1c,d and Supplementary Fig. 3b).

NKG2D co-stimulation regimen rescues CD4-unhelped $CD8^+$ T cell memory recall cytokine production and cytolytic responses

Based on 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 displayed complete rescue of granzyme B, IL-2, and IFN-γ production (Fig. 2a). The contribution of the memory boost vaccination (*i.e.*, a single vaccination) on naïve 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 CTL lysis (Fig. 2c,d). Importantly, administration of either Rae-1ε (in 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). Since $CD4^+$ T cells were present during boost vaccination, we analyzed their potential role in the memory recall responses observed. Addition of CD4 depletion during the memory boost resulted in decreased memory recall CTL lysis in all groups (Supplementary Fig. 5). While the greatest decrease was observed in the group primed without the NKG2D co-stimulation regimen and depleted of $CD4^+$ T cells both during priming and memory boost, rescue was still observed with the NKG2D co-stimulation regimen (Supplementary Fig. 5).

NKG2D on $CD8^+$ T cells is necessary for rescue of CD4-unhelped $CD8^+$ T cell memory recall responses

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, since NK and NKT cells express NKG2D, we determined their role in NKG2D-mediated rescue. C57BL/6 mice were depleted of $NK1.1^+$ cells[23] and Thy1.1-marked OT-I $CD8^+$ T cells were transferred into these mice. 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 $CD8^+$ T cells. Here, priming of $CD8^+$ T cells occurred after NK1.1 depletion antibody was 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-ngagement on $CD8^+$ T cells, we employed vaccination of $CD45.1^+$ 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 of CD4-unhelped CD8$^+$ T cells is not independent of IL-15

Roles for IL-15 in NKG2D upregulation[25] and signaling[26], as well as rescue of CD4-unhelped CD8$^+$ T cells[27], have been reported. Therefore, we investigated IL-15 presentation (via dendritic cell IL-15Rα expression) and found it to be equal under the 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. 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).

NKG2D co-stimulation regimen does not rescue CD4-unhelped CD8$^+$ T cell effector responses

We sought to understand the process by which NKG2D mediates rescue of memory responses by investigating CD8$^+$ T cell responses at the effector phase. A significant decrease in expansion of CD4-unhelped OVA-specific CD8$^+$ T cells was observed at the effector phase (day 15) and, unexpectedly, not augmented by the NKG2D co-stimulation regimen (Fig. 1b and Supplementary Fig. 7).

A significant increase in effector phase granzyme B production (Supplementary Fig. 8a) and lysis (Supplementary Fig. 8b) was observed in non-depleted mice receiving the NKG2D co-stimulation regimen. However, lack of CD4 help resulted in un-rescued minimal target lysis with OVA/Empty, OVA/Rae-1ε, and OVA/H60 vaccination (Supplementary Fig. 8c,d).

NKG2D engagement results in JNK2-mediated suppression of T-bet in CD4-unhelped CD8$^+$ T cells

CD4-unhelped CD8$^+$ T cells express higher levels of T-bet 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 if effector phase T-bet expression correlates with NKG2D-mediated rescue of CD4-unhelped CD8$^+$ T cell memory recall responses, we determined T-bet levels in OVA-specific CD8$^+$ T cells. In the context of CD4 help, OVA-specific CD8$^+$ T cells consistently expressed low T-bet levels throughout the effector and memory phases (Fig. 4a–c). Conversely, CD4-unhelped OVA-specific CD8$^+$ T cells primed without the NKG2D co-stimulation regimen expressed significantly increased levels of T-bet (Fig. 4a–c). Importantly, T-bet levels in CD4-unhelped CD8$^+$ T cells were significantly reduced (45%) with the NKG2D co-stimulation regimen, remained similar to T-bet levels of CD4-helped CD8$^+$ T cells throughout the effector phase, and were significantly lower during the memory phase (Fig. 4a–c). Corroborating these findings, T-bet levels were elevated in NKG2D-deficient versus WT OVA-specific CD8$^+$ T cells transferred into a WT host, especially under CD4-depletion conditions (Fig. 4d), and elevated when compared to endogenous CD8$^+$ T cells (Supplementary Fig. 9a). Likewise, T-bet levels in OVA-specific CD8$^+$ T cells were 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 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 elevated T-bet (Fig. 5a) and decreased 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 decreased pJNK2 levels (Fig. 5b). Based on these observations 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 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).

Augmented NKG2D co-stimulation confers protection in CD4-dependent infectious disease models

To address the condition in which $CD4^+$ T cells remain continuously low or absent, we characterized the ability of NKG2D to rescue $CD8^+$ T cell responses under continuous CD4 antibody depletion administered every 5 d (days −2 to 43) (Fig. 6a). Here, $CD8^+$ T cell memory expansion was significantly reduced (Fig. 6b), and T-bet further increased (Fig. 6c) compared to CD4 depletion only during priming (Figs. 1 and 4). Further, $CD8^+$ T cells primed with the NKG2D co-stimulation regimen and continuously depleted of $CD4^+$ T cells exhibited rescued 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 utilized a lethal Influenza-PR/8 infection model, in which the virus expresses OVA and clearance is CD4-dependent (Fig. 6d). Absence of the NKG2D co-stimulation regimen and presence of continuous CD4 depletion in mice resulted in 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 significantly-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 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 plays 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 long-term non-progressor (LTNP) HIV-positive donors whose maintained $CD4^+$ T cell help leads to potent anti-HIV $CD8^+$ T cell responses[37] (Supplementary Table 1). To replicate the conditions used in our *in vivo* mouse studies, donor PBMCs were stimulated with pooled HIV peptides in the presence or absence of exogenous NKG2D stimulating antibody, rested for 2 d, and re-stimulated 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 reduced 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, NKG2D co-stimulation rescued recall $CD8^+$ T cell responses in chronically-infected HIV-positive donors to resemble the phenotype and functionality associated with HIV-positive long-term non-progressor responses (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 as to how memory recall responses can be rescued in the absence of effector responses. We discovered that, through augmentation of phosphorylated JNK2, augmented NKG2D co-stimulation results in CD4-unhelped $CD8^+$ T effector cells with reduced expression of T-bet, a transcription factor which at low levels drives formation of effector cells with increased memory potential[28–31]. This leads us to propose that 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, work by Janssen *et al.*[5, 38] shows a comparable (*i.e.*, 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 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. However, 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, memory responses, albeit defective, were observed. These preserved memory responses are attributable to the presence 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]. Importantly, even with continuous CD4 depletion during 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 dependent, albeit partially, 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[43]. Future investigation may be warranted in determining whether partial rescue demonstrated in IL-15-deficient mice may be mediated by NKG2D signaling through alternative molecular pathways[44] or if other common gamma 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, NKG2D co-stimulation conferred mice with rescued memory recall $CD8^+$ T cells and a high rate of survival. Similar NKG2D-mediated survival was observed in a model of chronic LCMV (clone 13) infection under CD4-deficient conditions (personal observation). These findings raise the possibility of including an NKG2D co-stimulation regimen in human vaccination protocols. Specifically in the context of influenza vaccination, which in a non-adjuvant form does not drive strong CD4 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 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]. While increased T-bet

expression by Nef peptide-stimulated IFN-γ-producing $CD8^+$ T cells from HIV elite controllers (a subset of long-term non-progressors) has been reported[48], our study demonstrates that NKG2D co-stimulation reduced T-bet expression of Gag 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 $CD8^+$ T cell responses from chronically-infected HIV-positive donors to resemble the phenotype and functionality associated with HIV-positive long-term non-progressor $CD8^+$ 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.

Our work here demonstrates that NKG2D co-stimulatory signaling during priming may have therapeutic value, specifically in the development of optimal memory recall responses and reversal of the impotent state of CD4-unhelped $CD8^+$ T cells. These NKG2D-mediated findings are important from the perspective of 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 (*e.g.*, HIV/AIDS), exhausted or suppressed as a sequela of the given pathology (*e.g.*, chronic infection, cancer, etc.), or therapeutically depleted to remove immune suppression (*e.g.*, cancer). In such situations, delivery of NKG2D ligand can become a feasible substitute for CD4 help. These findings address limitations of current T cell regimens against intracellular pathogens and cancer by generating better memory recall responses even under severely CD4-compromised conditions.

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 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 HIV^+ for at least 5 y without extended antiretroviral therapy, $CD4^+$ T cell count < 500 cells per mm^3, and uncontrolled plasma HIV-1 RNA levels) and long-term non-progressor donors (documented HIV^+ for at least 5 y without extended antiretroviral therapy, $CD4^+$ 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 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 fetal bovine serum (Atlanta Biologicals), 2mM L-glutamine (Mediatech), and 1% penicillin/streptomycin (Mediatech).

In vitro culture

Mouse EL-4 target cells were loaded (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) ± 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 ± NKG2D stimulation antibody (1D11, 2 μg ml^{-1}), rested them for 2 d in media with minimal IL-2 (10 U ml^{-1}, and restimulated them similarly with HIV peptides (no NKG2D 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) 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, weighed, and given Influenza A virus (PR/8; strain A/Puerto Rico/8/1934 H1N1 modified to express OVA) via intranasal administration (80,000 EID_{50}). We euthanized mice on day 3 of infection for spleen recovery, or weighed them throughout the infection and euthanized them when they reached < 70% pre-infection weight.

CTL lysis assays

C57BL/6 splenocyte target cells were loaded (1μg ml^{-1} of SIINKEKL [$OVA_{257–264}$] or irrelevant peptide [KVPRNQDWL, $hgp100_{25–33}$]), CFSE-labeled (0.5 and 8μM, respectively), and adoptively transferred (1:1 ratio, 2×10^7 cells total) via retroorbital injection. Eighteen hours later spleen cells were analyzed by flow cytometry for CFSE loss and specific lysis was calculated[51].

Flow cytometry

We purchased all flow cytometry antibodies from Ebioscience, except mCD3 (BD Biosciences), m/hT-bet (BioLegend), and pJNK2 (Santa Cruz Biotechnology, Inc.). OVA MHC-I Tetramer-PE (SIINFEKL), HIV Gag HLA-A2 Tetramer-PE (KAFSPEVIPMF), and HIV Pol HLA-A2 Tetramer-PE (ILKEPVHGV) were purchased from Beckman Coulter. We performed flow cytometric antibody staining and analysis as previously described[49, 52, 53, 54]. Cell staining data were acquired using a 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[55, 56].

Antibody depletions, blocking, and stimulation

We delivered depletion/blocking monoclonal antibodies (CD4 antibody [GK1.5; BioXcell and The University of Chicago Frank W. Fitch Monoclonal Antibody Facility]; NK1.1 antibody [PK136, BioXcell]; and NKG2D 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/CD314 antibody (1D11, BioLegend).

Adoptive $CD8^+$ T cell transfer

We magnetically isolated cells from mouse spleens via negative selection using the MACS $CD8\alpha^+$ 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 not expressing Rae-1ε, as described previously[50]. Control EL4 cells were transfected with empty pCRAN.

Statistical analyses

We used the logrank 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.

Supplementary Material

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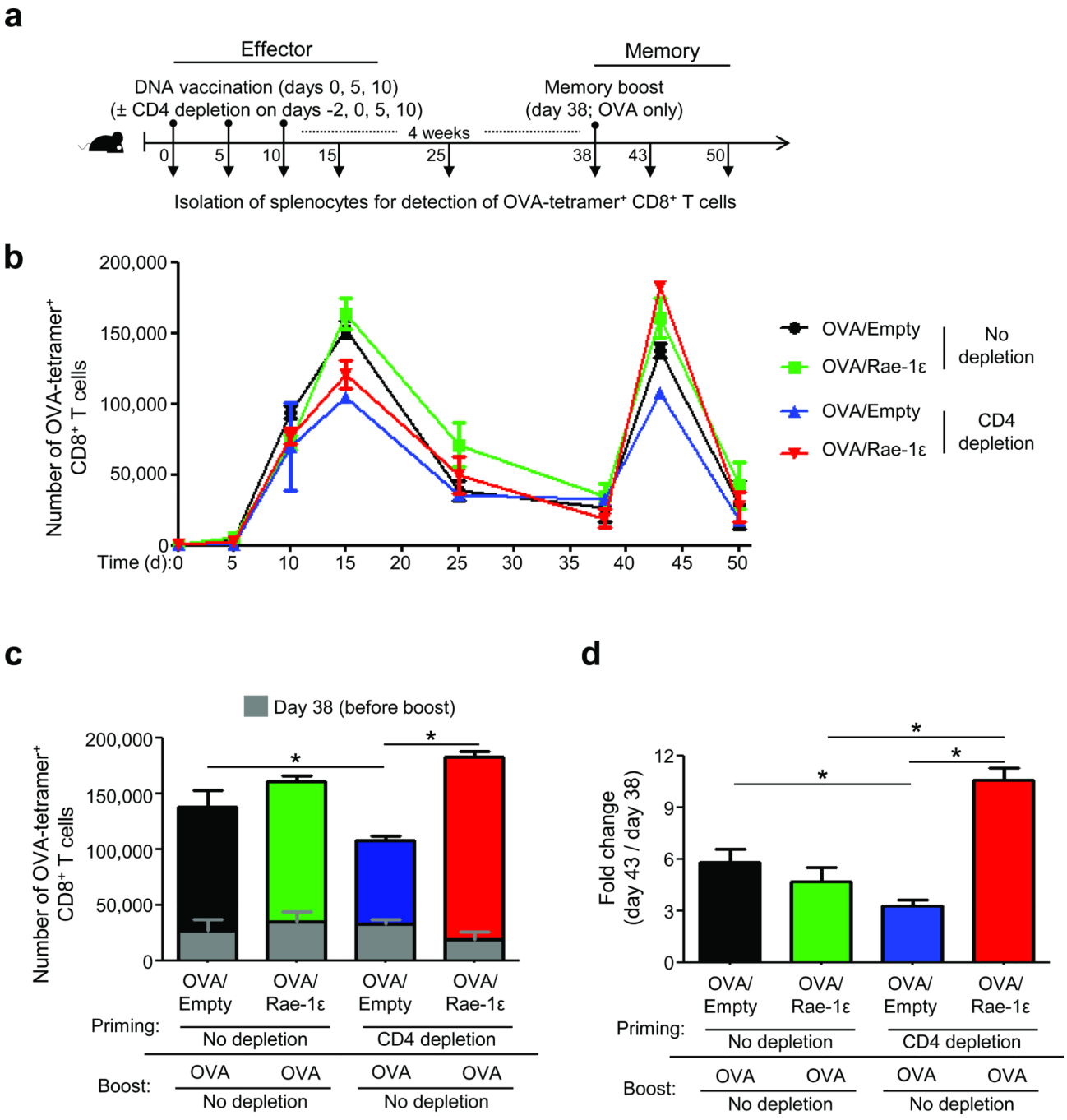

Figure 1.

NKG2D engagement by Rae-1ε rescues CD4-unhelped CD8$^+$ T cell memory recall expansion. (**a**) Experimental design for vaccination and CD4 depletion. (**b**) Expansion kinetics of OVA-tetramer$^+$ CD8$^+$ T cells (±SEM) calculated per spleen. (**c**) Mean number of OVA-tetramer$^+$ CD8$^+$ T cells (+SEM) per spleen on days 38 and 43. (**d**) Data from (**c**) shown as a fold change. Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results. *$P < 0.05$.

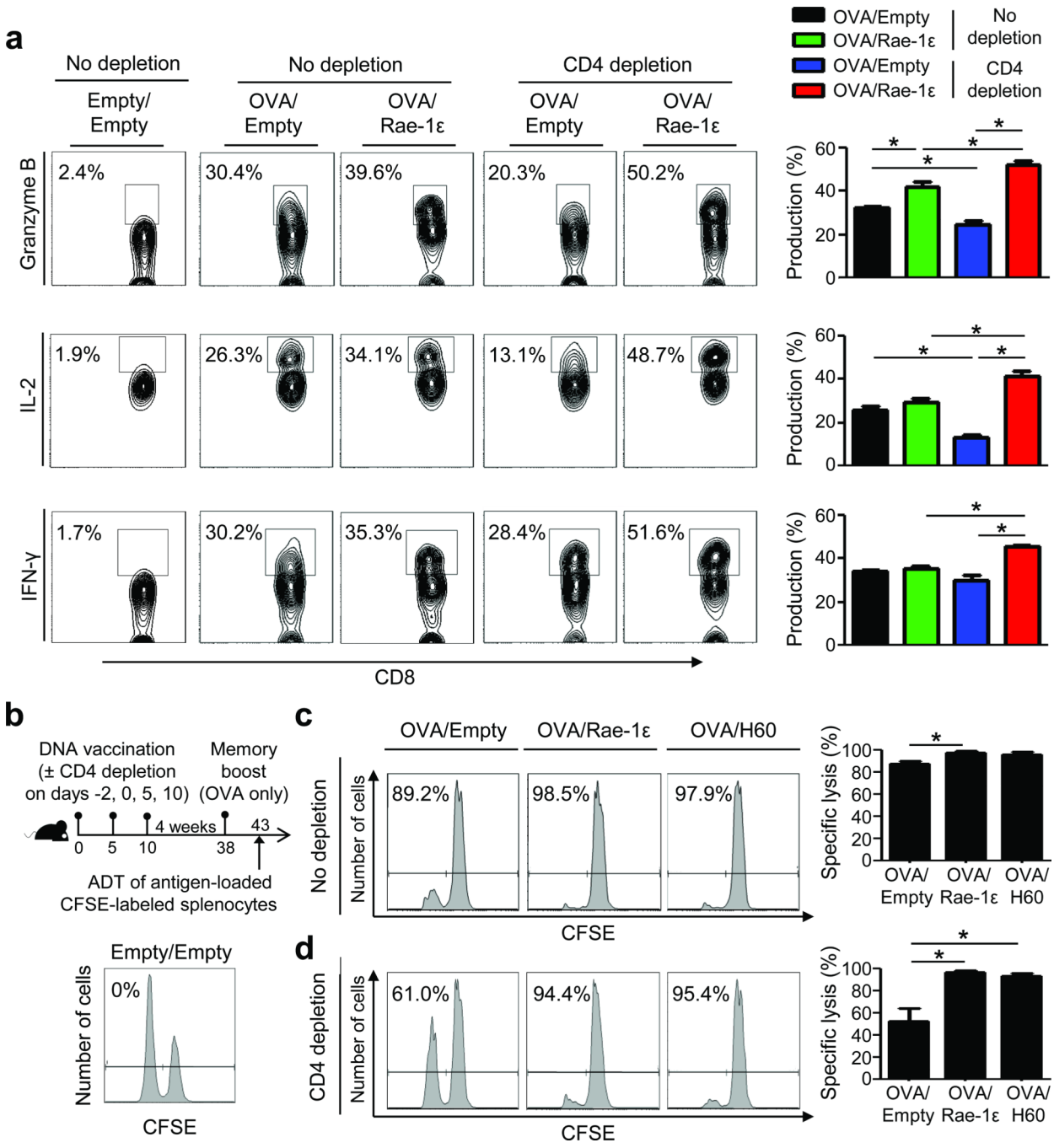

Figure 2.

NKG2D-mediated rescue of CD4-unhelped CD8$^+$ T cell memory recall responses involves cytokine and lytic molecule production and CTL lysis. (**a**) Splenic OVA-tetramer$^+$ CD8$^+$ T cell production of granzyme B, IL-2 and IFN-γ on day 43 as described in Fig. 1a. (**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 (Empty/Empty) mouse. (**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. Data are

representative of 3–5 mice analyzed individually per group per experiment from at least three experiments conducted with similar results. *$P < 0.05$.

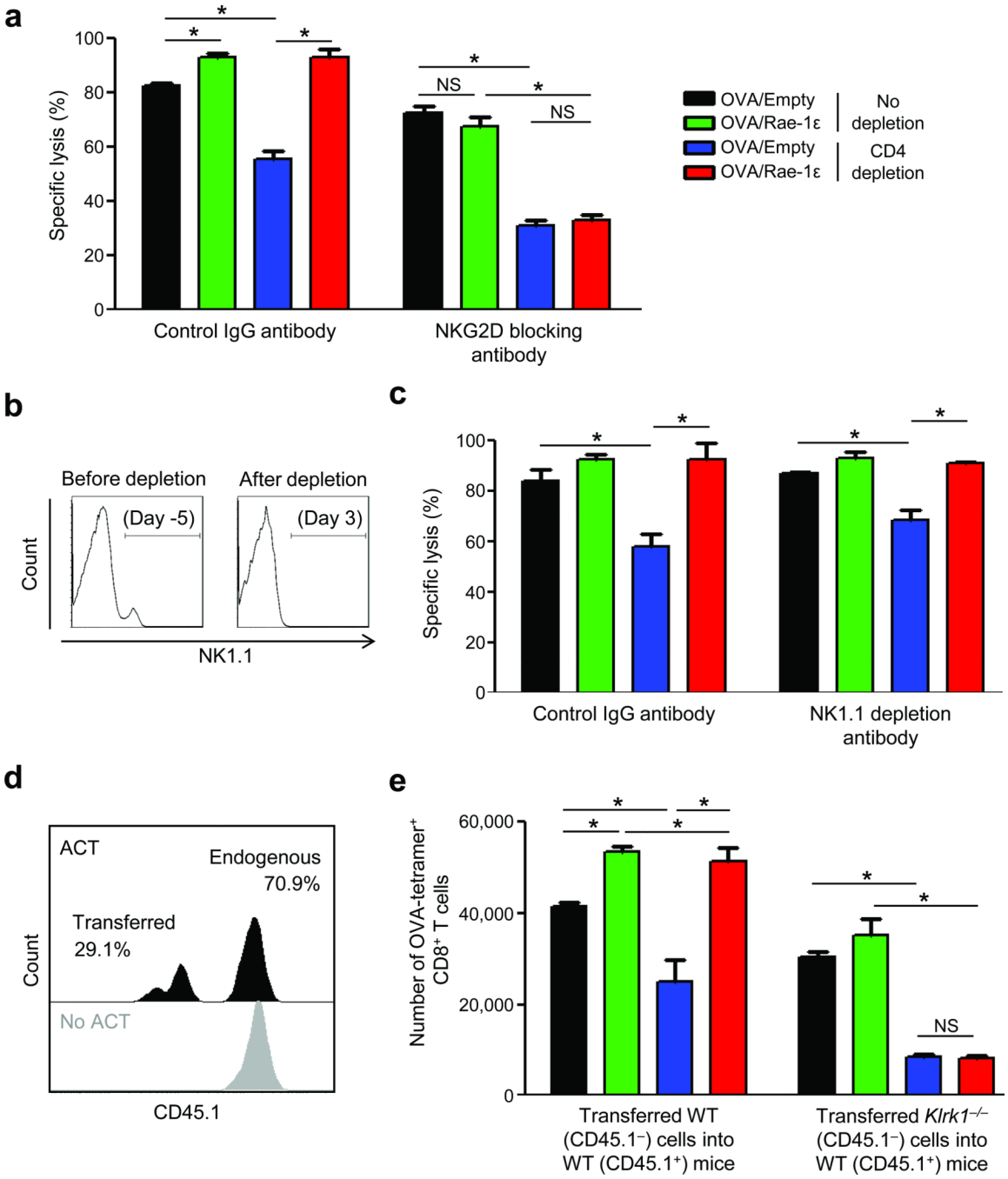

Figure 3.

Rescue of CD4-unhelped $CD8^+$ T cell memory recall responses is dependent on NKG2D expression on $CD8^+$ T cells. (**a**) Memory recall (day 43) specific lysis (% $^+$ SEM) of C57BL/6 mice vaccinated as described in Fig. 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 (% $^+$ SEM) (**c**) of C57BL/6 mice depleted of NK1.1 cells on days −5 and −3, adoptively transferred Thy1.1-marked naïve OT-I ($OVA_{257–264}$-specific) $CD8^+$ T cells ($2*10^6$ per mouse) on day −1, and vaccinated on day 0 ± 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; WT = wild type C57BL/6. (**e**) Mean number of OVA-tetramer$^+$ CD8$^+$ T cells (+SEM) from (**d**) per spleen on day 43. Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results. *$P < 0.05$; NS: $P > 0.05$.

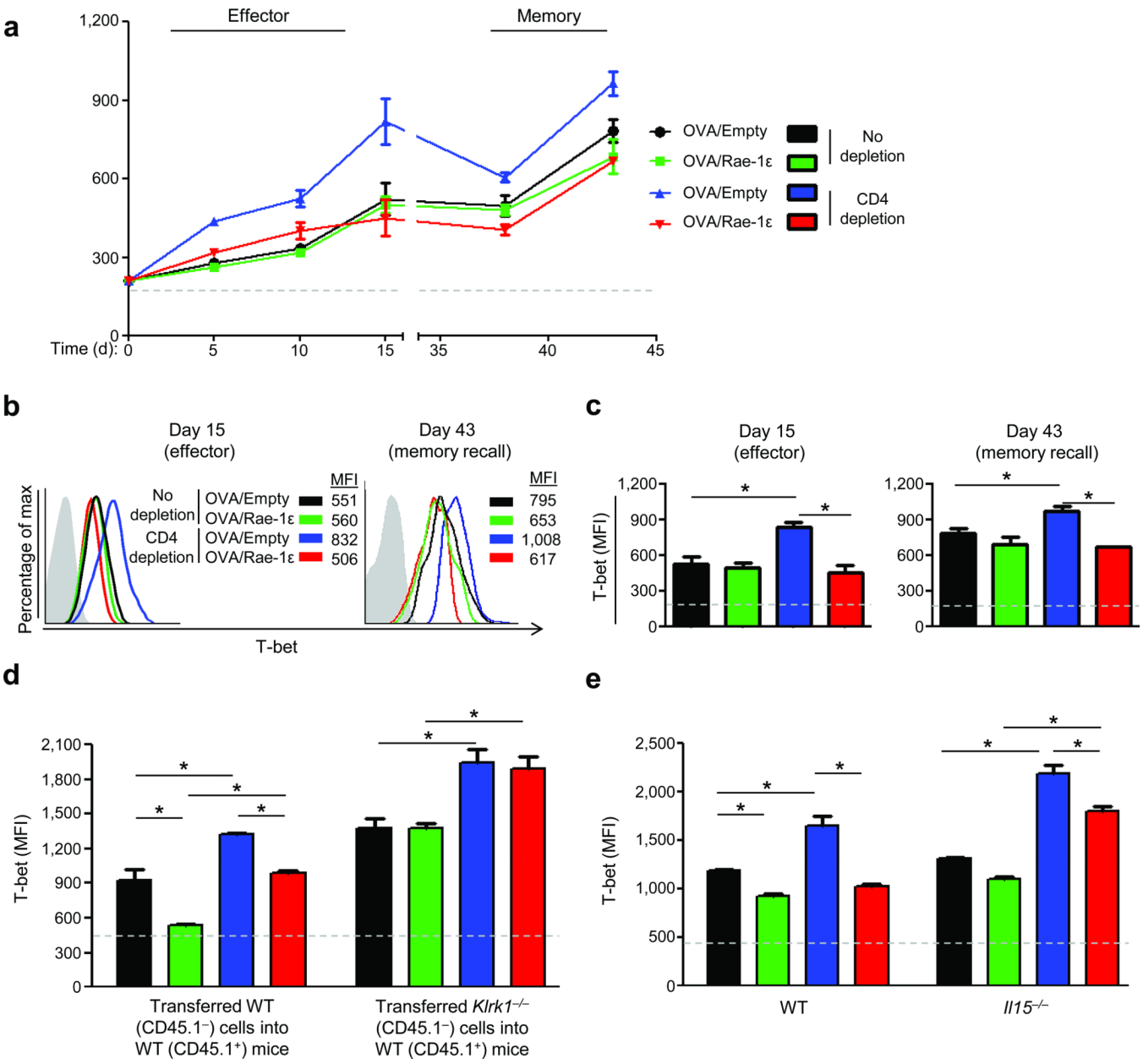

Figure 4.

NKG2D engagement reverses the elevated T-bet expression associated with CD4-unhelped CD8$^+$ T cells. (**a**) T-bet expression MFI (±SEM) in OVA-tetramer$^+$ CD8$^+$ T cells from C57BL/6 mice vaccinated as described in Fig. 1a. (**b**) T-bet staining on days 15 and 43. MFI = mean fluorescent intensity; gray curve = isotype. (**c**) T-bet expression from (**a**) on days 15 and 43. (**d**) Memory recall (day 43) T-bet expression on OVA-specific CD8$^+$ T cells transferred from CD45.1$^-$ NKG2D-deficient (*Klrk1*$^{-/-}$) or wild type C57BL/6 (WT) 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 Fig. 1a. Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results. Dashed lines represent MFI of background flow cytometric staining for T-bet. *$P < 0.05$; NS: $P > 0.05$.

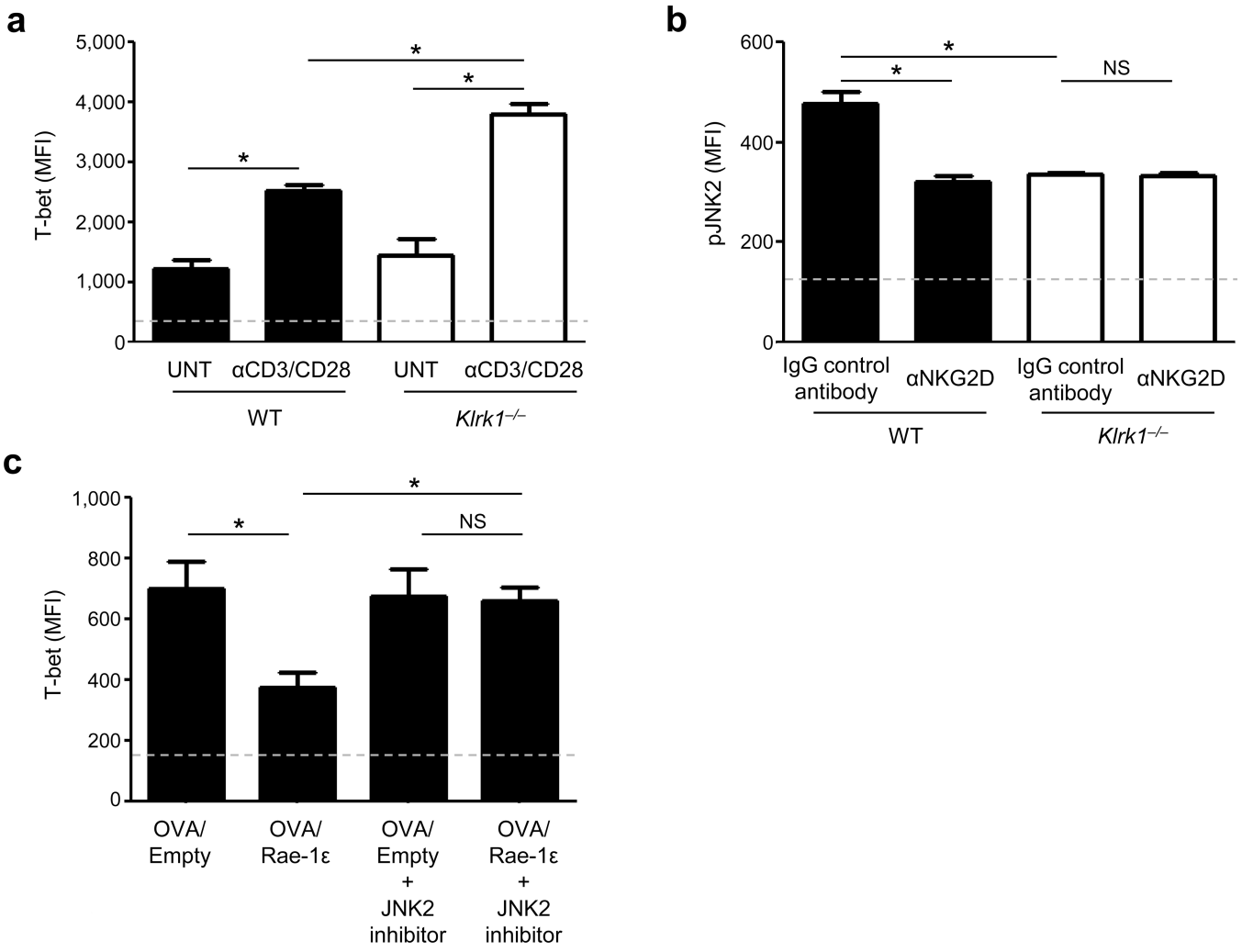

Figure 5.

Suppression of T-bet by NKG2D/Rae-1ε engagement is mediated through JNK2. (**a**) $CD8^+$ T cell T-bet MFI (+SEM) from NKG2D-deficient (*Klrk1*$^{-/-}$) and wild type C57BL/6 (WT) spleens stimulated *in vitro* with anti-CD3/CD28 antibodies (1 μg ml^{-1} each) for 72 h or left untreated. (**b**) pJNK2 MFI (+SEM) from $CD8^+$ T cells stimulated as in (**a**) ± NKG2D blocking antibody (αNKG2D). (**c**) T-bet MFI (+SEM) 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 a JNK2 inhibitor (JNK inhibitor IX; 25 nM,). Data are representative of 3–5 mice analyzed individually per group per experiment from at least three experiments conducted with similar results. Dashed lines represent MFI of background flow cytometric staining for the respective markers. *$P < 0.05$; NS: $P > 0.05$.

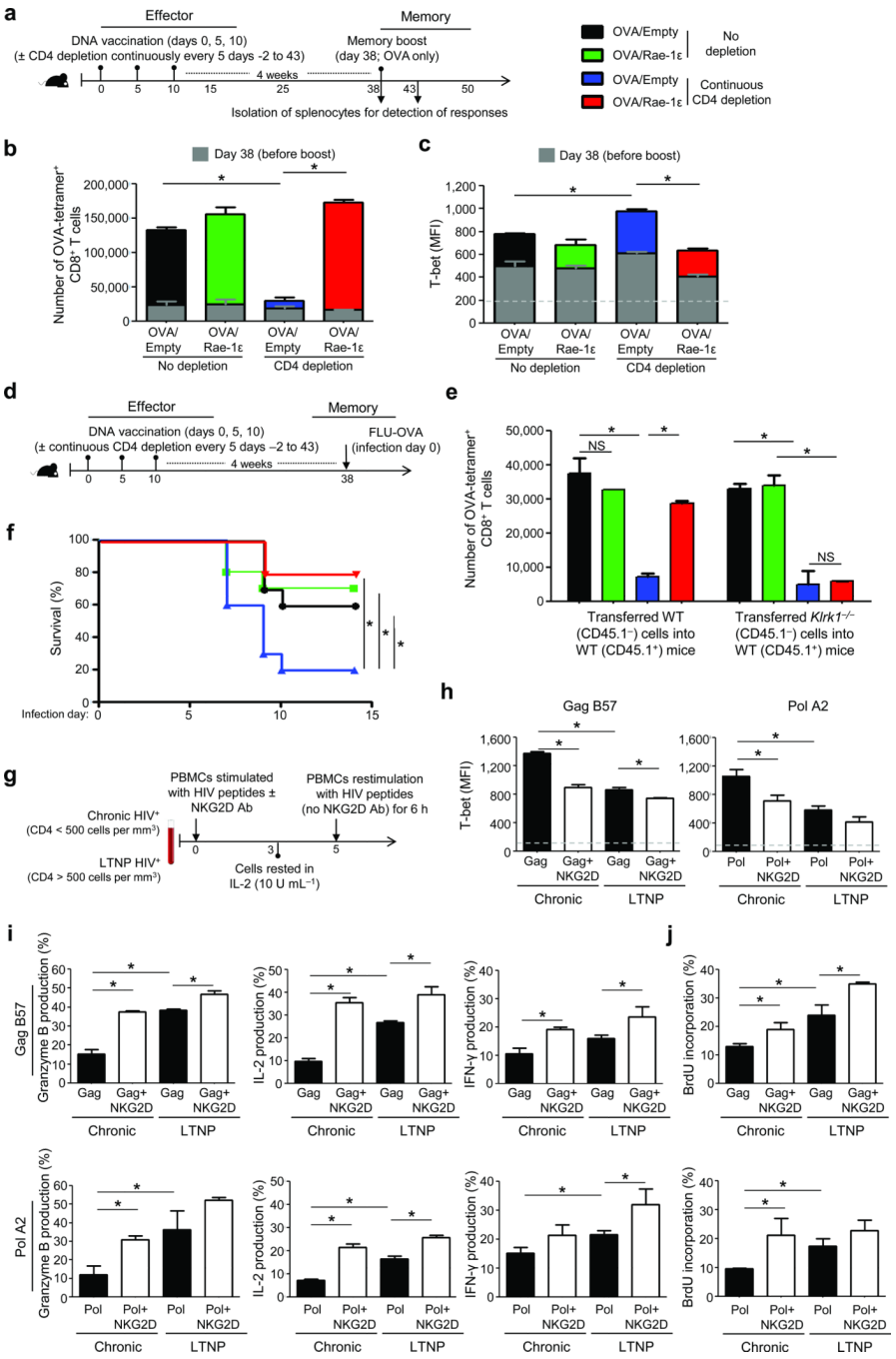

Figure 6.

NKG2D co-stimulation confers protection in CD4-dependent infectious disease models. (**a**) Experimental design for C57BL/6 mice vaccinated as described in Fig. 1a and continuously CD4 depleted. (**b,c**) Mean number (**c**) and T-bet MFI (+SEM) (**d**) of OVA-tetramer$^+$ CD8$^+$ T cells (+SEM) per spleen 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 NKG2D-deficient (*Klrk1*$^{-/-}$) CD8$^+$ T cells were adoptively transferred into WT hosts and treated as described in (**d**). (**f**) Survival curve for experiment described in (**d**) and repeated twice with similar results. (**g**) Experimental design for

stimulation of HIV-positive chronic (Chronic) and long-term non-progressor (LTNP) donor cells with pooled HIV peptides. (**h–j**) HIV-tetramer^{+} CD8^{+} T cell MFI (+SEM) of T-bet (**h**), mean % producing granzyme B, IL-2, and IFN-γ (+SEM) (**i**), and mean % incorporating BrdU (+SEM) (**j**), from at least three donors per group. Dashed lines represent MFI of background flow cytometric staining for the respective markers. *$P < 0.05$; NS: $P > 0.05$.