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Research Article

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The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice

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In order to prime T cells, DCs integrate signals emanating directly from pathogens and from their noxious action on the host. DNGR-1 (CLEC9A) is a DC-restricted receptor that detects dead cells. Therefore, we investigated the possibility that DNGR-1 affects immunity to cytopathic viruses. DNGR-1 was essential for cross-presentation of dying vaccinia virus-infected (VACV-infected) cells to CD8⁺ T cells in vitro. Following injection of VACV or VACV-infected cells into mice, DNGR-1 detected the ligand in dying infected cells and mediated cross-priming of anti-VACV CD8⁺ T cells. Loss of DNGR-1 impaired the CD8⁺ cytotoxic response to VACV, especially against those virus strains that are most dependent on cross-presentation. The decrease in total anti-VACV CTL activity was associated with a profound increase in viral load and delayed resolution of the primary lesion. In addition, lack of DNGR-1 markedly diminished protection from infection induced by vaccination with the modified vaccinia Ankara (MVA) strain. DNGR-1 thus contributes to anti-VACV immunity, following both primary infection and vaccination. The non-redundant ability of DNGR-1 to regulate cross-presentation of viral antigens suggests that this form of regulation of antiviral immunity could be exploited for vaccination.

Introduction

Pressure to detect infection has shaped the evolution of the immune system. Several families of pathogen recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) and activate myeloid cells to induce innate and adaptive immunity (1, 2). However, myeloid cells also sense and respond to tissue-derived signals, which potentially cooperate with signals from PRRs to tailor the immune response (3, 4). Cell damage is one source of tissue-derived signals: mediators released from dead cells, such as uric acid, HMGB1, and ATP, can promote inflammation and in some cases lead to antigen-specific adaptive immune responses (5–9). Notably, many pathogens, including viruses, induce cell death and are therefore likely to cause release of these mediators during infection (10). As viruses also possess PAMPs, an appealing hypothesis is that myeloid cells might integrate recognition of cell damage signals and recognition of PAMPs, so that immune responses to infection would be dually regulated by PRRs and damage-sensing systems (11, 12).

DCs are crucial for adaptive immune responses to infection. Of the various DC subsets, mouse CD8 α ⁺ DCs in lymphoid organs and CD103⁺ DCs in non-lymphoid tissues constitute an ontogenetically related family that excels in the uptake of apoptotic or dead cell material and the subsequent extraction and cross-presentation of cell-associated antigens on MHC class I molecules (13–21). These properties suggest that CD8 α ⁺-like DCs might be ideally placed to integrate sensing of cell death and PAMP sensing for regulation of CD8⁺ T cell priming. Interestingly, CD8 α ⁺ DCs and their human equivalents express high levels of DNGR-1, also known as CLEC9A (22, 23), an innate immune receptor for dead cells. DNGR-1 detects a preformed intracellular ligand that

is exposed upon loss of membrane integrity, when cells undergo primary or secondary necrosis (24). DNGR-1 is a type II transmembrane C-type lectin receptor (CLR) containing a single extracellular C-type lectin-like domain (CTLD) and a cytoplasmic tail with a hemi-immunoreceptor tyrosine-based activation motif (hemITAM) motif that allows binding to and signaling via spleen tyrosine kinase (Syk) (23). Notably, DNGR-1-deficient mice are partially deficient in CTL responses against antigens borne by dead cells (24). DNGR-1 might thus be a receptor that allows CD8 α ⁺-like DCs to respond to tissue damage signals generated during infection. However, DNGR-1-deficient mice have not yet been tested for impaired immunity to cytopathic pathogens.

Vaccinia virus (VACV) is a complex dsDNA virus of the Poxviridae family that induces necroptosis of host cells via the RIP1-RIP3 complex (25). Necroptosis leads to a rapid loss of membrane integrity (10) and could therefore lead to concomitant exposure of DNGR-1 ligands and viral PAMPs. VACV triggers a CD4⁺ T cell response that stimulates the generation of antibodies and a CTL response that destroys virus-infected cells. CTLs can be primed by direct infection of the APC or, alternatively, by APC-mediated cross-presentation of antigens derived from infected cells (26, 27). Importantly, the relative contribution of each of these pathways to the global CD8⁺ T cell response depends on the particular VACV strain and the infection route (28, 29). For example, the Western Reserve (WR) VACV strain strongly relies on direct presentation rather than cross-presentation for the generation of a CTL response (26, 27). In contrast, a deletion mutant WR (Δ B13R) lacking the apoptosis inhibitor B13R (Spi2) induces greater levels of early cell death when infecting an APC and may therefore shunt more antigen into the cross-presentation pathway (30). Similarly, CTL responses to the attenuated modified vaccinia Ankara (MVA) strain are known to depend strongly on cross-presentation (28). With regard to the infection route, skin scarification (s.s.), the pre-

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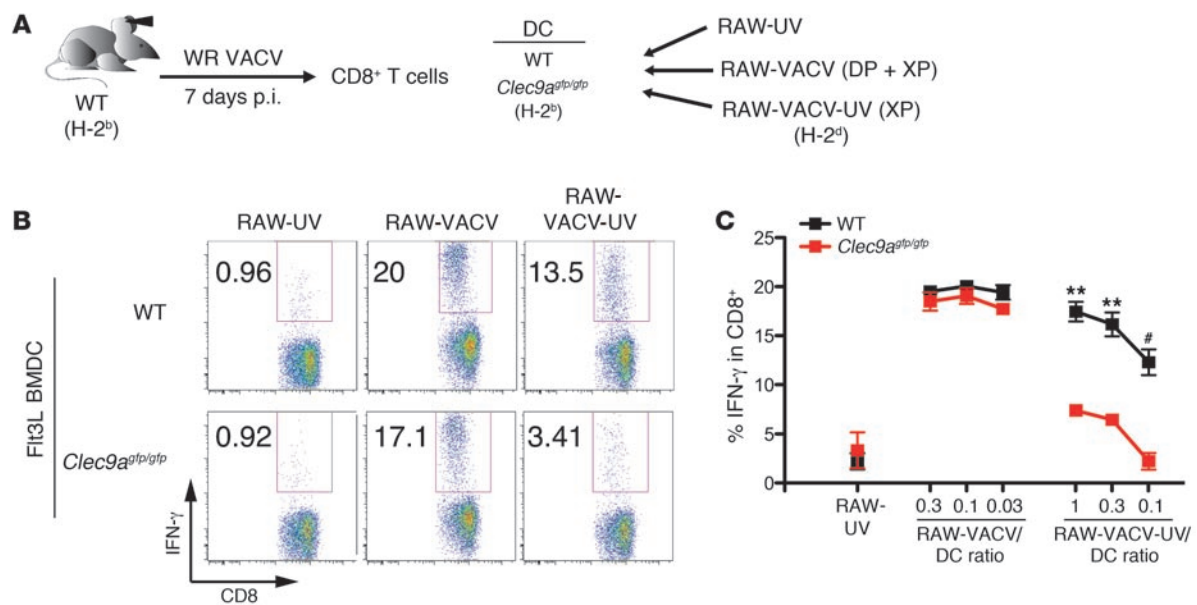


Figure 1 Lack of DNGR-1 blocks cross-presentation of vaccinia antigens in infected cells. (A–C) Production of IFN- γ by VACV-specific CD8⁺ effector T cells in response to cross-presented vaccinia antigens is severely impaired by the absence of DNGR-1 in Flt3L BMDCs. (A) For antigen presentation, RAW cells were either UV irradiated without infection (RAW-UV), infected with VACV without UV irradiation (RAW-VACV) to allow both direct presentation (DP) and cross-presentation (XP), or infected and subsequently irradiated to inactivate the virus (RAW-VACV-UV) to allow only cross-presentation. After 16 hours, RAW cells were exposed to Flt3L BMDCs from WT or *Clec9a*^{gfp/gfp} mice. As a readout of the restimulation ability of DCs, IFN- γ production was measured in CD8⁺ T cells from lymphoid organs of WT mice i.d. injected with WR VACV. (B) Representative set of dot plots. (C) Production of IFN- γ (mean \pm SEM) from a representative experiment ($n = 3$ biological replicates) of 3 performed. ** $P < 0.01$, # $P < 0.001$, unpaired 2-tailed Student's t test.

ferred method for vaccination with poxviruses (31), results in a response that is highly dependent on cross-presentation (29), and the same is therefore likely to be true of the related intradermal (i.d.) challenge model of VACV infection. Finally, even for a given virus and infection route, some CTL epitopes may be more or less dependent on cross-presentation. For example, MHC class I-restricted epitopes derived from the early VACV protein B8R can rely more on direct presentation, in contrast to ones derived from the late protein A3L (32).

For all the above reasons, we decided to analyze the contribution of DNGR-1 to immunity against VACV following primary i.d. infection with different VACV strains or after s.s. vaccination with MVA. We report that DNGR-1-deficient DCs are fully activated following interaction with VACV-infected cells but are blocked in their capacity to cross-present VACV antigens. This results in a weakened CD8⁺ effector T cell response in DNGR-1-deficient mice, which delays resolution of primary lesions, results in higher virus titers, and impairs CD8⁺ T cell memory responses induced by vaccination. Tissue damage sensing by DNGR-1 is thus a key component of anti-VACV CTL responses, suggesting that DCs integrate tissue damage signals with those originating from pathogens to induce an effective adaptive immune response to infection.

Results

DNGR-1-deficient DCs show normal activation but reduced cross-presenting ability upon exposure to VACV-infected cells. DNGR-1 deficiency impairs the ability of DCs to stimulate the proliferation and effector differentiation of CD8⁺ T cells in response to antigens borne

by uninfected dead cells (24). To determine whether the same is true in an infectious situation, we used a model of VACV infection. We compared Flt3L bone marrow-derived DCs (Flt3L BMDCs; a source of CD8 α ⁺-like DCs) from WT mice (H-2^b) and DNGR-1-deficient (*Clec9a*^{gfp/gfp}) mice (24). As an antigen source, we used VACV-infected RAW macrophages (H-2^d) (RAW-VACV), which can transmit virus to DCs, resulting in infection of the latter and direct antigen presentation by H-2^b MHC class I molecules but can also serve as a source of cell-associated antigen for cross-presentation. Alternatively, we treated infected RAW cells with UV (RAW-VACV-UV) to inactivate the virus, blocking direct infection of DCs and leaving available only the cross-presentation route. As a control for antigen specificity, we used uninfected RAW cells treated with UV (RAW-UV). Flt3L BMDCs from WT or *Clec9a*^{gfp/gfp} mice were exposed to VACV-infected or control cells and used to stimulate CD8⁺ T cells purified from immune WT mice (previously infected with VACV) (Figure 1A). When RAW-VACV cells were used as the virus source, production of IFN- γ by vaccinia-specific effector CD8⁺ T cells was unaffected by loss of DNGR-1 (Figure 1, B and C). In contrast, when RAW-VACV-UV cells were used, antigen-specific CD8⁺ T cell stimulation was markedly reduced in the absence of DNGR-1 (Figure 1, B and C). The same result was observed with total DCs obtained from mouse LNs or with purified CD8 α ⁺ DCs from mouse spleen, indicating that it was not restricted to the use of Flt3L BMDCs (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI60660DS1).

The use of preactivated CD8⁺ T cells as a readout in these assays made it unlikely that the observed effect was due to an

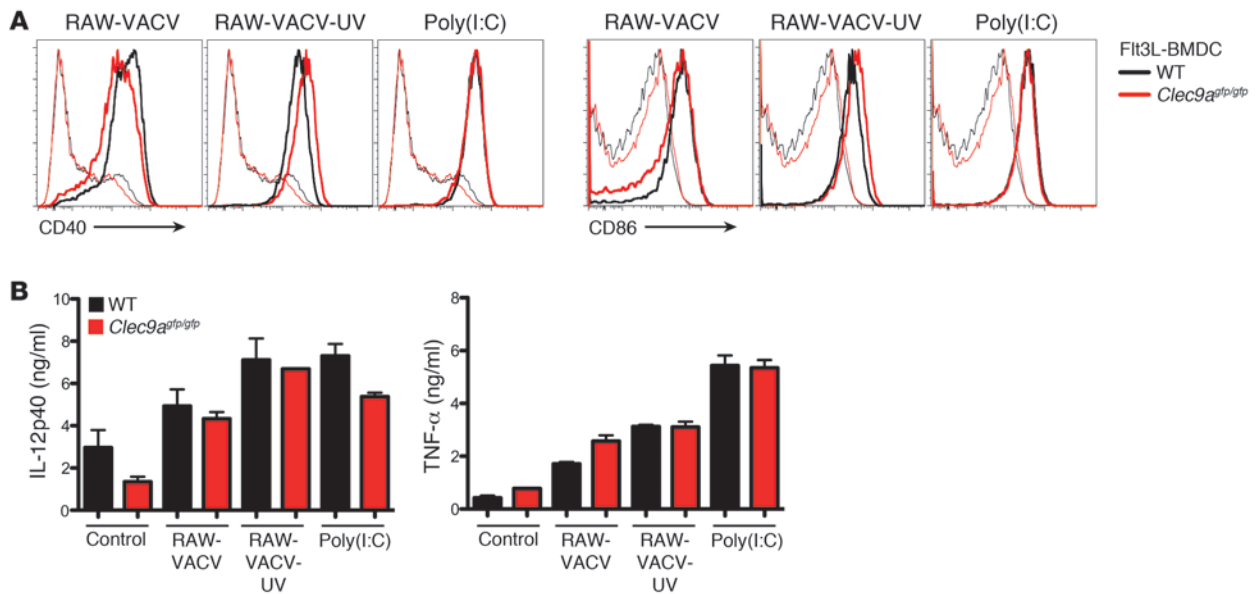


Figure 2 DNGR-1 does not affect DC activation in response to vaccinia-infected cells. Flt3L BMDCs from WT or *Clec9a^{gfp/gfp}* mice were untreated (control) or exposed for 20 hours to RAW-VACV or RAW-VACV-UV. (A) Induction of co-stimulatory molecules in DCs upon exposure to VACV-infected cells is not affected by the absence of DNGR-1. A representative set of histogram overlays is shown for CD40 (left) and CD86 (right). (B) Cytokine production induced in DCs by vaccinia-infected cells is not affected by the absence of DNGR-1. Data shown are from a representative experiment of 3 performed and are presented as mean \pm SEM ($n = 3$ biological replicates). Differences were not statistically significant.

impairment of DC activation. Nevertheless, because DNGR-1 has been reported to act as a myeloid activating receptor (22), we tested whether its absence affected activation of DCs in response to RAW-VACV or RAW-VACV-UV cells. Independent of UV irradiation, VACV-infected cells induced strong DC activation, measured at the level of either costimulatory molecule upregulation (CD40, CD86) or cytokine production (TNF- α , IL-12p40). Notably, this activation was not affected by DNGR-1 deficiency (Figure 2, A and B, and Supplemental Figure 2). Moreover, lack of DNGR-1 had no effect on the expression of MHC class I (Supplemental Figure 2). These results suggest that DNGR-1, rather than determining DC activation by dead cells, regulates a non-redundant step in cross-presentation by CD8 α^+ -like DCs of pathogen antigens associated with dead cells.

Dying VACV-infected cells expose DNGR-1 ligands. We next analyzed whether DNGR-1 ligands were exposed during VACV infection. Time-course analysis showed that infection of EL-4 cells with the WR VACV strain in vitro exposed the ligand in cells expressing VACV proteins as early as 24 hours after infection (Figure 3A and data not shown). All ligand-expressing cells were positive for annexin V and permeable to Hoechst 33258 staining, with a proportion of mid-positive Hoechst 33258 cells that corresponded to the transition of late apoptotic to early necrotic cells and bright positive Hoechst 33258 cells that were fully necrotic (Figure 3A).

To extend these findings in vivo, we injected WR VACV i.d. into mouse ears and analyzed the presence of DNGR-1 ligand in ear cell suspensions 4 days later. A fraction of the cells from infected mice stained positive for both DNGR-1 ligand and vaccinia proteins, revealing that some infected cells expose the ligand in vivo (Figure 3B). All DNGR-1 ligand-positive cells were stained with annexin V and permeable to Hoechst 33258 – in this case the predominant fraction was cells that stained positive for Hoechst

at intermediate levels (early necrotic), as the late necrotic cells had probably been removed in vivo.

VACV-infected dying cells bearing exposed DNGR-1 ligands could potentially be encountered by dermal DCs. Our analysis of DNGR-1 expression in ear cell suspensions detected a subset of CD11c⁺CD24^{hi} dermal DCs that expressed DNGR-1 (Figure 3C). Cells in this subset were related to the CD8 α^+ DCs in lymphoid tissues and, like these cells, had an elevated capacity to cross-present exogenous antigens to CD8⁺ T cells (19, 21, 33).

DNGR-1 is crucial for cross-presentation of VACV antigens in vivo. To determine the contribution of DNGR-1 to cross-presentation of viral antigens in vivo, we used an established model in which mice are injected i.p. with RAW-VACV-UV cells, so that the generated CD8⁺ T cell response depends only on cross-presentation of VACV antigens (34). After 6 days, we restimulated effector peritoneal CD8⁺ T cells with MHC class I-restricted epitopes derived from the early VACV protein B8R or the late protein A3L (35) (Figure 4A). Notably, we found that DNGR-1 deficiency greatly reduced the effector CD8⁺ T cell response in this model (Figure 4, B and C).

To confirm that the ex vivo restimulated effector CD8⁺ T cell responses reflected systemic CTL activity in vivo, we performed an in vivo cytotoxicity assay in which the targets were syngeneic splenocytes pulsed with the specific VACV peptides and labeled with different doses of CellTraceViolet or CFSE to allow discrimination by flow cytometry. Mice were injected with the targets on day 5 after primary i.p. challenge with the RAW-VACV-UV cells (Figure 4A). Analysis of killing activity in vivo 16 hours after transfer of the targets confirmed the impairment of CTL activity toward both VACV peptides (Figure 4, D and E). Thus, DNGR-1 deficiency impairs CTL activity in response to cross-presented VACV antigens.

High DNGR-1 expression is tightly restricted to CD8 α^+ -like DCs. To unequivocally determine whether the deficiency in the

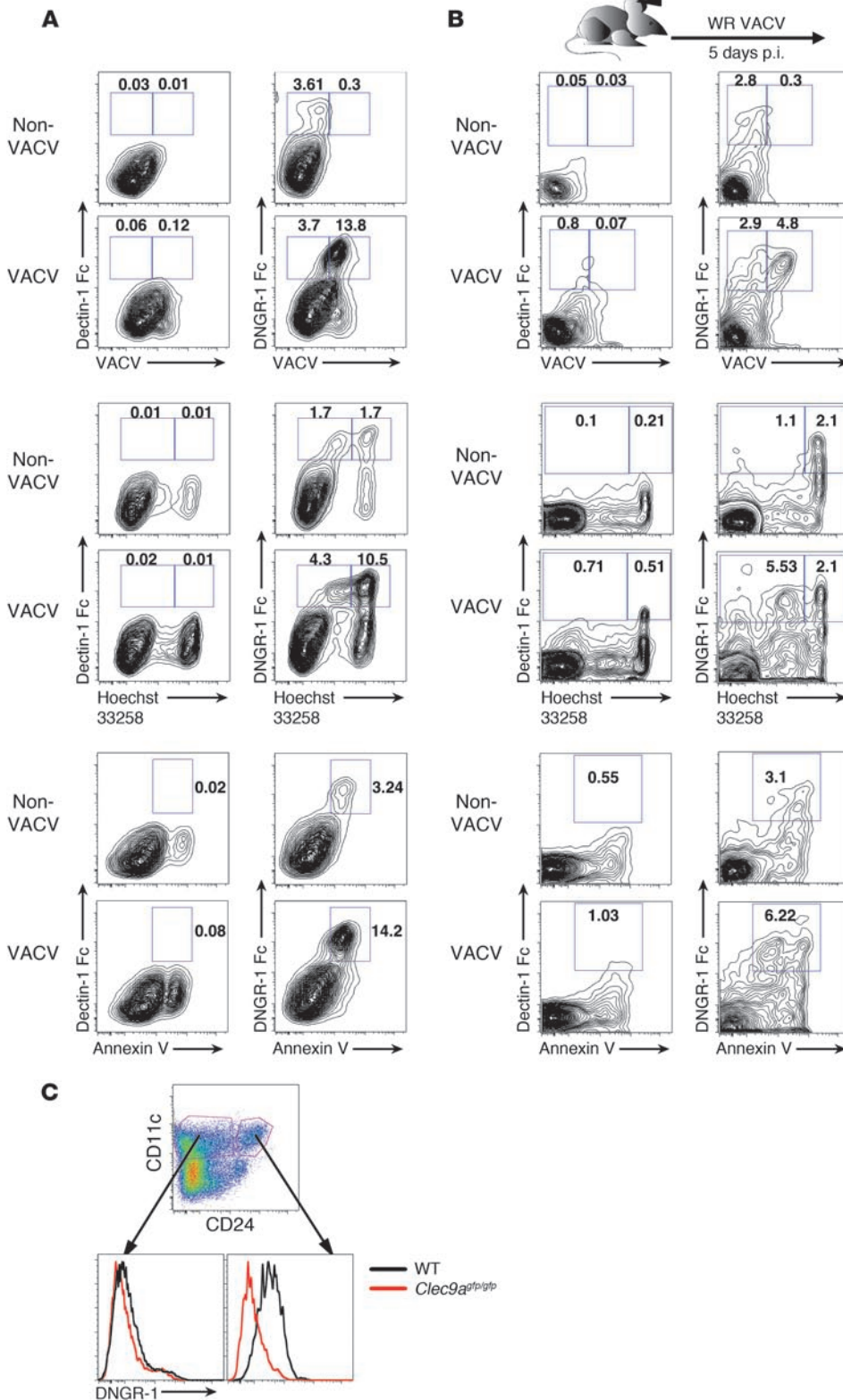


Figure 3

Cells infected with vaccinia virus expose DNGR-1 ligand in vitro and in vivo. **(A)** Infection with VACV in vitro exposes DNGR-1 ligand. EL-4 cells were infected with WR VACV for 48 hours and stained with anti-vaccinia antibody and with control (Dectin-1-Fc) or DNGR-1-Fc constructs to detect the ligand and counterstained with annexin V and Hoechst 33258 (5 μg/ml). **(B)** Infection of C57BL/6 mice with VACV exposes DNGR-1 ligand simultaneously with vaccinia antigens in infected ear cells. WR VACV was injected i.d. in the ear, and, after 5 days, dermal cell suspensions were prepared as indicated in Methods. Staining and data are displayed as in **A**. **(C)** DNGR-1 is expressed in DCs locally in the ear. Cell suspensions from the ears of WT or *Clec9a^{gfp/gfp}* mice were semi-purified for CD11c⁺ cells by positive selection and stained for CD11c, CD24, and DNGR-1. A subset of CD11c⁺CD24^{hi} DCs expresses DNGR-1. The dot plots in all panels are a replicate set of 3 from a representative experiment of 3 performed.

CTL response was due to lack of DNGR-1 expression on CD8α⁺ DCs, we carried out adoptive transfer experiments using CD8α⁺ spleen DCs from WT or DNGR-1-deficient (*Clec9a^{gfp/gfp}*) mice. After incubation of splenocytes for 2 hours with RAW-VACV-UV

cells, the CD8α⁺ spleen DCs were purified and transferred adoptively into *Clec9a^{gfp/gfp}* recipient mice (Figure 5A). After 7 days, ex vivo restimulation of splenocytes with B8R or A3L VACV peptides demonstrated that WT, but not DNGR-1-deficient, CD8α⁺ DCs

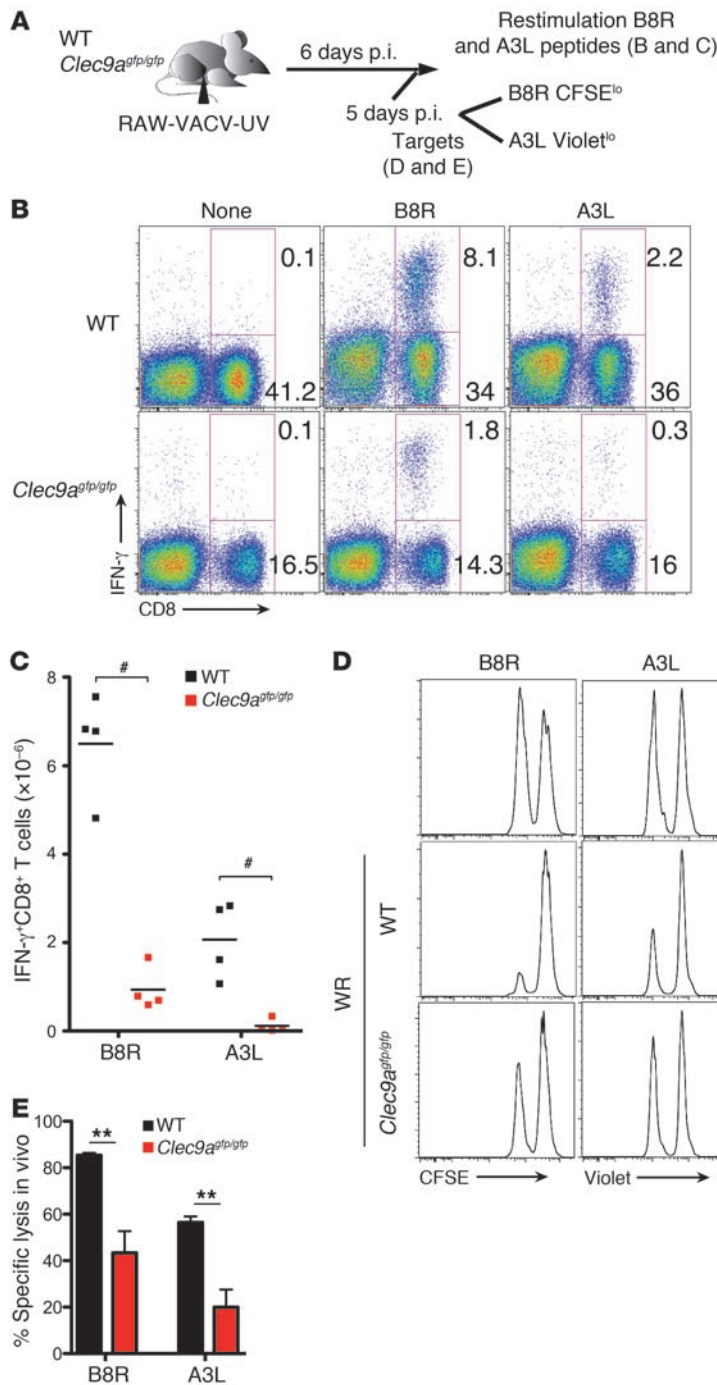


Figure 4

Lack of DNDR-1 blocks cross-presentation of VACV antigens in vivo. (A) RAW cells were infected with WR VACV and UV treated to inactivate the virus (RAW-VACV-UV) and then transferred i.p. (10^7 cells per mouse) to WT and *Clec9a^{gfp/gfp}* mice. (B and C) Absence of DNDR-1 impairs the CD8⁺ effector T cell response to cross-presented VACV peptides. After 6 days, peritoneal cells were extracted and restimulated with B8R or A3L VACV peptides. (B) Representative dot plot set. (C) Absolute numbers of IFN- γ -producing CD8⁺ T cells found in peritoneal washes, shown as individual data from a representative experiment ($n = 4$ biological replicates) of 3 performed. (D and E) Lack of DNDR-1 reduces CTL killing activity in vivo against cross-presented vaccinia peptides. On day 5 after transfer, splenocytes from syngeneic mice were loaded with the early peptide B8R or the late peptide A3L (CFSE^{lo} or CellTraceViolet^{lo}, respectively) or no peptide (CFSE^{hi} or CellTraceViolet^{hi}) and transferred i.p. The peritoneal lavage was analyzed 16 hours later for specific killing of targets. (D) Representative histogram set. Control histograms from noninfected mice show the proportion of transferred targets. (E) Percentage specific killing in a representative experiment of 3 performed. Data are presented as mean \pm SEM ($n = 4$ biological replicates). ** $P < 0.01$, # $P < 0.001$, unpaired 2-tailed Student's t test.

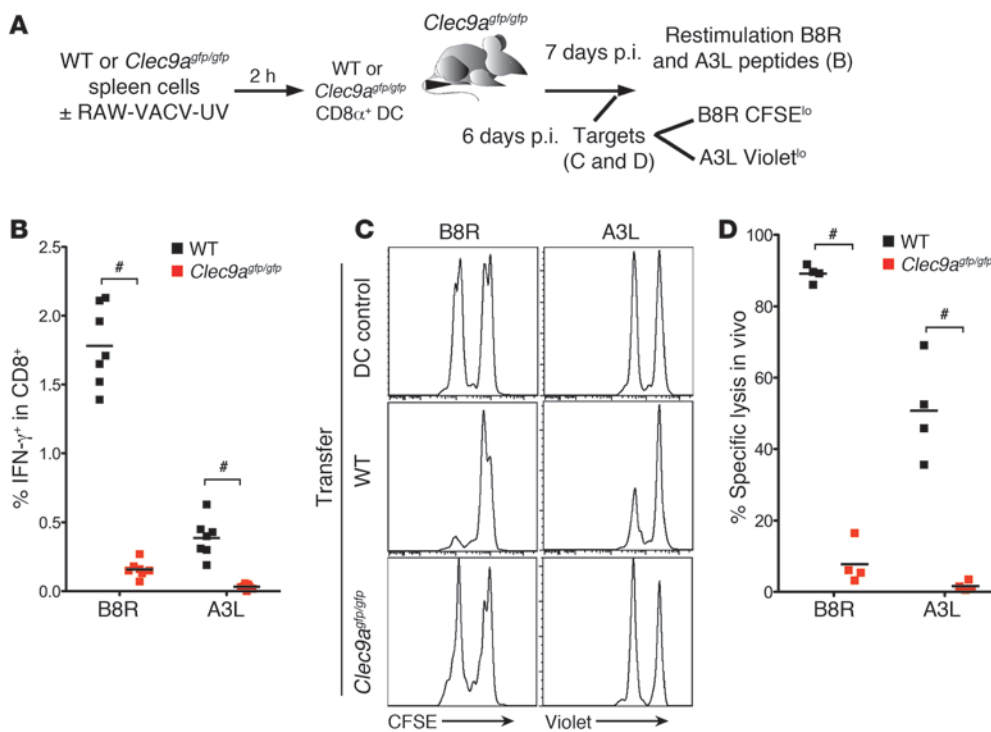
ears ex vivo with WT DCs and VACV antigens to determine the frequency of effector T cells in skin (Figure 6A). As a source of antigen for restimulations, we used RAW-VACV or RAW-VACV-UV cells (see above) and assessed IFN- γ production separately in CD8⁺ and CD4⁺ T cells. Interestingly, when RAW-VACV cells were used, CD4⁺ and CD8⁺ T cell responses were equivalent in WT and DNDR-1-deficient mice (Figure 6, B and C). In contrast, restimulation with RAW-VACV-UV cells, which permits MHC class I presentation only via the cross-presentation route, revealed a partial blockade of the VACV-specific CD8⁺ T cell response in the absence of DNDR-1, while the CD4⁺ T cell response was unaffected (Figure 6, B and C). Thus, only the CD8⁺ T cell effector response to antigens cross-presented from WR VACV was partially impaired in DNDR-1-deficient mice.

We next tested the Δ B13R VACV strain, which lacks an inhibitor of apoptosis (30). Dermal cells were obtained from WT or DNDR-1-deficient mouse ears infected with Δ B13R VACV for 7 days and restimulated as above (Figure 6D). In contrast to infection with the parental WR strain, the virus-specific CD8⁺ T cell response to Δ B13R VACV was significantly impaired in DNDR-1-deficient mice, whether assessed by restimulation with RAW-VACV or RAW-VACV-UV (Figure 6E). As with the WR strain, the CD4⁺ T cell response against Δ B13R VACV was normal in the absence of DNDR-1 (Figure 6F). Moreover, the antibody response against WR or Δ B13R VACV was also identical in WT and DNDR-1-deficient mice (Supplemental Figure 3, A and B). In sum, DNDR-1 deficiency selectively decreased the CD8⁺ T cell effector response to VACV antigens that rely on cross-presentation.

Loss of DNDR-1 reduces the CD8⁺ T cell effector response and killing activity in vivo against VACV epitopes. To further test the effect of DNDR-1 deficiency on the effector CD8⁺ T cell response, we infected WT or DNDR-1-deficient mice i.d. with the WR or Δ B13R VACV strain

efficiently transferred the ability to cross-present VACV antigens to CD8⁺ T cells (Figure 5B). These results were confirmed by measurement of systemic CTL activity 6 days after transfer of CD8 α^+ DCs (Figure 5, C and D). These data show that lack of DNDR-1 expression in CD8 α^+ DCs was responsible for the observed defective cross-presentation of VACV-derived antigens in vivo.

DNDR-1 deficiency impairs the CD8⁺ T cell effector response to cross-presented VACV antigens. To test whether DNDR-1 contributes to the CTL response in the context of live virus infection, we injected the WR VACV strain i.d. into the ears of WT and DNDR-1-deficient mice. After 7 days, we restimulated cells from the infected

**Figure 5**

DNGR-1 expression on transferred $CD8\alpha^+$ spleen DCs is crucial for their ability to cross-present VACV antigens. (A) Splenocytes from WT or *Clec9a^{Δfp/gfp}* mice were extracted and cultured for 2 hours with RAW cells infected with WR VACV and irradiated with UV to inactivate the virus (RAW-VACV-UV). $CD8\alpha^+$ DCs were purified as indicated in Methods and transferred into the hind paws of *Clec9a^{Δfp/gfp}* mice (2×10^6 $CD8\alpha^+$ DCs per mouse). (B) WT but not DNGR-1-deficient $CD8\alpha^+$ DCs cross-present VACV-derived antigens. After 7 days, splenocytes were extracted and restimulated with B8R or A3L VACV peptides. Production of IFN- γ is shown as individual data from a representative experiment ($n = 7$ biological replicates) of 3 independent experiments performed. (C and D) Cross-presentation of VACV antigens by WT but not DNGR-1-deficient $CD8\alpha^+$ DCs results in CTL killing activity against vaccinia epitopes in vivo. On day 6 after transfer, splenocytes from syngeneic mice were loaded with the early peptide B8R, the late peptide A3L, or no peptide and labeled as in Figure 4 and transferred i.v. Splenocytes were analyzed 16 hours later for specific killing of targets. (C) Representative histogram set. DCs transferred without preincubation with RAW-VACV-UV show the proportion of transferred targets. (D) Percentage specific killing shown as individual data from a representative experiment ($n = 4$ biological replicates) of 3 performed. # $P < 0.001$, unpaired 2-tailed Student's t test.

and measured IFN- γ production by skin T cells 7 days later after ex vivo restimulation with B8R and A3L peptides (Figure 7A). In mice infected with WR, the $CD8^+$ T cell response to the early epitope B8R was not significantly affected, whereas the response to the late epitope A3L was reduced by DNGR-1 deficiency (Figure 7B). This correlates with the fact that VACV antigens from early promoters can be directly presented, whereas direct presentation does not occur for antigens driven by late promoters (32). In contrast, responses to both epitopes were impaired by DNGR-1 deficiency in mice infected with the $\Delta B13R$ VACV strain, in line with the greater dependence of this strain on cross-presentation (Figure 7C).

To determine systemic CTL activity in vivo, we injected syngeneic splenocyte targets loaded with B8R, A3L, or no peptide and labeled as before into mice on day 6 after primary i.d. challenge with the WR or $\Delta B13R$ VACV strains (Figure 7D). The results of analysis of killing activity in vivo after 16 hours mirrored those of ex vivo restimulation, with DNGR-1 deficiency having little effect on CTL activity against the early peptide but decreasing killing activity

against targets loaded with the late peptide in animals challenged with WR VACV (Figure 7, E and F). The DNGR-1-dependent killing activity against both peptides was greater in the case of infection with the $\Delta B13R$ VACV strain, consistent with the ex vivo restimulation assay. Thus, DNGR-1 deficiency impairs overall CTL activity against VACV.

Loss of DNGR-1 delays the resolution of primary infection by vaccinia strains. We tested whether the deficiency in the $CD8^+$ T cell response to VACV antigens in the absence of DNGR-1 affected the control of the lesion caused by the virus. WR and $\Delta B13R$ VACV strains were injected i.d. into the ears of WT or DNGR-1 KO mice, and the size of the lesion was monitored over 22 days. Primary expansion of the virus is controlled by innate immunity, and the virus load peaks at days 4–5, when adaptive immunity begins to take over (36). The adaptive immune response initiates lesion resolution by day 8–10, and healing is complete by 3–4 weeks. Notably, DNGR-1 deficiency did not affect WR VACV lesions during the innate phase (up to day 8) but significantly affected subsequent resolution (Figure 8A). Similar effects were seen with the $\Delta B13R$ VACV strain, which induced larger and more persistent lesions (Figure 8B),

as reported previously (37). DNGR-1 thus plays a non-redundant role in the resolution of infection by two distinct VACV strains.

To monitor the effect of the adaptive response on viral replication, we measured viral titers in the ears on day 7, at the onset of the primary adaptive response, and on day 16, well into the resolution phase. Lack of DNGR-1 did not affect viral titers on day 7 (Figure 8, C and D), showing that DNGR-1 does not impact the early innate response to the virus. However, the viral load in the ears of mice infected with either the WR or the $\Delta B13R$ VACV strain increased more than 10-fold in the absence of DNGR-1 by day 16 (Figure 8, C and D). Lack of DNGR-1 thus strongly impairs the control of virus load and lesion resolution, likely through its effects on the $CD8^+$ T cell effector response.

DNGR-1 deficiency impairs the secondary response following vaccination with MVA. To extrapolate these findings to a vaccination setting, we first investigated whether the response to the MVA vaccine was also dependent on DNGR-1. The response of $CD8^+$ T cells, but not $CD4^+$ T cells, was decreased in MVA-immunized DNGR-1-defi-

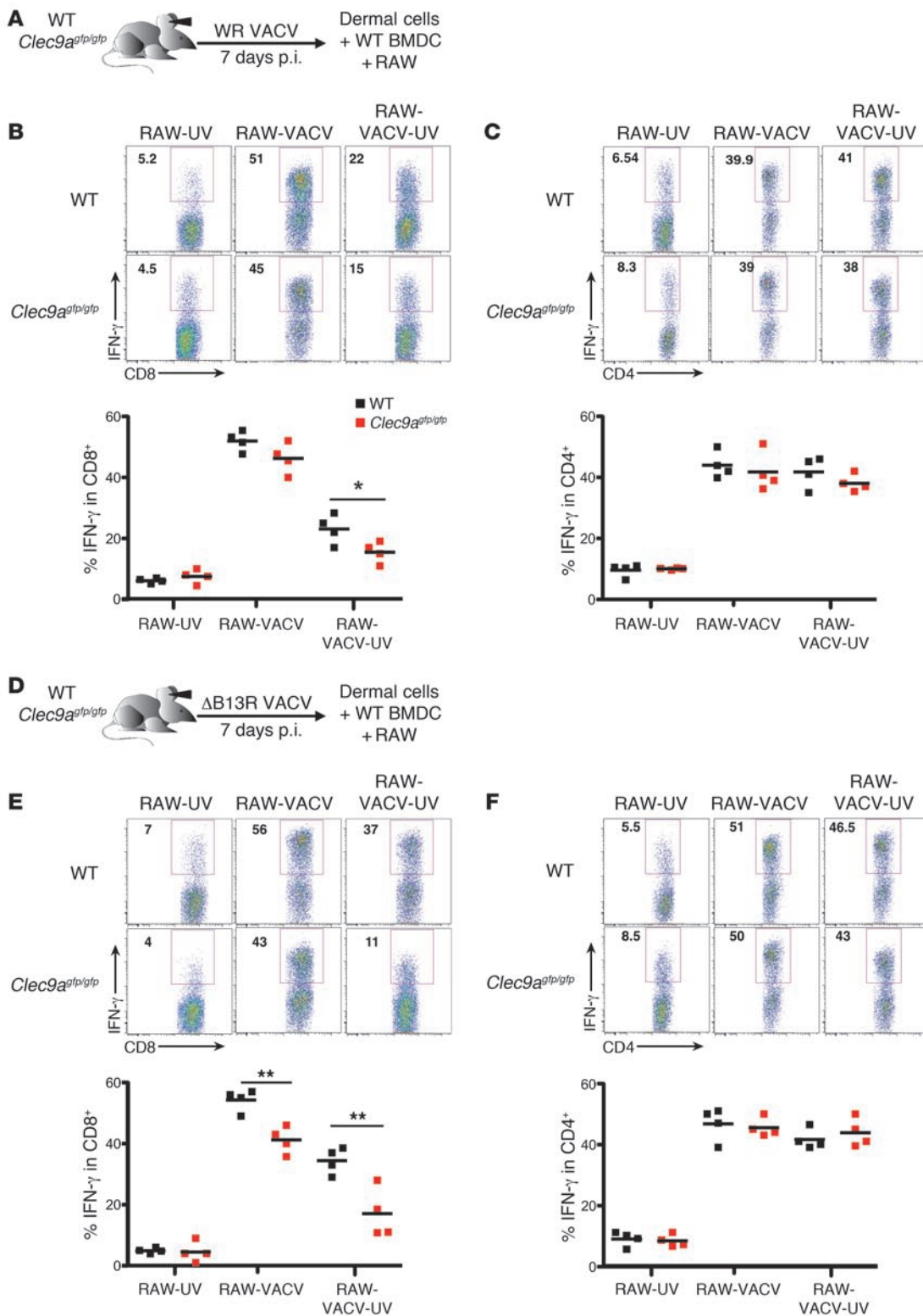


Figure 6

DNGR-1 deficiency impairs the CD8⁺ T cell effector response to vaccinia virus infection. WT or DNGR-1–deficient mice were infected i.d. in the ear with WR (A) or Δ B13R (D) VACV strains. On day 7 p.i., ear dermal cell suspensions containing effector T cells were restimulated for IFN- γ production in the presence of WT Flt3L BMDCs pretreated with RAW-VACV or RAW-VACV-UV cells. The effector response of CD8⁺ T cells (B and E) but not CD4⁺ T cells (C and F) to cross-presented antigens from WR (B and C) or Δ B13R (E and F) VACV is reduced in the absence of DNGR-1. Upper panels show representative dot plot sets. Lower panels show individual data for production of IFN- γ in CD8⁺ T cells and CD4⁺ T cells from a representative experiment ($n = 4$ biological replicates) of 3 performed. * $P < 0.05$, ** $P < 0.01$, unpaired 2-tailed Student's t test.

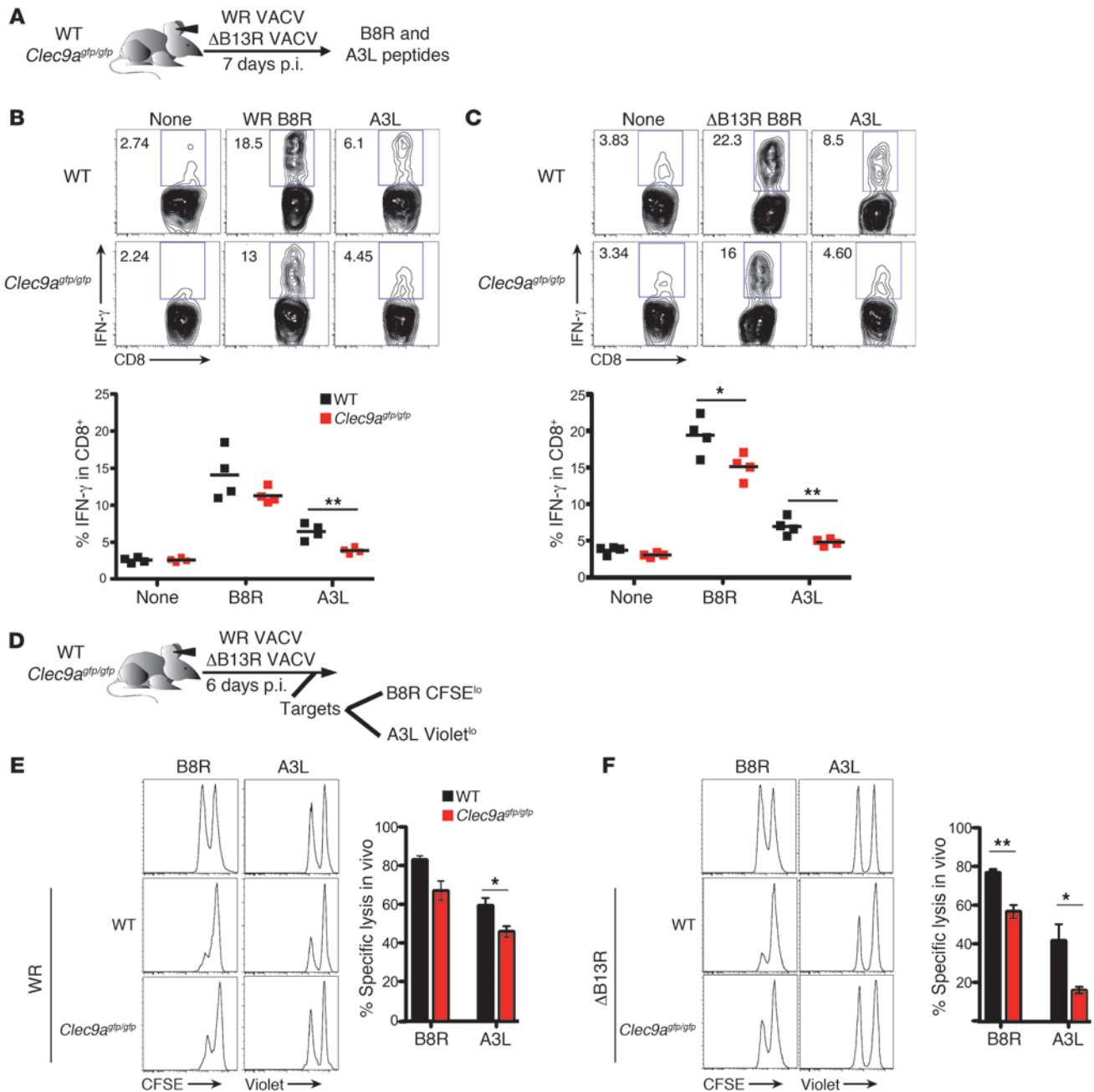


Figure 7

The CD8⁺ T cell effector response to vaccinia epitopes is decreased in the absence of DNGR-1. (A–C) Absence of DNGR-1 impairs the CD8⁺ T cell effector response to early and late vaccinia peptides. (A) WT or DNGR-1-deficient mice were infected i.d. in the ear with WR (B) or Δ B13R (C) VACV strains, and 7 days later, ear dermal cell suspensions were obtained and restimulated with B8R and A3L VACV peptides. Upper panels show representative dot plot sets. Lower panels show individual data for production of IFN- γ from a representative experiment ($n = 4$ biological replicates) of 3 performed. (D–F) CTL killing activity in vivo against vaccinia epitopes is reduced in DNGR-1-deficient mice. (D) WT or DNGR-1-deficient mice were infected with WR (E) or Δ B13R (F) VACV i.d. in the ear, and killing assays were conducted on day 6 as in Figure 4. Upper panels show representative histogram sets. Control histograms from non-infected mice to show the proportion of transferred targets. Lower panels show the percentage specific killing in a representative experiment of 3 performed is shown. Data are presented as mean \pm SEM ($n = 4$ biological replicates). * $P < 0.05$, ** $P < 0.01$, unpaired 2-tailed Student's t test.

cient mice, whether measured by restimulation with RAW-VACV, RAW-VACV-UV, or the B8R- and A3L-specific peptides (Figure 9, A and B, and data not shown). As expected, the use of *Clec9a*^{gfp/gfp} BMDcs further impaired restimulation by RAW-VACV or RAW-

VACV-UV cells (Supplemental Figure 4). Furthermore, DNGR-1 deficiency also impaired in vivo killing activity against targets pulsed with B8R or A3L peptides (Figure 9C). In fact, DNGR-1 appeared to contribute more significantly to immunity against

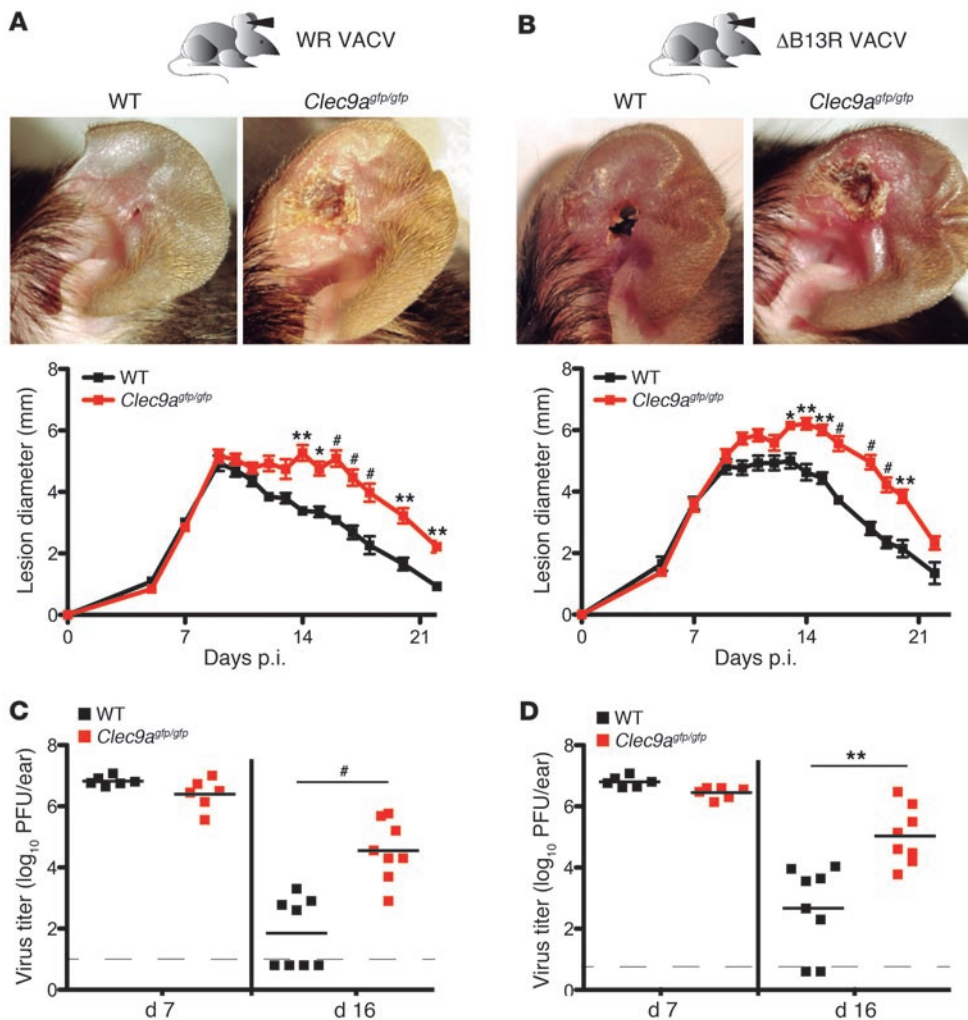


Figure 8

Loss of DNDR-1 delays the resolution of primary infection by vaccinia strains. WT or DNDR-1-deficient mice were infected i.d. in the ear with WR (A and C) or ΔB13R (B and D) VACV strains. (A and B) DNDR-1-deficient mice show increased lesion size and a delay in the resolution of primary infection. Upper panels show representative pictures at day 15. Lower panels show the temporal development of lesion size (mean ± SEM; n = 12) from a representative experiment of 3 performed. (C and D) Primary infection in the absence of DNDR-1 results in higher viral load during the resolution phase. Viral load in the ears on days 7 and 16 is shown as individual data and the mean from a representative experiment of 3 performed. *P < 0.05, **P < 0.01, #P < 0.001, unpaired 2-tailed Student's t test.

MVA than to the other replicative VACV strains (compare Figure 9 with Figures 6 and 7).

To address the role of DNDR-1 in the generation of memory responses against VACV, a relevant issue for vaccination with poxviruses, we vaccinated WT or DNDR-1-deficient mice with the MVA VACV strain by s.s. of the base of the tail (31) (Figure 10A). After 21 days, mice were injected i.d. in the ears with the WR VACV strain, and lesion size, effector T cell response, and viral titers were measured. During a secondary response, virus load can be controlled from the outset by the adaptive immune response. Consistent with this notion, viral titers at day 5 after ear injection were significantly lower in vaccinated mice compared with non-vaccinated mice used as controls (Figure 10B). Remarkably, DNDR-1 deficiency resulted in a 10-fold higher viral titer in vaccinated mice, suggesting a defective secondary CTL response. To confirm this, we obtained CD8⁺ T cells from draining LNs on day 5 after challenge and tested them for their response to B8R and A3L VACV epitopes in the ex vivo restimulation assay. Cells from vaccinated and subsequently challenged DNDR-1-KO mice displayed a markedly weaker secondary response to the vaccinia peptides (Figure 10C). The reduced CD8⁺ T cell effector response and higher viral titers also manifested themselves in increased lesion size in DNDR-1-deficient mice (Figure 10D). These results show that DNDR-1 is crucial for the generation

of an effective memory CTL response following vaccination with an attenuated VACV vaccine strain.

Syk deficiency in CD11c⁺ cells impairs the CD8⁺ T cell effector response to VACV infection. DNDR-1 signals via the kinase Syk (22, 24), and we therefore examined the contribution of Syk to the capacity of DCs to cross-present antigens from VACV-infected cells (Figure 11A). Comparison of the cross-presentation capacity of Flt3L BMDCs from WT and CD11c-Cre × *Syk^{fl/fl}* mice (38) revealed that Syk deficiency in DCs impaired the CD8⁺ T cell response to VACV antigens that were cross-presented but not to those that were directly presented (Figure 11, B and C).

To test the influence of Syk on the CD8⁺ T cell effector response in vivo, we injected WT or CD11c-Cre × *Syk^{fl/fl}* mice i.d. with WR VACV, and dermal cells obtained after 7 days were restimulated with B8R and A3L VACV peptides (Figure 11, D-F). Syk deficiency in CD11c⁺ cells in vivo impaired the CD8⁺ T cell effector response against VACV peptides, suggesting that activation of Syk kinase is a non-redundant step in the signaling pathway downstream of DNDR-1 that regulates cross-presentation of antigens from VACV-infected cells.

Inhibitors of lysosomal activity restore the cross-presentation ability of DNDR-1-deficient DCs. Since DNDR-1 is located in non-lysosomal compartments (24), we hypothesized that DNDR-1 might retain the cargo from VACV-infected cells in a pre-lysosomal compart-

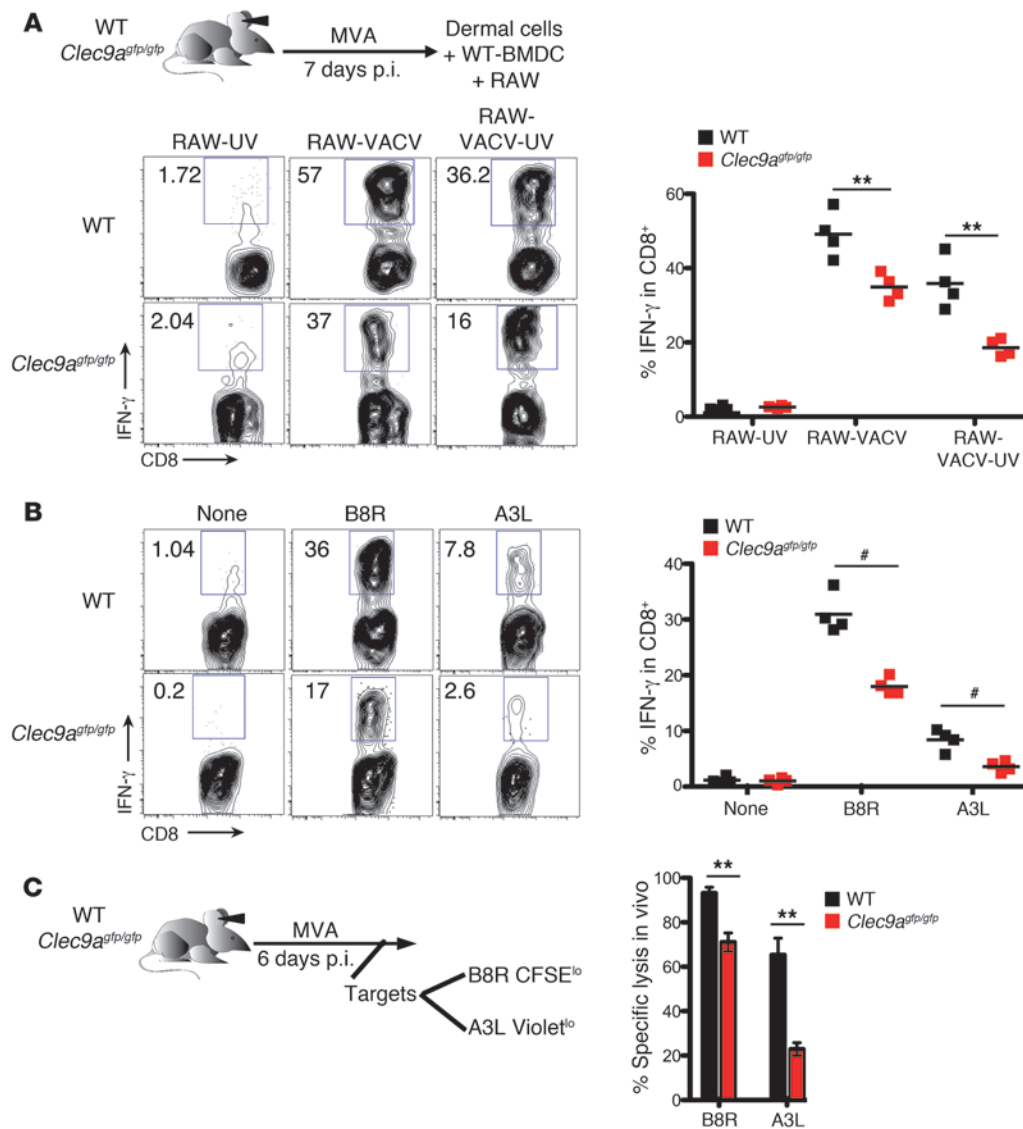


Figure 9

DNGR-1 deficiency impairs the effector response induced by MVA virus strain. WT or DNGR-1-deficient mice were infected with the MVA VACV strain. The CD8⁺ T cell effector response to MVA VACV is impaired in the absence of DNGR-1. On day 7 p.i., ear dermal cell suspensions containing effector T cells were restimulated for IFN- γ production in the presence of (A) WT Flt3L BMDCs pretreated with RAW-VACV or RAW-VACV-UV cells or (B) B8R or A3L peptides. Left panels show representative dot plot sets. Right panels show individual data for production of IFN- γ in CD8⁺ T cells from a representative experiment ($n = 4$ biological replicates) of 3 performed. (C) CTL killing activity in vivo against vaccinia epitopes is reduced in DNGR-1-deficient mice. WT or DNGR-1-deficient mice were infected i.d. in the ear with MVA VACV, and killing assays were conducted on day 6 as in Figure 4. Data show percentage specific killing as mean \pm SEM for a representative experiment ($n = 4$ biological replicates) of 3 performed. ** $P < 0.01$, # $P < 0.001$, unpaired 2-tailed Student's t test.

ment with limited proteolytic activity, thereby favoring cross-presentation of antigens (39–41) (Figure 12A). To test this hypothesis, we examined the effects of inhibitors of lysosomal protease activity (leupeptin plus pepstatin) and of lysosome acidification (bafilomycin A1) (41). These drugs did not significantly affect the cross-presentation ability of WT Flt3L BMDCs assayed as in Figure 1 (Figure 12B). However, both treatments restored the cross-presentation capacity of DNGR-1-deficient DCs to levels similar to those of WT DCs (Figure 12B). Direct presentation was not affected by either treatment (Figure 12C). We also tested a proteasome inhibitor (MG-132), which blocked cross-presentation by WT DCs and

did not affect the already inhibited cross-presentation by DNGR-1 deficient DCs, indicating that the cross-presentation pathway in this setting was proteasome dependent (data not shown). These results suggest that DNGR-1 might promote the retention of viral cargo in a prelysosomal compartment with low proteolytic activity, thereby permitting antigen cross-presentation.

Discussion

In addition to directly sensing pathogens, the innate immune system also responds to alterations to homeostasis. Necrosis is a drastic example of non-homeostatic cell death. Necrotic cells have

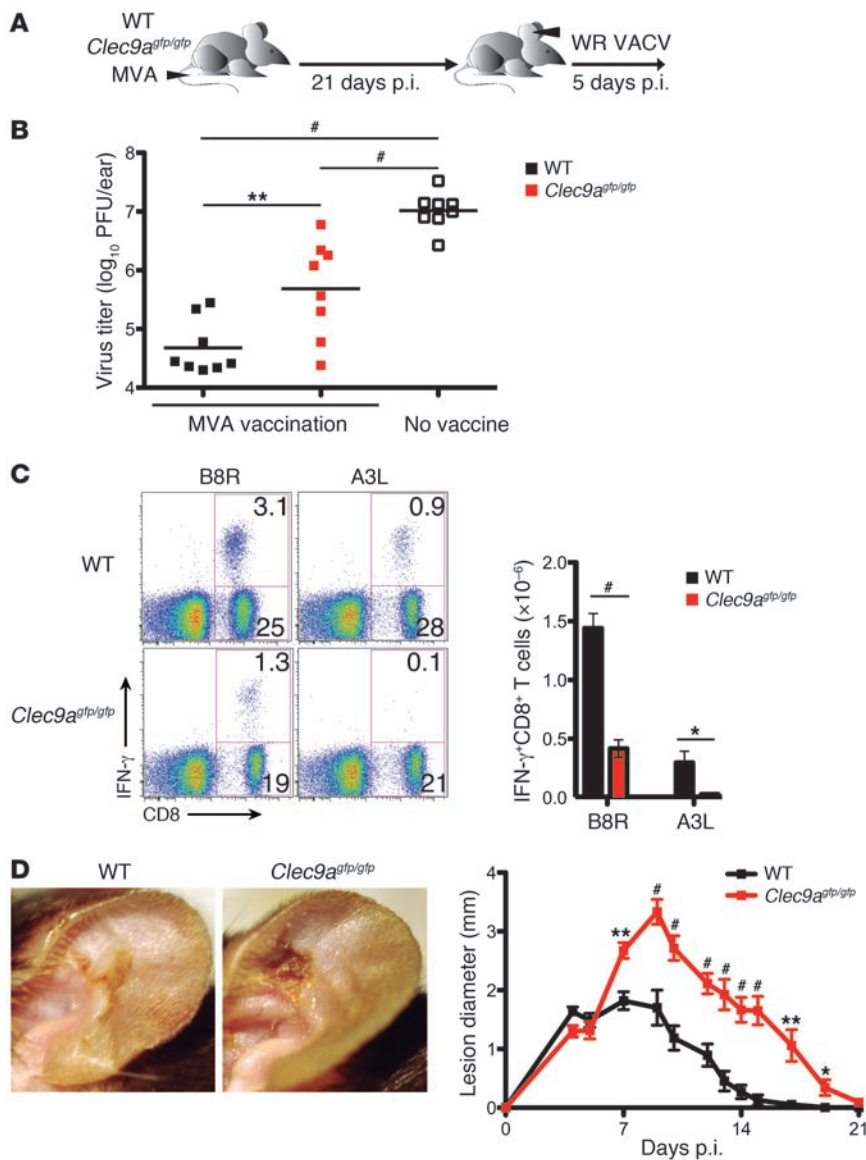


Figure 10

DNDR-1 deficiency blocks the secondary effector response after vaccination with the MVA virus strain. (A) WT or DNDR-1-deficient mice were infected with MVA VACV and challenged 21 days later with the WR VACV strain. (B) DNDR-1 deficiency results in a higher viral load during secondary challenge following vaccination. Viral load in the ears on day 5 after secondary challenge is shown as individual data and the mean in a representative experiment of 3 performed. (C) The CD8⁺ T cell secondary response to early and late vaccinia peptides is defective in DNDR-1-deficient mice. Cell suspensions obtained from draining LNs on day 7 were tested against B8R and A3L VACV peptides, and the response was analyzed and depicted as in Figure 5. Results are shown from a representative experiment of 3. (D) DNDR-1 deficiency increases the lesion size upon secondary VACV challenge following MVA vaccination. Left panels show representative images at day 10 after secondary challenge. Right panel shows the temporal development of lesion size (mean ± SEM; n = 14) from a representative experiment of 3 performed. *P < 0.05, **P < 0.01, #P < 0.001, unpaired 2-tailed Student's t test.

proinflammatory properties, and antigens contained within them often provoke an adaptive immune response (9, 42, 43). Thus, necrosis is potentially an important component of immunity. However, most responses to cell death have been studied in a non-infectious context (44), and the contribution of damage-related signals to the immune response to infection is poorly understood (11, 12). We previously found that CD8α⁺ DCs can sense dead cells through DNDR-1 and that this receptor favors cross-priming of CTLs against antigens derived from non-infectious dead cells (24). Here, we tested whether DNDR-1 also modulates CTL responses to virus infection. Remarkably, we found that DNDR-1 deficiency results in defective cross-presentation of VACV antigens to CD8⁺ T cells and reduces the overall CTL response against the virus, delaying the resolution of virus-induced lesions. Lack of DNDR-1 also impaired the secondary response following vaccination with MVA. These results show that DNDR-1 is an essential component of the CTL response to cytopathic viruses, suggesting that detection of cell and tissue damage has been co-opted during evolution as an important component of adaptive immunity to infection.

The similarity of DNDR-1 to the related myeloid activating CLR Dectin-1 suggested that DNDR-1's involvement in cross-priming might stem from its ability to act as a "danger" receptor (45), translating dead cell recognition into DC activation. Consistent with that idea, zymosan stimulation of a chimeric receptor comprising the extracellular domain of Dectin-1 and the intracellular tail and transmembrane domain of DNDR-1 induced TNF-α production in a macrophage cell line (22). However, our results are not consistent with a crucial DC-activating function for DNDR-1 (see also ref. 46), as we show that DNDR-1-deficient DCs are activated normally in response to VACV-infected cells (46). Nonetheless, stimulation of VACV-specific pre-primed CD8⁺ T cells by DNDR-1-deficient DCs was impaired, especially in conditions where cross-presentation was the only option for generating the appropriate MHC class I peptides. Thus, DNDR-1 specifically impairs cross-presentation of cell-associated antigens during viral infection. The fact that DNDR-1 deficiency is not compensated by signals from PRRs shows that sensing of damage via DNDR-1 and the detection of

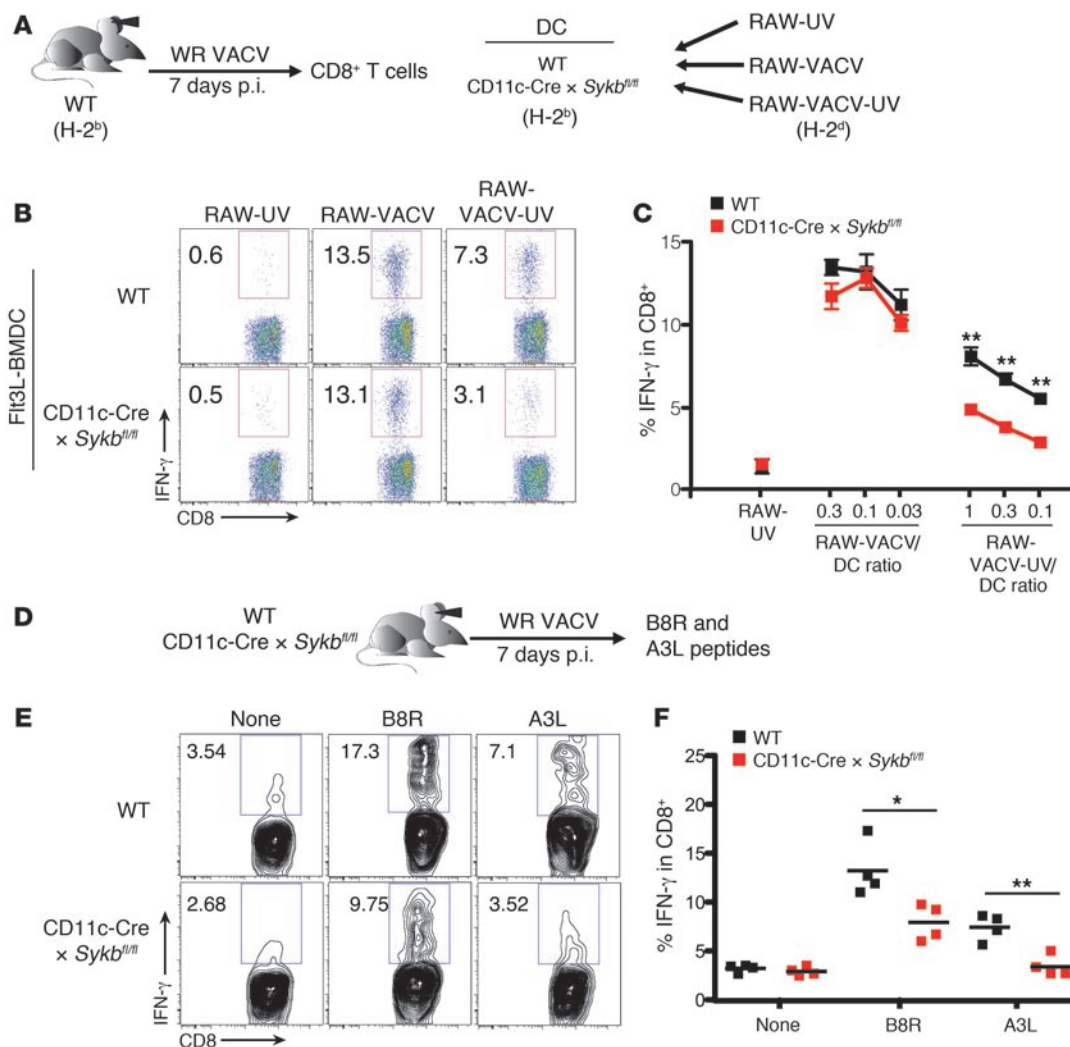


Figure 11

Syk deficiency in CD11c⁺ cells impairs the CD8⁺ T cell effector response to VACV infection. (A–C) CD11c-Cre × Sykb^{fl/fl} DCs show deficient cross-presentation of vaccinia antigens from infected cells. (A) Flt3L BMDCs from WT or CD11c-Cre × Sykb^{fl/fl} mice were exposed to RAW-UV, RAW-VACV, or RAW-VACV-UV as in Figure 1. IFN-γ production was measured in CD8⁺ T effector cells in response to lymphoid cells of WT mice i.d. injected with WR VACV. (B) Representative set of dot plots. (C) Production of IFN-γ (mean ± SEM) from a representative experiment (n = 3 biological replicates) of 3 performed. (D–F) Lack of Syk in CD11c⁺ cells impairs the CD8⁺ T cell effector response to early and late vaccinia peptides. (D) WT or CD11c-Cre × Sykb^{fl/fl} mice were infected i.d. in the ear with WR VACV, and 7 days later, ear dermal cell suspensions were obtained and restimulated with B8R and A3L VACV peptides. (E) Representative dot plot set. (F) Production of IFN-γ is shown as individual data from a representative experiment (n = 4 biological replicates) of 3 performed. *P < 0.05, **P < 0.01, unpaired 2-tailed Student’s t test.

PAMPs act at different levels but in concert to effectively prime CTL responses against cytopathic viruses.

We found that i.d. infection with VACV exposed DNGR-1 ligand in vivo on dermal cells also expressing VACV proteins. Ligand-expressing cells were positive for annexin V and permeable, with different levels of expression of Hoechst 33258, suggesting expression of the ligand in the transition from late apoptosis to necrosis. Most tissue damage caused by viral replication was confined to the injection area, and it is not clear how DNGR-1, expressed at high levels selectively by CD8α⁺ DCs in lymphoid organs (23), could participate in the sensing of local damage. Adoptive transfer experiments demonstrated that when CD8α⁺ DCs expressed DNGR-1, they gained the capacity to cross-pres-

ent antigens from VACV-infected cells in vivo. It was proposed that migratory DCs can carry viral antigens to draining LNs and transfer them to CD8α⁺ DCs for local cross-presentation (47, 48). Consistent with this possibility, we found some VACV proteins and DNGR-1 ligands in cells in the draining LNs (data not shown), although not to the same extent as in the dermis. Alternatively, if expressed by any of the dermal DC subsets, DNGR-1 might detect its ligand locally in the dermis. Indeed, we found expression of DNGR-1 in a CD11c⁺CD24^{hi} subset of dermal cells, corresponding to the dermal CD103⁺ DC subset specialized in cross-presentation (19, 21, 33). Thus, DNGR-1 is specifically expressed by all DC subsets with functional capacity for cross-presentation, in both mouse and human (23).

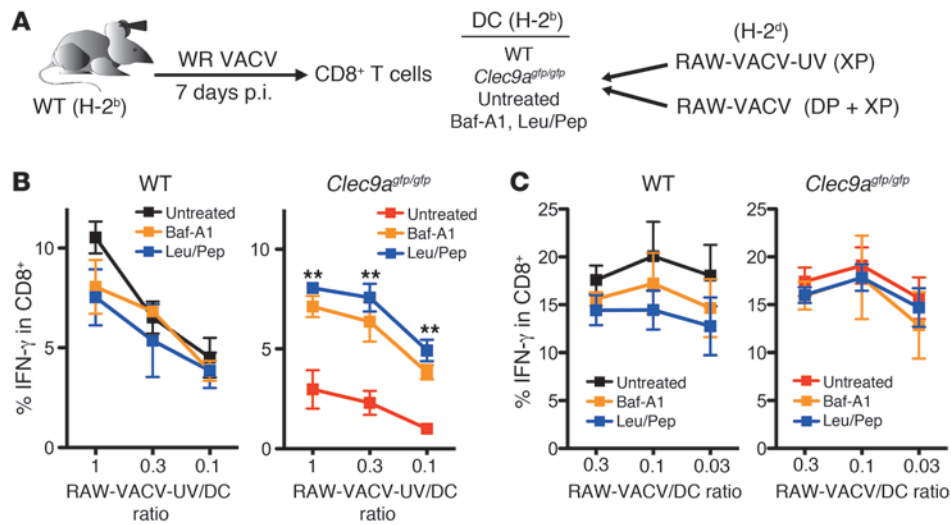


Figure 12

Inhibitors of lysosomal activity restore the cross-presentation ability of DNGR-1-deficient DCs. (A) Flt3L BMDCs from WT or *Clec9a*^{gfp/gfp} mice were left untreated or treated with bafilomycin A1 (Baf-A1) or leupeptin plus pepstatin (Leu/Pep). To analyze direct presentation or cross-presentation, the DCs were then cocultured for 2 hours with RAW-VACV (B) or RAW-VACV-UV (C) as in Figure 1. As a readout of the restimulation ability of the DCs, IFN- γ production in polyclonal CD8⁺ T cells specific to VACV antigens, as in Figure 1. Production of IFN- γ is shown as mean \pm SEM from a representative experiment ($n = 3$ biological replicates) of 3 experiments performed. Both Baf-A1 and Leu/Pep restore cross-presentation ability to *Clec9a*^{gfp/gfp} DCs. $**P < 0.01$, unpaired 2-tailed Student's t test.

Our observations indicate that DNGR-1 controls much of the CD8⁺ T cell response to antigens cross-presented in vivo from cells infected with non-replicative VACV, but only a fraction of the total CD8⁺ T cell response following primary VACV infection. The contribution of DNGR-1 is VACV strain specific, being most noticeable with the MVA strain, followed by the Δ B13R and the WR strains. The dependence of the CD8⁺ T cell effector response on DNGR-1 seems to reflect the relative contribution of direct or cross-presentation to anti-VACV responses (26, 27, 49). This is likely to be affected by the VACV strain used and the infection route. For instance, the MVA strain can infect APCs (28, 50) and subsequently might induce direct priming as well as cross-priming. However, these attenuated viruses lack many caspase inhibitors, reducing the potential lifespan of any directly infected APC. Indeed, MVA-infected DCs undergo apoptosis and inhibition of cellular protein synthesis much earlier than DCs infected with the WR strain (51), and CTL responses to MVA depend strongly on cross-presentation (28). Similarly, B13R (Spi2) inhibits apoptosis (52), and therefore responses to the B13R deletion mutant (Δ B13R), because of the reduced lifespan of the directly infected APC, are likely to rely to a greater extent on cross-presentation. Regarding the infection route, CTL responses to VACV i.p. or i.v. challenge strongly depend on direct presentation, whereas those to s.c. or i.d. infection rely mainly on cross-presentation (29). This might explain why we found a contribution of DNGR-1 to the immunity against VACV triggered by i.d. challenge. Nevertheless, we believe that DNGR-1 is likely to play a general, non-redundant role in immunity to infection by other cytopathic viruses in situations that depend on cross-priming for induction of CD8⁺ T cell responses, such as West Nile virus, CMV, or influenza virus (18, 49, 53).

VACV infection in DCs is abortive, leading to expression of early but not late viral genes (54, 55). The relative dependence on DNGR-1 is higher for the response to the late VACV peptide A3L than to

the early peptide B8R. Although both peptides are cross-presented (34), responses to early peptides reflect a higher contribution of direct presentation, whereas those to late peptides, which cannot be directly presented in an aborted APC (32), would rely to a greater extent on cross-presentation. The relative contribution of CTLs compared with other adaptive immune mechanisms to the control of primary viral infection also appears to depend on the infection route (56, 57). Lack of DNGR-1 had a remarkable impact on immunity against the virus, with viral loads 100-fold higher on day 16 after infection with the WR and Δ B13R strains. The increased viral load in the absence of DNGR-1 delayed the resolution of the lesion. Remarkably, this effect seems to be specifically CD8⁺ T cell dependent, as CD4⁺ T cell and antibody responses to VACV were unaffected. Our data are consistent with previous results showing that targeting of viral antigens to DNGR-1 results in enhanced cross-presentation and robust CD8⁺ T cell response (58).

The precise mechanism by which DNGR-1 controls cross-presentation of antigens associated with infected and damaged cells remains unclear. Syk signaling in DCs is essential for cross-presentation of these antigens, and Syk deficiency in CD11c⁺ cells impairs effector CD8⁺ T cell responses to VACV infection in vivo. Endocytosed DNGR-1 is preferentially located in non-lysosomal compartments (24), and therefore DNGR-1 might mediate the retention of dead cell-associated cargo in prelysosomal compartments. The diversion of cargo to such a compartment has been shown to occur with the mannose receptor, another CLR (59). The limited proteolytic activity in this compartment would result in partial processing of antigens, favoring their transition to the cytosol for cross-presentation (39–41). Cross-presentation ability was restored in DNGR-1-deficient DCs by inhibition of lysosomal protease activity with leupeptin plus pepstatin or by inhibition of the lysosome-acidifying V-ATPase with bafilomycin A1. DNGR-1 might signal to prevent acidification of this prelysosomal com-



partment, for example, via NOX2-dependent production of reactive oxygen species (41).

Clinical interest in VACV arises from the prospect of using MVA and its derivatives as viral vectors for vaccination against infectious diseases and tumors (60, 61). MVA induces strong CD8⁺ T cell immunity, which is crucial for protection against secondary challenge with VACV (56, 62). Being a defective virus, MVA does not replicate in mammalian cells, and it was not clear to what extent MVA would induce a cytopathic effect that would expose the DNDR-1 ligand. However, we found that MVA's capacity to trigger exposure of DNDR-1 ligand to be similar to that of the other VACV strains tested (data not shown). In addition, we found that MVA immunogenicity depends strongly on DNDR-1: the CD8⁺ T cell memory response was poor in the absence of the receptor and resulted in diminished protection against secondary challenge with VACV. Although our results were obtained in the mouse, they nevertheless suggest that DNDR-1 involvement is an important parameter to consider in the clinical development of poxvirus vectors for human vaccination.

In conclusion, our data show that DNDR-1 is a non-redundant receptor in antiviral immunity both in a primary infection and in a vaccination setting. DNDR-1 acts in concert with but at a different level from PRRs, and specifically controls cross-presentation of cargo associated with damaged cells. This mechanism in the control of immune responses is a promising target for vaccination strategies designed to induce CTL responses.

Methods

Mice. *Clec9a^{egfp/egfp}* mice (DNDR-1-deficient) on the C57BL/6 background were backcrossed more than 10 times to C57BL/6J-Cr1 mice. *Sykb^{fl/fl}* mice were a gift from Alexander Tarakhovskiy (The Rockefeller University, New York, New York, USA) (63), and CD11c-Cre BAC transgenic mice were provided by Boris Reizis (Columbia University, New York, New York, USA) (64). All mice were bred at the CNIC in specific pathogen-free conditions.

Cell culture and purification. Culture medium for cell lines (EL-4) and primary cells was RPMI 1640 supplemented with 5 mM glutamine, penicillin, streptomycin, 2-mercaptoethanol (all from Invitrogen), and 10% heat-inactivated fetal bovine serum (Sigma-Aldrich). Mouse Flt3L BMDCs were generated by culturing BM cells in the presence of 50 ng/ml Flt3L (R&D Systems) and replacing medium on days 5 and 10 (65). After 12–14 days, most cells had a typical DC morphology and a CD8 α -like phenotype (MHC class II⁺, CD11c⁺, CD24^{hi}, CD11b^{lo}, B220⁻, DNDR-1⁺). CV-1 cells, RAW macrophages, and BHK-21 cells were grown in DMEM supplemented as above. Single-cell suspensions of LNs, spleens, and ears were prepared by liberase/DNase digestion. When further purification of CD8⁺ T cells was required, cell suspensions were negatively selected using a cocktail of biotin-conjugated antibodies (anti-CD11c, -CD11b, -B220, -MHC II, -CD4, -NK1.1), followed by incubation with streptavidin-microbeads (Miltenyi Biotec). For purification of DCs from LNs, anti-CD11c microbeads (Miltenyi Biotec) were used. To purify CD8 α ⁺ DCs for restimulation or adoptive transfer, splenocytes were first obtained by negative selection with biotin-SIRP- α and streptavidin microbeads, followed by positive selection with anti-CD11c microbeads. Typical yields by FACS staining were more than 95% pure.

Antibodies, flow cytometry, ELISA. Samples were stained with the appropriate antibody cocktails in ice-cold PBS supplemented with 2 mM EDTA, 1% FCS, and 0.2% sodium azide. Anti-mouse antibodies to CD4, CD8 α , CD11b, CD11c, CD24, CD40, CD44, CD62L, CD86, CD103, I-A^b (MHC II), H-2K^b (MHC I), B220, and NK1.1 were used as conjugates to FITC, PE, PerCP-Cy5.5, or APC and were obtained from eBioscience. Purified anti-

Fc γ RIII/II (2.4G2) was used to block unspecific Ab binding. Non-cell-permeant Hoechst 33258 (5 μ g/ml) was used as a counterstain to detect necrotic cells. Anti-DNDR-1 1F6 antibody has been previously described (23). Anti-vaccinia-FITC (Acris Antibodies) was used to detect vaccinia proteins expressed in infected cells. Recombinant hCLEC9AFc chimera (R&D Systems) and anti-human Fc-biotin were used to stain DNDR-1 ligand, together with the control Dectin-1-Fc chimera (24). APC-anti-IFN- γ was from Miltenyi Biotec. Events were acquired using a FACSCanto flow cytometer (BD), and data were analyzed with FlowJo software (Tree Star). Representative plots are shown in the figures. The percentage of positive cells was calculated and is indicated in dot plots. Each experiment contained a minimum of 3 biological replicates, and a minimum of 3 independent experiments was performed. Percentage and MFI data from sets of experiments are graphed as mean \pm SEM. Antibody pairs for ELISA (IL-12p40 and TNF- α) were from BD, and ELISAs were performed according to the manufacturer's instructions. ELISAs were developed using ExtrAvidin-alkaline phosphatase and pNPP alkaline phosphatase substrate from Sigma-Aldrich, with absorbance measured at 405 nm. Anti-VACV antibody in the sera of infected mice was measured 28 days after infection (p.i.) by standard ELISA techniques. VACV-specific IgG was captured in wells coated overnight with UV-inactivated virus (originally equivalent to 10⁶ PFU in 50 μ l per well) and detected using rabbit anti-mouse IgG conjugated to horseradish peroxidase. After development with SIGMA-FAST *o*-phenyl-enediamine dihydrochloride, the reaction was stopped with sulfuric acid and the optical density was read at 450 nm.

Virus and peptides. The WR VACV strain was a gift from Jonathan W. Yewdell and Jack R. Bennink (NIH, Bethesda, Maryland, USA). Stocks were grown in CV-1 monolayers and used as clarified sonicated cell extracts. The WR VACV mutant lacking B13R (AB13R) was provided by Geoffrey Smith (Imperial College, London, United Kingdom). MVA was a gift from Bernard Moss (Laboratory of Viral Diseases, NIAID, NIH, Bethesda, Maryland, USA). MVA stocks were generated as described previously (66) in BHK-21 cells provided by Mariano Esteban (Centro Nacional de Biotecnología, Madrid, Spain).

The peptides ²⁰TSYKFESV²⁷ (B8R), from vaccinia soluble IFN- γ receptor homolog, and ²⁷⁰KSYNYMLL²⁷⁷ (A3L), from vaccinia precursor of core protein 4b (67), were a gift from Hisse M. Van Santen (Centro de Biología Molecular "Severo Ochoa," Madrid) and were synthesized in an Applera 433A peptide synthesizer, purified, and determined to be homogeneous by HPLC.

Viral infections and virus titration. Mice were infected i.d. in the ears with 5 \times 10⁴ PFU of the required VACV strain (68). The development of the lesion was monitored by its diameter with a digital caliper. To study the secondary response to MVA immunization, WT or DNDR-1-deficient mice were skin scarified with MVA VACV (2 \times 10⁶ PFU/mouse) and challenged after 21 days with WR VACV (5 \times 10⁴ PFU/mouse) i.d. in the ear. To detect DNDR-1 ligands in vivo, dermal cells were purified 5 days p.i. and analyzed with DNDR-1-Fc, Dectin-1-Fc, or CD69-Fc followed by biotin anti-human Fc (Jackson ImmunoResearch Laboratories Inc.) and SA-PE (eBioscience). Cells were counterstained with anti-vaccinia-FITC, annexin V-APC, and Hoechst 33258.

For virus titration, the ventral and dorsal dermal sheets of infected mouse ears were separated with forceps and incubated with 50 μ g/ml liberase CI (Roche) for 1 hour at 37°C. After 5 freeze-thaw cycles, ear homogenates were serially diluted and inoculated into the culture medium of CV-1 cells. Cells were stained 24 hours later with crystal violet; the detection limit was 6.6 PFU/ear. Each dot in the figures represents the virus titer in one ear of an individual mouse, and the horizontal bars represent the mean values for each group.

Transfer of virally infected cells in vivo and adoptive transfer of CD8 α ⁺ DCs. To study cross-presentation of VACV in vivo, we adapted a previously estab-



lished model (34). RAW cells were infected with WR VACV by 1 hour of adsorption and 4 hours of infection before UV irradiation. After 16 hours, cells (10^7 /mouse) were transferred i.p. Targets for in vivo cytotoxicity were transferred i.p. as indicated below. After 6 days, peritoneal cells were restimulated with B8R or A3L peptides for detection of intracellular IFN- γ .

For adoptive transfer of CD8 α^+ DCs, WT or *Clec9a^{flp/flp}* mice were s.c. injected with the B16-F10 cell line secreting Flt3L (provided by Glenn Dranoff, Harvard Medical School, Boston, Massachusetts, USA) (69) to expand CD8 α^+ DCs in the spleen. After 2 weeks, splenocytes were incubated for 2 hours with RAW-VACV-UV cells and then purified as indicated above to obtain more than 95% pure CD8 α^+ DCs. DCs (2×10^6 /mouse) were transferred via the footpad. After 6 days, targets for in vivo cytotoxicity were transferred i.v. as indicated below. After 7 days, spleen cells were restimulated with B8R or A3L peptides for detection of intracellular IFN- γ .

In vitro analysis of stimulation and antigen presentation. DCs were stimulated by coculture with VACV-infected RAW cells treated with or without UV irradiation to inactivate the virus. Maturation was assessed by upregulation of CD40 and CD86 co-stimulatory molecules and cytokine release measured by ELISA as indicated above.

To test DC cross-presenting ability, RAW cells were irradiated with UVC (240 mJ/cm^2) either without exposure to VACV (RAW-UV) or after incubation with VACV for 4 hours (RAW-VACV-UV). Alternatively, infected RAW cells were left un-irradiated (RAW-VACV). Sixteen hours after UV irradiation, RAW cells were cocultured for 4 hours with WT, CD11c-Cre \times *Syke^{fl/fl}*, or *Clec9a^{flp/flp}* Flt3L BMDCs, LN-DCs, or spleen CD8 α^+ DCs purified as described above. To the cocultures we then added CD8 $^+$ T cells purified from splenocytes of mice i.d. injected 7 days earlier with WR VACV to the cultures for 6 hours, brefeldin A (Sigma-Aldrich, $5 \mu\text{g/ml}$) being added for the last 4 hours of culture. Cells were then stained with FITC-anti-CD4 and PE-anti-CD8 α , fixed in 4% PFA, and incubated with APC-anti-IFN- γ during permeabilization with 0.1% saponin. An average of 10,000 of each T cell subset was analyzed in each sample. Background activation obtained with CD8 $^+$ T cells not pulsed with any peptide (0%–0.3%) was subtracted.

To test the effect of inhibitors on the cross-presenting ability of Flt3L BMDCs, bafilomycin A1 ($0.05 \mu\text{M}$) or leupeptin/pepstatin ($5 \mu\text{M}$) was added to the coculture of Flt3L BMDCs with RAW-VACV or RAW-VACV-UV cells. Bafilomycin A1, but not leupeptin/pepstatin, was washed out after this incubation, and CD8 $^+$ T cells with polyclonal specificity against VACV antigens were then added for restimulation and IFN- γ measurement as above.

For restimulation of effector T cells from WT or DNNGR-1-deficient mice *ex vivo*, ear dermal cells from VACV-infected WT or DNNGR-1-KO mice were purified on day 7 and added to Flt3L BMDCs from WT mice treated with RAW cells as above. Where indicated, T cells were restimulated with Flt3L BMDCs from *Clec9a^{flp/flp}* mice. The T cell effector response was measured by intracellular IFN- γ , as above. For restimulation of effector cells with peptides, ear dermal cell suspensions or splenocytes were exposed to an excess of peptide ($1 \mu\text{M}$), and intracellular IFN- γ was measured as above.

In vivo cytotoxicity assay. Splenocytes were used as targets and split into two populations, labeled with either a high or a low concentration of CFSE or CellTraceViolet (Invitrogen) and washed. CFSE^{lo} cells ($0.125 \mu\text{M}$ CFSE) were pulsed with B8R peptide, whereas Violet^{lo} cells ($0.25 \mu\text{M}$ CellTraceViolet) were pulsed with A3L peptide. Cells were then mixed with unpulsed CFSE^{hi} ($0.85 \mu\text{M}$) or Violet^{hi} ($1 \mu\text{M}$) cells and injected i.p. into syngeneic recipients (70). The peritoneal lavage was obtained 16 hours later and analyzed by flow cytometry to measure in vivo killing. Specific killing was calculated using the formula $100 \times (1 - [\% \text{ CFSE (or Violet) peptide} / \% \text{ CFSE (or Violet) no peptide}])$, as described previously (23).

Statistics. Statistical analysis was performed using Prism software (GraphPad Software Inc.). Statistical significance for comparison between 2 groups of samples with a normal distribution (Shapiro-Wilk test for normality) was determined using the unpaired 2-tailed Student's *t* test. A *P* value less than 0.05 was considered significant. Values within dot plots represent percentages.

Study approval. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by the CNIC Ethical Committee for Animal Welfare and by the Spanish Ministry for Rural and Marine Environment.

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