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**A DC receptor for damaged cells mediates cross-priming of CTL during vaccinia virus infection in mice.**

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**List of non-standard abbreviations: ΔB13R:** B13R WR VACV deletion mutant. **DNGR-1,** dendritic cell NK lectin group receptor 1. **Flt3L**: FMS-like tyrosine kinase 3 ligand. **i.d.:** intradermal(ly). **MVA:** modified vaccinia Ankara. **p.i.**: post-infection. **VACV:** vaccinia virus. **WR:** Western Reserve VACV.

**Abstract**

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 and might, therefore, impact immunity to cytopathic viruses. Here we report a non-redundant role for DNGR-1 in cross-presentation of viral antigens from vaccinia virus (VACV)-infected cells. We show that, following intradermal injection of VACV into mouse ears, dying infected cells expose DNGR-1 ligand and can be detected by DNGR-$1^+$ dermal DCs, resulting in cross-priming of anti-VACV $CD8^+$ T cells. Loss of DNGR-1 impairs 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 results in a profound increase in viral load and delayed resolution of the primary lesion. In addition, lack of DNGR-1 markedly diminishes protection from infection induced by vaccination with the modified vaccinia Ankara (MVA) strain. DNGR-1 thus contributes to anti-VACV immunity, both following primary infection and vaccination. The finding that DNGR-1 regulates cross-presentation of viral antigens and is non-redundant with other innate receptors that detect viral infection uncovers a novel form of regulation of antiviral immunity that 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 or 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 both PRRs and damage-sensing systems (11, 12).

Dendritic cells (DCs) are crucial for adaptive immune responses to infection. Of the various DC subsets, mouse CD8α$^{+}$ 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 CD8α$^{+}$-like DCs might be ideally placed to integrate sensing of cell death and PAMP sensing for regulation of CD8$^{+}$ T cell priming. Interestingly, CD8α$^{+}$ 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 pre-formed 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 hemITAM motif that allows binding

to and signaling via spleen tyrosine kinase (Syk) (23). Notably, DNGR-1 deficient mice are partially deficient in cytotoxic T lymphocyte (CTL) responses against antigens borne by dead cells (24). DNGR-1 might thus be a receptor that allows $CD8\alpha^+$-like DCs to respond to tissue damage signals generated during infection. However, DNGR-1 deficient mice have not yet been tested for impairment of immunity to cytopathic pathogens.

Vaccinia virus (VACV) is a complex dsDNA virus from 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 (ΔB13R) 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 preferred 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 be directly presented, 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 if 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 $CD8\alpha^+$ -like DCs) from wild type mice (WT; H-$2^b$) and DNGR-1 deficient (Clec9a$^{gfp/gfp}$) mice (24). As an antigen source, we used VACV-infected RAW 264.7 (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, infected RAW cells were treated 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). 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 1A, B). 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 1A and B). The same result was observed with total DCs obtained from mouse lymph nodes or with purified $CD8\alpha^+$ DCs from mouse spleen, indicating that it was not restricted to the use of Flt3L BMDCs (Supplemental Figure 1).

The use of pre-activated $CD8^+$ T cells as a readout in these assays made it unlikely that the observed effect was due to an 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. Independently of UV irradiation, VACV-infected cells induced strong DC activation, measured either at the level of costimulatory molecule upregulation (CD40, CD86) or cytokine production (TNF-α, IL12 p40). Notably, this activation was not affected by DNGR-1 deficiency (Figure 2A 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 $CD8\alpha^+$-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 of infection of EL-4 cells with the WR VACV strain in vitro showed that exposure of the ligand could be detected in cells expressing VACV proteins as early as 24h after infection (Figure 3A and not shown). All ligand-expressing cells are positive for Annexin V and permeable to Hoechst 33258 staining, showing a proportion of mid-positive Hoechst 33258 cells that correspond to the transition of late apoptotic to early necrotic cells and bright positive Hoechst 33258 cells that are fully necrotic (Figure 3A).

To extend these findings in vivo, we injected WR VACV intradermally (i.d.) into mouse ears and analyzed the presence of DNGR-1 ligand in ear cell suspensions 4d 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 are stained with Annexin V and permeable to Hoechst 33258, being in this case predominant the fraction with mid levels of Hoechst (early necrotic) as the late necrotic cells are probably 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 expresses DNGR-1 (Figure 3C). Cells in this subset are related to the $CD8\alpha^+$ DCs in lymphoid tissues and, like these cells, possess 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 intraperitoneally (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). Notably, we found that DNGR-1 deficiency greatly reduced the effector $CD8^+$ T cell response in this model (Figure 4A and B).

To confirm that the ex vivo restimulated effector $CD8^+$ T cell responses reflect 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 CellTrace violet 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. Analysis of killing activity in vivo 16h after transfer of the targets confirmed the impairment of CTL activity toward both VACV peptides (Figure 4C and D). Thus, DNGR-1 deficiency impairs CTL activity in response to cross-presented VACV antigens.

High DNGR-1 expression is tightly restricted to $CD8\alpha^+$-like DCs. To unequivocally determine whether the deficiency in the CTL response was due to lack of DNGR-1 expression on $CD8\alpha^+$ DCs, we carried out adoptive transfer experiments using $CD8\alpha^+$ spleen DCs from WT or DNGR-1 deficient ($Clec9a^{gfp/gfp}$) mice. After incubation of splenocytes for 2h with RAW-VACV-UV cells, the $CD8\alpha^+$ spleen DCs were purified and transferred adoptively into $Clec9a^{gfp/gfp}$ recipient mice (Figure 5). After 7 days, ex vivo restimulation of splenocytes with B8R or A3L VACV peptides demonstrated that WT, but not DNGR-1 deficient, $CD8\alpha^+$ DCs efficiently transfer the ability to cross-present VACV antigens to $CD8^+$ T cells (Figure 5A). These results were confirmed by measurement of systemic CTL activity 6 days after transfer of $CD8\alpha^+$ DCs (Figure 5C and D). These data show that lack of DNGR-1 expression in $CD8\alpha^+$ DCs is responsible for the observed defective cross-presentation of VACV-derived antigens in vivo.

**DNGR-1 deficiency impairs the $CD8^+$ T cell effector response to cross-presented VACV antigens.**

To test whether DNGR-1 contributes to the CTL response in the context of live virus infection, we injected the WR VACV strain i.d. in the ears of WT and DNGR-1 deficient mice. After 7d, cells from the infected ears were restimulated ex vivo with WT DCs and VACV antigens to determine the frequency of effector T cells in skin. 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 DNGR-1 deficient mice (Figure 6A and B). 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 DNGR-1, while the $CD4^+$ T cell response was unaffected (Figure 6A and B). Thus, only the $CD8^+$ T cell effector response to antigens cross-presented from WR VACV is partially impaired in DNGR-1 deficient mice.

We next tested the ΔB13R VACV strain that lacks an inhibitor of apoptosis (30). Dermal cells were obtained from WT or DNGR-1 deficient mouse ears infected with ΔB13R VACV for 7d and restimulated as above. In contrast to infection with the parental WR strain, the virus-specific $CD8^+$ T cell response to ΔB13R VACV was significantly impaired in DNGR-1 deficient mice, whether assessed by restimulation with RAW-VACV or RAW-VACV-UV (Figure 6C). As with the WR strain, the $CD4^+$ T cell response against ΔB13R VACV was normal in the absence of DNGR-1 (Figure 6D). Moreover, the antibody response against WR or ΔB13R VACV was also identical in WT and DNGR-1 deficient mice

(Supplemental Figure 3A and B). In sum, DNGR-1 deficiency selectively decreases the CD8$^+$ T cell effector response to VACV antigens that rely on cross-presentation.

**Loss of DNGR-1 reduces the CD8$^+$ T cell effector response and killing activity in vivo against VACV epitopes.**

To further test the effect of DNGR-1 deficiency on the effector CD8$^+$ T cell response, WT or DNGR-1 deficient mice were infected i.d. with the WR or ΔB13R VACV strain and IFN-γ production by skin T cells was measured 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 7A). 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 ΔB13R VACV strain, in line with the greater dependence of this strain on cross-presentation (Figure 7B).

To determine systemic CTL activity in vivo, syngeneic splenocyte targets loaded with B8R, A3L or no peptide and labeled as before were injected into mice on day 6 after primary i.d. challenge with the WR or ΔB13R VACV strains. Analysis of killing activity in vivo after 16h mirrored the results 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 7C and D). The DNGR-1-dependent killing activity against both peptides was greater in the case of infection with the ΔB13R 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 ΔB13R 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 impacted subsequent resolution (Figure 8A). Similar effects were seen with the ΔB13R VACV strain, which induced larger and more persistent lesions (Figure 8B), as reported (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 8C 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 ΔB13R VACV strain was increased more than 10 fold in the absence of DNGR-1 by day 16 (Figure 6C 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-deficient mice, whether measured by restimulation with RAW-VACV, RAW-VACV-UV or the B8R and A3L specific peptides (Figure 9A, B and not shown). As expected, the use of Clec9a$^{gfp/gfp}$ BMDCs further impaired restimulation by RAW-VACV or RAW-VACV-UV (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 appears to contribute more significantly to immunity against MVA than to the other replicative VACV strains (compare Figure 9 with Figures 6 and 7).

To address the role of DNGR-1 in the generation of memory responses against VACV, a relevant issue for vaccination with poxviruses, we vaccinated WT or DNGR-1 deficient mice with the MVA VACV strain by skin scarification of the base of the tail (31). After 21d, 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 10A). Remarkably, DNGR-1 deficiency resulted in a 10-fold higher viral titer in vaccinated mice, suggesting a defective secondary CTL response. To confirm this, CD8$^+$ T cells were obtained from draining LNs on day 5 post-challenge and tested for their response to B8R and A3L VACV epitopes in the ex vivo restimulation assay. Cells from vaccinated and subsequently challenged DNGR-1 KO mice displayed a markedly weaker secondary response to the vaccinia peptides (Figure 10B). The reduced CD8$^+$ T cell effector response and higher viral titers also manifested themselves in increased lesion size in DNGR-1-deficient mice (Figure 10C). These results show that DNGR-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.**

DNGR-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. Comparison of the cross-presentation capacity of Flt3L BMDCs from WT and CD11cCre x Syk$^{fl/fl}$ mice (38) revealed that Syk deficiency in DCs impairs the CD8$^+$ T cell response to VACV antigens that are cross-presented but not to those that are directly presented (Figure 11A and B).

To test the influence of Syk on the CD8$^+$ T cell effector response in vivo, WT or CD11cCre x Syk$^{fl/fl}$ mice were i.d. injected with WR VACV, and dermal cells obtained after 7 days were restimulated with B8R and A3L VACV peptides (Figure 11C and D). 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 DNGR-1 that regulates cross-presentation of antigens from VACV-infected cells.

**Inhibitors of lysosomal activity restore the cross-presentation ability of DNGR-1 deficient DCs.**

Since DNGR-1 is located in non-lysosomal compartments (24), we hypothesized that DNGR-1 might retain the cargo from VACV-infected cells in a pre-lysosomal compartment with limited proteolytic activity, thereby favoring cross-presentation of antigens (39-41). To test this hypothesis, we examined the effects of inhibitors of lysosomal protease activity (leupeptin + 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 12A, left panel). However, both treatments restored the cross-presentation capacity of DNGR-1 deficient DCs to levels similar to those of WT DCs (Figure 12A, right panel). Direct presentation was not affected by either treatment (Figure 12B). 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 is proteasome-dependent (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 pro-inflammatory properties and antigens contained within them often provoke an adaptive immune response (9, 42, 43). As such, 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 $CD8\alpha^+$ DCs can sense dead cells through DNGR-1 and that this receptor favors cross-priming of CTLs against antigens derived from non-infectious dead-cells (24). Here, we tested whether DNGR-1 also modulates CTL responses to virus infection. Remarkably, we found that DNGR-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 DNGR-1 also impaired the secondary response following vaccination with MVA. These results show that DNGR-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 DNGR-1 to the related myeloid activating CLR Dectin-1 suggested that DNGR-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 DNGR-1 induced TNF-α production in a macrophage cell line (22). However, our results are not consistent with a crucial DC activating function for DNGR-1, as we show that DNGR-1-deficient DCs are activated normally in response to VACV-infected cells (see also accompanying paper by Zelenay et al.). Nonetheless, stimulation of VACV-specific pre-primed $CD8^+$ T cells by DNGR-1-deficient DCs was impaired, especially in conditions where cross-presentation is the only option for generating the appropriate MHC class I peptides. Thus DNGR-1 specifically impairs cross-presentation of cell-associated antigens during viral infection. The fact that DNGR-1 deficiency is not compensated by signals from PRRs shows that sensing of damage via DNGR-1 and the detection of 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 is 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 demonstrate that DNGR-1expression by CD8α$^+$ DCs confers them the capacity to cross-present antigens from VACV-infected cells in vivo. It was proposed that migratory DCs can carry viral antigens to draining lymph nodes and transfer them to CD8α$^+$ DCs for local cross-presentation (46, 47). Consistent with this possibility, we found some VACV proteins and DNGR-1 ligands in cells in the draining LNs (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 find 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, both in mouse and human (23).

Our observations show 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 ΔB13R 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, 48). This is likely to be affected by the VACV strain used and the infection route. For instance, the MVA strain can infect APCs (28, 49), and subsequently might induce direct priming as well as cross-priming. However, these attenuated viruses lack many caspase inhibitors, reducing the potential lifetime 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 (50), and CTL responses to MVA depend strongly on cross-presentation (28). Similarly, B13R (Spi2) inhibits

apoptosis (51), and therefore responses to the B13R deletion mutant (ΔB13R), 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 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, 48, 52).

VACV infection in DCs is abortive, leading to expression of early but not late viral genes (53, 54). 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 (55, 56). Lack of DNGR-1 has a remarkable impact on immunity against the virus, with viral loads 100-fold higher on day 16 after infection with the WR and ΔB13R strains. The increased viral load in the absence of DNGR-1 delays 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 are unaffected. Our data concur with previous results showing that targeting of viral antigens to DNGR-1 results in enhanced cross-presentation and robust $CD8^+$ T cell response (57).

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 (58). 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 + pepstatin or by inhibition of the lysosome acidifying V-ATPase with bafilomycin-A1. DNGR-1 might signal to prevent acidification of this prelysosomal compartment, 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 (59, 60). MVA induces strong CD8$^+$ T cell immunity, which is crucial for protection against secondary challenge with VACV (55, 61). Being a defective virus, MVA does not replicate in mammalian cells and it was not clear to what extent it would induce a cytopathic effect that would expose the DNGR-1 ligand. However, we found that MVA has a similar capacity to trigger exposure of DNGR-1 ligand as the other VACV strains tested (not shown). In addition, we found that MVA immunogenicity depends strongly on DNGR-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 DNGR-1 involvement is an important parameter to consider in the clinical development of poxvirus vectors for human vaccination.

In conclusion, our data show that DNGR-1 is a non-redundant receptor in anti-viral immunity both in a primary infection and in a vaccination setting. DNGR-1 acts in concert but at a different level from PRRs, and specifically controls cross-presentation of cargo associated with damaged cells. This novel 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 (DNGR-1-deficient) on the C57BL/6 background were backcrossed more than 10 times to C57BL/6J-Crl. Syk$^{fl/fl}$ mice were a kind gift from Alexander Tarakhovsky (The Rockefeller University, New York) (62), and CD11c-Cre BAC transgenic mice were kindly provided by Boris Reizis (Columbia University, New York) (63). 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 5mM glutamine, penicillin, streptomycin, 2-mercaptoethanol (all from Invitrogen) and 10% heat-inactivated fetal bovine serum (Sigma). Mouse Flt3L-BMDCs were generated by culturing BM cells in the presence of 50 ng/ml Flt3L (R&D) and replacing medium on days 5 and 10 (64). After 12-14 days, most cells had a typical DC morphology and a CD8α-like phenotype (MHC class II$^+$, CD11c$^+$, CD24$^{hi}$, CD11b$^{lo}$, B220$^-$, DNGR-1$^+$). CV-1 cells, RAW264.7 macrophages (RAW), and BHK-21 cells were grown in DMEM supplemented as above. Single cell suspensions of lymph nodes (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) was used. To purify CD8α$^+$ 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 2mM EDTA, 1% FCS and 0.2% sodium azide. Anti-mouse antibodies to CD4, CD8α, CD11b, CD11c, CD24, CD40, CD44, CD62L, CD86, CD103, I-$A^b$ (MHC-II), H2Kb (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-FcγRIII/II (2.4G2) was used to block unspecific Ab binding. Non cell-permeant Hoechst 33258 (5 μg/ml) was used as a counterstain to detect necrotic cells. Anti-DNGR-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) and anti-human-Fc-biotin was used to stain DNGR-1 ligand, together with the control Dectin1Fc chimera (24). APC-anti-IFN-γ was from Miltenyi Biotec. Events were acquired using a FACSCanto flow cytometer (Becton Dickinson), 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 on dot plots. Each experiment contained a minimum of three biological replicates, and a minimum of three independent experiments was performed. Percentage and mean fluorescence intensity (MFI) data from sets of experiments are graphed as the mean ± SEM. Antibody pairs for ELISA (IL12p40 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, with absorbance measured at 405 nm. Anti-VACV antibody in the sera of infected mice was measured 28 days p.i. by standard ELISA techniques. VACV-specific IgG was captured in wells coated overnight with UV-inactivated virus (originally equivalent to $10^6$ PFU in 50 μl 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 kind gift from Jonathan W. Yewdell and Jack R. Bennink (National Institutes of Health, Bethesda, MD). Stocks were grown in CV-1 monolayers and used as clarified sonicated cell extracts. The WR VACV mutant lacking B13R (ΔB13R) was kindly provided by Professor Geoffrey Smith (Imperial College, London, UK). Modified vaccinia virus Ankara (MVA) was a gift from Bernard Moss (Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD). MVA stocks were generated as described (65) in BHK-21 cells kindly provided by Dr. M Esteban (Centro Nacional de Biotecnología, Madrid).

The peptides $^{20}$TSYKFESV$^{27}$ (B8R), from vaccinia soluble interferon-gamma receptor homologue, and $^{270}$KSYNYMLL$^{277}$ (A3L), from vaccinia precursor of core protein 4b, (66) were a kind 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 $5x10^4$ PFU of the required VACV strain (67). The development of the lesion was monitored by its diameter with a digital caliper. To study the secondary response to MVA immunization, WT or DNGR-1 deficient mice were skin scarified with MVA VACV ($2x10^6$ pfu/mouse) and challenged after 21d with WR VACV ($5x10^4$ pfu/mouse) i.d. in the ear. To detect DNGR-1 ligands in vivo, dermal cells were purified 5d p.i. and analyzed with DNGR-1-Fc, Dectin-Fc or CD69-Fc followed by biotin

anti-human-Fc (Jackson Immunoresearch) 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 50μg/ml liberase CI (Roche) for 1h 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 24h 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 thick horizontal bars represent the mean values for each group.

**Transfer of virally infected cells in vivo and adoptive transfer of CD8α$^+$ DCs.**

To study cross-presentation of VACV in vivo, we adapted a previously established model (34). RAW 264.7 cells were infected with WR-VACV by 1h of adsorption and 4h of infection before UV irradiation. After 16h, 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 CD8α$^+$ DCs, WT or Clec9a$^{gfp/gfp}$ mice were s.c. injected with the B16-F10 cell line secreting Flt3L (generously provided by Glenn Dranoff, Harvard Medical School)(68) to expand CD8α$^+$ DCs in the spleen. After two weeks, splenocytes were incubated for 2h with RAW-VACV-UV cells and then purified as indicated above to obtain >95% pure CD8α$^+$ DCs. DCs ($2x10^6$/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 co-culture with VACV infected RAW cells treated with or without UV irradiation to inactivate the virus. Maturation was assessed by up-regulation 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 4h (RAW-VACV-UV). Alternatively, infected RAW cells were left un-irradiated (RAW-VACV). Sixteen hours after UV irradiation, RAW cells were co-cultured for 4 h with WT, CD11cCre x $Syk^{fl/fl}$ or $Clec9a^{gfp/gfp}$ Flt3L BMDCs, LN-DCs or Spleen $CD8\alpha^+$ DCs purified as described above. To the co-cultures we then added $CD8^+$ T cells purified from splenocytes of mice i.d. injected 7d earlier with WR VACV were added to the cultures for 6h, being brefeldin A (Sigma, 5 μg/ml) added for the last 4h of culture. Cells were then stained with FITC-anti-CD4 and PE-anti-CD8α, 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 non-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 (0.05μM) or leupeptin/pepstatin (5μM) were added to the co-culture 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 re-stimulation of effector T cells from WT or DNGR-1 deficient mice ex vivo, ear dermal cells from VACV-infected WT or DNGR-1 KO mice were purified at 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$^{gfp/gfp}$ mice. The T cell effector response was measured by intracellular IFN-γ, as above. For re-stimulation of effector cells with peptides, ear dermal cell suspensions or splenocytes were exposed to an excess of peptide (1μM) and intracellular IFN-γ measured as above.

**In vivo cytotoxicity assay**

Splenocytes were used as targets and split into 2 populations, labeled with either a high or a low concentration of CFSE or CellTrace™ Violet and washed. CFSE$^{lo}$ cells (0.125 μM CFSE) were pulsed with B8R peptide, whereas Violet$^{lo}$ cells (0.25μM CellTrace™ Violet) were pulsed with A3L peptide. Cells were then mixed with unpulsed CFSE$^{hi}$ (0.85μM) or Violet$^{hi}$ (1 μM) cells and injected i.p. into syngeneic recipients (69). The peritoneal lavage was obtained 16h later and analyzed by flow cytometry to measure in vivo killing. Specific killing was calculated using the formula 100 × (1 – [% CFSE (or Violet) peptide / % CFSE (or Violet) no peptide]), as described (23).

**Statistical analysis**

Statistical analysis was performed using Prism software (GraphPad Software, Inc). Statistical significance for comparison between two groups of samples with a normal distribution (Shapiro-Wilk test for normality) was determined using the unpaired two-tailed Student's *t* test. A *p* value <0.05 was considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

**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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**References**

1. Janeway, C.A. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1:1-13.
2. Palm, N.W., and Medzhitov, R. 2009. Pattern recognition receptors and control of adaptive immunity. *Immunol Rev* 227:221-233.
3. Matzinger, P., and Kamala, T. 2011. Tissue-based class control: the other side of tolerance. *Nat Rev Immunol* 11:221-230.
4. Nish, S., and Medzhitov, R. 2011. Host defense pathways: role of redundancy and compensation in infectious disease phenotypes. *Immunity* 34:629-636.
5. Gallucci, S., Lolkema, M., and Matzinger, P. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5:1249-1255.
6. Shi, Y., Zheng, W., and Rock, K.L. 2000. Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses. *Proc Natl Acad Sci USA* 97:14590-14595.
7. Sauter, B., Albert, M.L., Francisco, L., Larsson, M., Somersan, S., and Bhardwaj, N. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 191:423-434.
8. Scaffidi, P., Misteli, T., and Bianchi, M.E. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418:191-195.
9. Rovere-Querini, P., Capobianco, A., Scaffidi, P., Valentinis, B., Catalanotti, F., Giazzon, M., Dumitriu, I.E., Müller, S., Iannacone, M., Traversari, C., et al. 2004. HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep* 5:825-830.

10. Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* 11:700-714.

11. Kono, and Rock. 2008. How dying cells alert the immune system to danger. *Nat Rev Immunol* 8:279-289.

12. Lazzaro, B.P., and Rolff, J. 2011. Immunology. Danger, microbes, and homeostasis. *Science* 332:43-44.

13. den Haan, J.M., Lehar, S.M., and Bevan, M.J. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192:1685-1696.

14. Schulz, O., and Reis e Sousa, C. 2002. Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells. *Immunology* 107:183-189.

15. Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R.M., and Inaba, K. 2002. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J Exp Med* 195:1289-1302.

16. Schnorrer, P., Behrens, G.M.N., Wilson, N.S., Pooley, J.L., Smith, C.M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., et al. 2006. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci USA* 103:10729-10734.

17. Dudziak, D., Kamphorst, A.O., Heidkamp, G.F., Buchholz, V.R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H.-W., Park, C.G., et al. 2007. Differential antigen processing by dendritic cell subsets in vivo. *Science* 315:107-111.

18. Hildner, K., Edelson, B.T., Purtha, W.E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B.U., Unanue, E.R., Diamond, M.S., et al. 2008. Batf3

deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322:1097-1100.

19. Ginhoux, F., Liu, K., Helft, J., Bogunovic, M., Greter, M., Hashimoto, D., Price, J., Yin, N., Bromberg, J., Lira, S.A., et al. 2009. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med* 206:3115-3130.

20. Henri, S., Poulin, L.F., Tamoutounour, S., Ardouin, L., Guilliams, M., de Bovis, B., Devilard, E., Viret, C., Azukizawa, H., Kissenpfennig, A., et al. 2009. CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med*.

21. Edelson, B.T., KC, W., Juang, R., Kohyama, M., Benoit, L.A., Klekotka, P.A., Moon, C., Albring, J.C., Ise, W., Michael, D.G., et al. 2010. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J Exp Med* 207:823-836.

22. Huysamen, C., Willment, J.A., Dennehy, K.M., and Brown, G.D. 2008. CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes. *J Biol Chem* 283:16693-16701.

23. Sancho, Mourão-Sá, Joffre, Schulz, Rogers, Pennington, Carlyle, and Reis E Sousa, C. 2008. Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest* 118:2098-2110.

24. Sancho, D., Joffre, O., Keller, A., Rogers, N., Martínez, D., Hernanz-Falcón, P., Rosewell, I., and Reis e Sousa, C. 2009. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458:899-903.

25. Cho, Y.S., Challa, S., Moquin, D., Genga, R., Ray, T.D., Guildford, M., and Chan, F.K.-M. 2009. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137:1112-1123.

26. Sigal, L.J., Crotty, S., Andino, R., and Rock, K.L. 1999. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 398:77-80.

27. Xu, R.-H., Remakus, S., Ma, X., Roscoe, F., and Sigal, L.J. 2010. Direct presentation is sufficient for an efficient anti-viral CD8+ T cell response. *PLoS Pathog* 6:e1000768.

28. Gasteiger, G., Kastenmuller, W., Ljapoci, R., Sutter, G., and Drexler, I. 2007. Cross-priming of cytotoxic T cells dictates antigen requisites for modified vaccinia virus Ankara vector vaccines. *J Virol* 81:11925-11936.

29. Shen, X., Wong, S.B.J., Buck, C.B., Zhang, J., and Siliciano, R.F. 2002. Direct priming and cross-priming contribute differentially to the induction of CD8+ CTL following exposure to vaccinia virus via different routes. *J Immunol* 169:4222-4229.

30. Kettle, S., Alcamí, A., Khanna, A., Ehret, R., Jassoy, C., and Smith, G.L. 1997. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. *J Gen Virol* 78 ( Pt 3):677-685.

31. Liu, L., Zhong, Q., Tian, T., Dubin, K., Athale, S.K., and Kupper, T.S. 2010. Epidermal injury and infection during poxvirus immunization is crucial for the generation of highly protective T cell-mediated immunity. *Nat Med* 16:224-227.

32. Bronte, V., Carroll, M.W., Goletz, T.J., Wang, M., Overwijk, W.W., Marincola, F., Rosenberg, S.A., Moss, B., and Restifo, N.P. 1997. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. *Proc Natl Acad Sci USA* 94:3183-3188.

33. Bedoui, S., Whitney, P., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R., Wojtasiak, M., Shortman, K., Carbone, F., et al. 2009. Cross-presentation of viral and self antigens by skin-derived CD103(+) dendritic cells. *Nat Immunol* 10:488-495.

34. Lev, A., Dimberu, P., Das, S.R., Maynard, J.C., Nicchitta, C.V., Bennink, J.R., and Yewdell, J.W. 2009. Efficient cross-priming of antiviral CD8+ T cells by antigen donor cells is GRP94 independent. *J Immunol* 183:4205-4210.

35. Yang, Z., Bruno, D.P., Martens, C.A., Porcella, S.F., and Moss, B. 2010. Simultaneous high-resolution analysis of vaccinia virus and host cell transcriptomes by deep RNA sequencing. *Proc Natl Acad Sci USA* 107:11513-11518.

36. Jacobs, N., Chen, R.A., Gubser, C., Najarro, P., and Smith, G.L. 2006. Intradermal immune response after infection with Vaccinia virus. *J Gen Virol* 87:1157-1161.

37. Tscharke, D.C., Reading, P.C., and Smith, G.L. 2002. Dermal infection with vaccinia virus reveals roles for virus proteins not seen using other inoculation routes. *J Gen Virol* 83:1977-1986.

38. Kool, M., Willart, M.A.M., van Nimwegen, M., Bergen, I., Pouliot, P., Virchow, J.C., Rogers, N., Osorio, F., Reis E Sousa, C., Hammad, H., et al. 2011. An Unexpected Role for Uric Acid as an Inducer of T Helper 2 Cell Immunity to Inhaled Antigens and Inflammatory Mediator of Allergic Asthma. *Immunity*.

39. Delamarre, L., Couture, R., Mellman, I., and Trombetta, E.S. 2006. Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. *J Exp Med* 203:2049-2055.

40. Delamarre, L., Pack, M., Chang, H., Mellman, I., and Trombetta, E.S. 2005. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 307:1630-1634.

41. Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I.C., Lennon-Duménil, A.-M., Seabra, M.C., Raposo, G., and Amigorena, S. 2006. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126:205-218.

42. Rock, K.L., and Kono, H. 2008. The inflammatory response to cell death. *Annu Rev Pathol* 3:99-126.

43. Shi, Y., Evans, J.E., and Rock, K.L. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425:516-521.

44. Zitvogel, L., Kepp, O., and Kroemer, G. 2010. Decoding cell death signals in inflammation and immunity. *Cell* 140:798-804.

45. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:991-1045.

46. Belz, G.T., Smith, C.M., Eichner, D., Shortman, K., Karupiah, G., Carbone, F.R., and Heath, W.R. 2004. Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses. *J Immunol* 172:1996-2000.

47. Smith, C.M., Belz, G.T., Wilson, N.S., Villadangos, J.A., Shortman, K., Carbone, F.R., and Heath, W.R. 2003. Cutting edge: conventional CD8 alpha+ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1. *J Immunol* 170:4437-4440.

48. Torti, N., Walton, S.M., Murphy, K.M., and Oxenius, A. 2011. Batf3 transcription factor-dependent DC subsets in murine CMV infection: differential impact on T cell priming and memory inflation. *Eur. J. Immunol.* (in press).

49. Kastenmuller, W., Drexler, I., Ludwig, H., Erfle, V., Peschel, C., Bernhard, H., and Sutter, G. 2006. Infection of human dendritic cells with recombinant vaccinia

virus MVA reveals general persistence of viral early transcription but distinct maturation-dependent cytopathogenicity. *Virology* 350:276-288.

50. Chahroudi, A., Garber, D.A., Reeves, P., Liu, L., Kalman, D., and Feinberg, M.B. 2006. Differences and similarities in viral life cycle progression and host cell physiology after infection of human dendritic cells with modified vaccinia virus Ankara and vaccinia virus. *J Virol* 80:8469-8481.

51. Dobbelstein, M., and Shenk, T. 1996. Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. *J Virol* 70:6479-6485.

52. Shen, L., Sigal, L.J., Boes, M., and Rock, K.L. 2004. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21:155-165.

53. Engelmayer, J., Larsson, M., Subklewe, M., Chahroudi, A., Cox, W.I., Steinman, R.M., and Bhardwaj, N. 1999. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J Immunol* 163:6762-6768.

54. Jenne, L., Hauser, C., Arrighi, J.F., Saurat, J.H., and Hugin, A.W. 2000. Poxvirus as a vector to transduce human dendritic cells for immunotherapy: abortive infection but reduced APC function. *Gene Ther* 7:1575-1583.

55. Xu, R., Johnson, A.J., Liggitt, D., and Bevan, M.J. 2004. Cellular and humoral immunity against vaccinia virus infection of mice. *J Immunol* 172:6265-6271.

56. Mota, B.E.F., Gallardo-Romero, N., Trindade, G., Keckler, M.S., Karem, K., Carroll, D., Campos, M.A., Vieira, L.Q., da Fonseca, F.G., Ferreira, P.C.P., et al. 2011. Adverse events post smallpox-vaccination: insights from tail scarification infection in mice with Vaccinia virus. *PLoS ONE* 6:e18924.

57. Idoyaga, J., Lubkin, A., Fiorese, C., Lahoud, M.H., Caminschi, I., Huang, Y., Rodriguez, A., Clausen, B.E., Park, C.G., Trumpfheller, C., et al. 2011. Comparable T

helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A. *Proc Natl Acad Sci U S A* 108:2384-2389.

58. Burgdorf, S., Kautz, A., Böhnert, V., Knolle, P.A., and Kurts, C. 2007. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316:612-616.

59. Draper, S.J., and Heeney, J.L. 2010. Viruses as vaccine vectors for infectious diseases and cancer. *Nat Rev Microbiol* 8:62-73.

60. Moss, B. 2011. Smallpox vaccines: targets of protective immunity. *Immunol Rev* 239:8-26.

61. Moutaftsi, M., Salek-Ardakani, S., Croft, M., Peters, B., Sidney, J., Grey, H., and Sette, A. 2009. Correlates of protection efficacy induced by vaccinia virus-specific CD8+ T-cell epitopes in the murine intranasal challenge model. *Eur J Immunol* 39:717-722.

62. Saijo, K., Schmedt, C., Su, I.-H., Karasuyama, H., Lowell, C.A., Reth, M., Adachi, T., Patke, A., Santana, A., and Tarakhovsky, A. 2003. Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development. *Nat Immunol* 4:274-279.

63. Caton, M.L., Smith-Raska, M.R., and Reizis, B. 2007. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med* 204:1653-1664.

64. Naik, S.H., Proietto, A.I., Wilson, N.S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M.H., O'Keeffe, M., Shao, Q.-x., Chen, W.-f., et al. 2005. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* 174:6592-6597.

65. Earl, P.L., Cooper, N., Wyatt, L.S., Moss, B., and Carroll, M.W. 2001. Preparation of cell cultures and vaccinia virus stocks. *Curr Protoc Protein Sci* Chapter 5:Unit5 12.

66. Moutaftsi, M., Peters, B., Pasquetto, V., Tscharke, D.C., Sidney, J., Bui, H.H., Grey, H., and Sette, A. 2006. A consensus epitope prediction approach identifies the breadth of murine T(CD8+)-cell responses to vaccinia virus. *Nat Biotechnol* 24:817-819.

67. Tscharke, D.C., and Smith, G.L. 1999. A model for vaccinia virus pathogenesis and immunity based on intradermal injection of mouse ear pinnae. *J Gen Virol* 80 ( Pt 10):2751-2755.

68. Mach, N., Gillessen, S., Wilson, S.B., Sheehan, C., Mihm, M., and Dranoff, G. 2000. Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* 60:3239-3246.

69. Medina, F., Ramos, M., Iborra, S., de Leon, P., Rodriguez-Castro, M., and Del Val, M. 2009. Furin-processed antigens targeted to the secretory route elicit functional TAP1-/-CD8+ T lymphocytes in vivo. *J Immunol* 183:4639-4647.