

Short Communication

Card9 controls Dectin-1-induced T-cell cytotoxicity and tumor growth in mice

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Activation of the C-type lectin receptor Dectin-1 by β -glucans triggers multiple signals within DCs that result in activation of innate immunity. While these mechanisms can potentially prime CD8⁺ cytotoxic T-cell (CTL) responses without additional adjuvants, the Dectin-1 effector pathways that control CTL induction remain unclear. Here we demonstrate that Dectin-1-induced CTL cross-priming in mice does not require inflammasome activation but strictly depends on the adapter protein Card9 *in vitro*. *In vivo*, Dectin-1-mediated Card9 activation after vaccination drives both expansion and activation of Ag-specific CTLs, resulting in long-lasting CTL responses that are sufficient to protect mice from tumor challenge. This Dectin-1-induced antitumor immune response was independent of NK cell function and completely abrogated in Card9-deficient mice. Thus, our results demonstrate that Dectin-1-triggered Card9 signaling but not inflammasome activation can potentially cross-prime Ag-specific CTLs, suggesting that this pathway would be a candidate for immunotherapy and vaccine development.

Keywords: Card9 · CD8⁺ cytotoxic T cells · Cross-priming · Dectin-1 · Tumor immunity



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Introduction

CD8⁺ cytotoxic T cells (CTLs) are central effectors of antitumor immunity. Owing to their strong cytolytic potency, CTL activation is tightly regulated and requires instruction by specialized APCs

like DCs. DCs control uptake, processing, and presentation of exogenous (tumor-derived) Ag in the context of MHC class I (MHC-I), which enables them to prime CTLs [1]. This process has been termed cross-priming and is a prerequisite for the induction of CTL-based antitumor immunity. Cross-priming

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requires DC maturation with upregulation of MHC-I and costimulatory molecules as well as the release of proinflammatory cytokines. Activation and maturation of DCs is mainly mediated by ligation of PRRs, which detect highly conserved PAMPs. Activation of PRRs triggers downstream signaling pathways that converge on nuclear translocation of different transcription factors like NF- κ B and IFN regulatory factors (IRFs) [2].

PAMPs associated with fungi such as β -glucans have long been recognized not only to be important for the induction of anti-fungal immunity, but to have antitumor activity [3]. Underlying mechanisms and molecular pathways were unclear but are beginning to emerge. β -glucans are high molecular weight polymers of glucose, consisting of a linear β -(1-3)-linked backbone and β -(1-6)-linked side chains that can be detected by the prototypical C-type lectin receptor (CLR) Dectin-1 [4]. Ligation of plasma membrane bound Dectin-1 on myeloid phagocytes like DCs by particulate β -glucans such as Curdlan leads to formation of the “phagocytic synapse” necessary to induce diversified downstream signaling [5, 6]. Recruitment of spleen tyrosine kinase (Syk) initiates the assembly of a multimolecular complex consisting of Card9, Bcl10, and Malt1, which triggers canonical activation of NF- κ B [7]. A later study identified an additional Dectin-1-mediated, Card9-independent pathway that leads to noncanonical NF- κ B activation via Syk, NF- κ B-inducing kinase (NIK) and IKK α [8]. Furthermore, Dectin-1/Syk/Card9 signaling has been shown to activate the NLRP3 inflammasome for caspase 1 dependent processing of bioactive IL-1 β in both mice [9] and human macrophages [10].

In addition to its direct antifungal activity through induction of phagocytosis and production of ROS in phagocytes, Dectin-1 ligation results in Syk/Card9-dependent activation of DCs with release of proinflammatory cytokines that promote the differentiation of murine T_H1 and T_H17 cells [11]. Furthermore, Dectin-1-mediated DC maturation can trigger potent expansion and differentiation of Ag-specific CTLs in mice [12]. Also in human DCs, Curdlan-mediated Dectin-1 activation has been shown to promote the release of a variety of proinflammatory cytokines, T_H1 and T_H17 differentiation as well as expansion and increased cytolytic activity of CTLs [13].

Overall, CLR-mediated interconnection of innate and adaptive immune activity is a cornerstone of immunity against numerous fungal pathogens including *Candida albicans*, *Pneumocystis carinii*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* [14]. In fact, polymorphisms in the gene encoding Dectin-1 [15] and mutations in Card9 [16] are tightly associated with defective fungal clearance in humans. As inducers of all arms of adaptive immunity, CLR ligands, including those for Dectin-1, are interesting candidates to enhance vaccine strategies and have been used successfully to trigger and improve Ag-specific T-cell responses [14]. However, signaling pathways downstream of Dectin-1/Syk that mediate CTL cross-priming and vaccine results yet remain undefined. Using genetically deficient mice and targeted depletion of effector cells, we show here that Card9 is a critical signaling hub for Dectin-1-mediated cross-priming of CTLs and that Card9-mediated CTL induction is essential for control of tumor growth and long-lasting

antitumor immunity after Dectin-1 ligand-based prophylactic vaccination.

Results and discussion

Dectin-1-mediated cross-priming of CD8⁺ T cells requires Card9 in DCs in vitro

To evaluate the molecular requirements for Dectin-1-mediated cross-priming of CD8⁺ CTLs, we cocultured BM-derived DCs with T cells derived from transgenic OT-I mice. These CD8⁺ T cells have a uniform T-cell receptor specific for the immune-dominant OVA peptide (SIINFEKL). As described previously [11], stimulation with the Dectin-1 agonist Curdlan resulted in rapid maturation of BM-derived DCs (BMDCs) with upregulation of the costimulatory molecule CD80, a process that critically depended on Card9 (Fig. 1A). Following addition of OVA protein, Curdlan-mediated activation of BMDCs induced Card9-dependent proliferation and potent cross-priming of cocultured Ag-specific T cells (1B and Supporting Information Fig. 1). CTL priming with robust IFN- γ release was mediated by DC-intrinsic Dectin-1 and downstream Card9 activation but did not require MyD88, a central adapter molecule for TLR signaling.

During *C. albicans* infection, Dectin-1 ligation can additionally result in the activation of the NLRP3 inflammasome and subsequent processing of pro-IL-1 β into its bioactive form [9]. Indeed, we observed that DCs that genetically lack the inflammasome adapter molecule apoptosis-associated speck-like protein containing a CARD (ASC) failed to produce IL-1 β in response to Curdlan-mediated Dectin-1 activation (Fig. 1C), but showed no defect in CTL cross-priming (Fig. 1D and E). These findings are in line with a previous study demonstrating that NLRP3-deficient mice showed regular CTL function following Curdlan-based vaccination in vivo [17]. Taken together, these data show that DC activation and subsequent cross-priming of CTLs following Dectin-1 activation by its specific ligand Curdlan is mediated via Card9 but is independent of MyD88 signaling or formation of the NLRP3 inflammasome.

Card9-dependent CTL cross-priming and antitumor immunity in vivo by Dectin-1-mediated vaccination

We next investigated the role of Card9 activity for cross-priming of CTLs in vivo. As described previously [12], s.c. protein vaccination with OVA and the Dectin-1 ligand Curdlan, but not OVA alone increased the frequency and cytolytic activity of MHC-I-SIINFEKL Tetramer⁺ CD8⁺ T cells in treated mice (Fig. 2A and B). Consistent with our in vitro findings, Dectin-1-induced, systemic expansion and activation of OVA-specific CTLs was largely abrogated in Card9-deficient mice. Compared to Card9-independent controls, both frequency and cytolytic activity of Curdlan-induced specific CTLs were inferior to those achieved by the TLR9 ligand CpG.

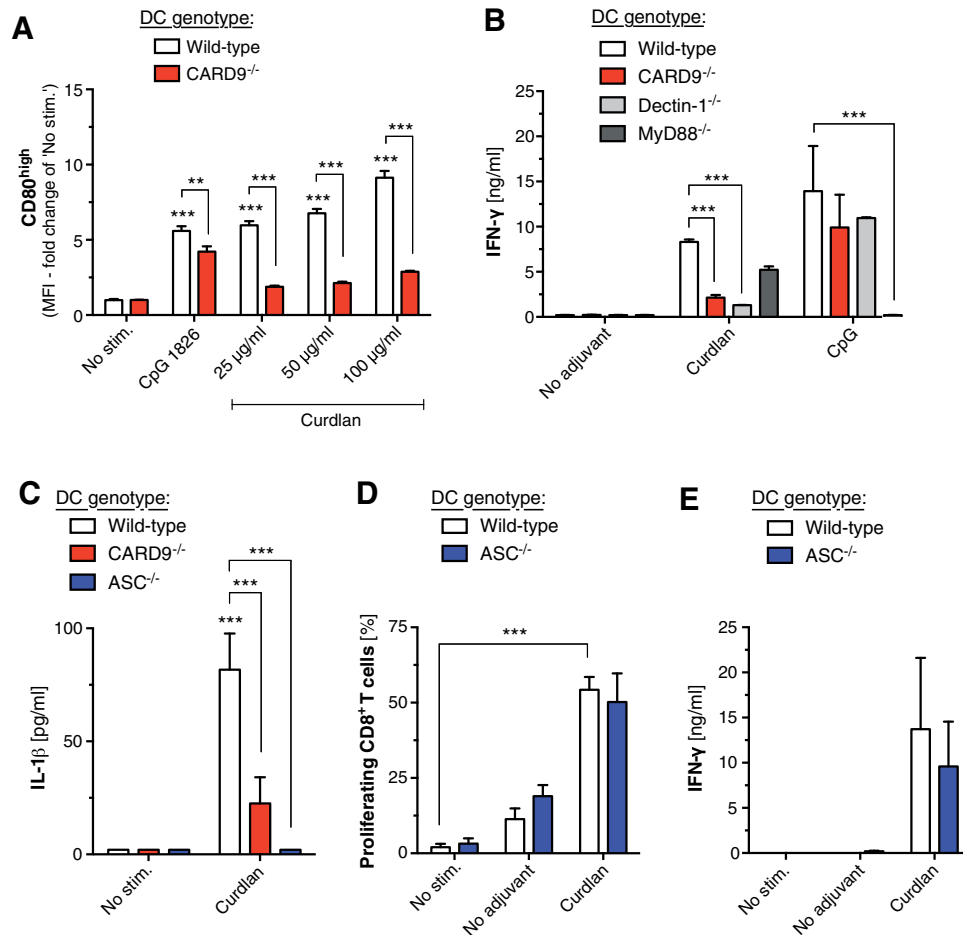


Figure 1. Dectin-1-mediated cross-priming of CD8⁺ T cells in vitro requires Card9 signaling in DCs. (A) BM-derived DCs (BMDCs) from WT and Card9-deficient (Card9^{-/-}) donor mice were stimulated with increasing concentrations of Curdlan or the TLR9 ligand CpG 1826. CD80 expression on BMDCs was analyzed by flow cytometry. An asterisk without brackets indicates comparison to the WT “No stim.” condition. (B) BMDCs from WT, Card9^{-/-}-deficient, Dectin-1-deficient (Dectin-1^{-/-}), and MyD88-deficient (MyD88^{-/-}) donor mice were stimulated as described above and were cocultured with magnetically purified, CFSE-labeled CD8⁺ OT-I T cells in the presence of OVA protein. IFN-γ release into the supernatant was analyzed by ELISA. (C–E) BMDCs from WT, Dectin-1^{-/-}, or ASC-deficient (ASC^{-/-}) mice were stimulated as described above and (C) IL-1β release was determined by ELISA. (D) After coculture with CD8⁺ OT-I T cells in the presence of OVA protein, T-cell proliferation was determined by flow cytometry and (E) IFN-γ release into the supernatant was analyzed by ELISA. (A, B, D, and E) Data are shown as mean ± SEM of at least triplicate samples and are representative of at least two independent experiments. Data in (C) give mean values ± SEM of *n* = 4 individual samples (CpG stimulation in WT and Card9^{-/-}, *n* = 7) that were pooled from four independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001; one-way ANOVA with Bonferroni posttests.

To investigate whether Card9-mediated cross-priming of CTLs would translate into antitumor immunity in a tumor prevention model, we immunized mice s.c. with a single dose of OVA protein either alone or mixed with different adjuvants. One week later, mice were challenged with OVA-expressing B16 melanoma cells injected into the contralateral flank (Fig. 3A). We observed that Curdlan-boosted vaccination resulted in significant tumor growth delay with complete rejection of OVA-expressing tumors in 50% of the treated animals (Fig. 3B and Supporting Information Fig. 2A). In contrast, no protection from melanoma growth was observed in mice immunized with OVA Ag alone. The development of Dectin-1- but not TLR9-induced antitumor immunity was completely dependent on Card9, as Card9-deficient recipient animals failed to control tumor growth following vaccination with Curdlan

(Fig. 3B). Only in WT but not Card9^{-/-} mice, Curdlan-induced antitumor immunity was associated with prolonged survival of most treated animals (Fig. 3C).

Despite relatively rare occurrence and low cytolytic activity of OVA-specific circulating CD8⁺ T cells in Curdlan-vaccinated WT mice (Fig. 2A and B), Dectin-1/Card9-induced antitumor immunity was exclusively mediated by CTLs as Ab-mediated CD8⁺ T cell but not NK cell depletion resulted in abrogation of Curdlan-based antitumor immunity (Fig. 3D and Supporting Information Fig. 3). Emphasizing the crucial role of Ag-specific CTLs for tumor control in our model, the frequency of circulating OVA-specific CTLs correlated with the efficacy of the antitumor response. Independent of genotype and treatment, high CTL numbers were associated with reduced tumor volume and prolonged survival (Supporting

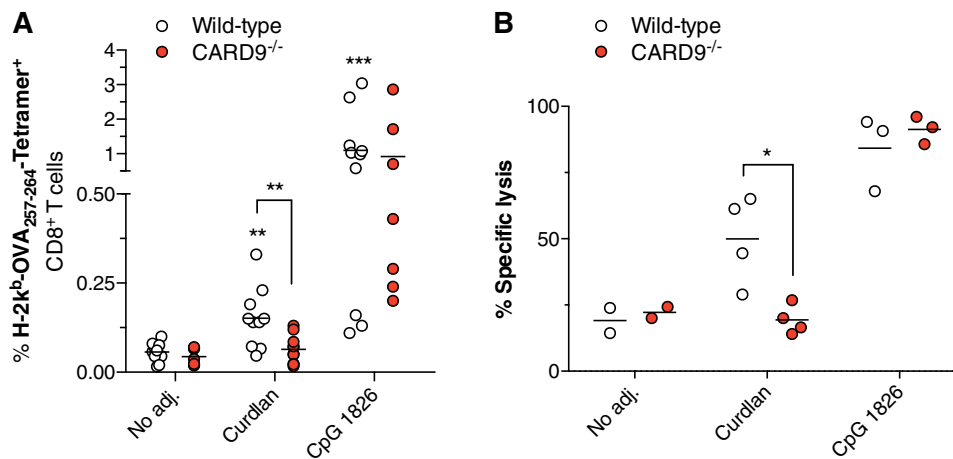


Figure 2. Robust cross-priming of cytotoxic T cells following Dectin-1 activation in vivo depends on Card9. WT and Card9^{-/-} mice were injected s.c. with OVA protein in combination with different adjuvants (Curdlan or CpG). (A) Frequency of H-2K^b-SIINFEKL Tetramer⁺ (OVA-specific) cytotoxic T cells in the peripheral blood was determined by flow cytometry. An asterisk without brackets indicates comparison to the WT “No adj.” group. (B) In vivo cytotoxic activity was measured by target cell elimination of fluorescently labeled, SIINFEKL peptide-pulsed syngenic splenocytes. The graph shows mean specific target cell lysis in the draining LN of individual mice. (A and B) Each circle represents a single replicate. Data are pooled from two independent experiments (A) or show representative data from one of two identical experiments (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Bonferroni posttests.

Information Fig. 2B and C). Four weeks after initial tumor induction, all surviving WT animals were rechallenged i.v. with OVA-expressing B16 melanoma cells. This resulted in rapid growth of macroscopically visible pseudometastases in the lungs of naïve, untreated WT mice (Fig. 3E). In contrast, recipient animals that had been vaccinated with protein in combination with either Curdlan or CpG 1826 and had thus rejected s.c. tumors were resistant to subsequent, systemic tumor rechallenge.

Taken together, our data identify Card9 as central regulator of Dectin-1-mediated cross-priming of CTLs and subsequent antitumor immunity. Long-lasting antitumor CTL responses critically depended on signaling through the Card9 pathway. We could exclude a dominant role of the inflammasome adapter ASC in Dectin-1-mediated CTL priming. This is in line with previous findings that cytolytic CTL function following a Curdlan-based vaccine was independent of the NLRP3 inflammasome in vivo [17]. Generally, type I IFNs (IFN- α and IFN- β) have been found to play a critical role in DC-mediated cross-priming of tumor-specific CTLs [18, 19]. We have previously shown that Dectin-1 and downstream signaling via SYK/Card9 and the transcription factor IRF5 can induce potent IFN- β production in DCs [20]. Future studies will need to evaluate the impact of this pathway on Dectin-1-mediated CTL cross-priming, especially in the context of antitumor immunity.

Dectin-1 seems an attractive target for tumor immunotherapy, since Dectin-1-mediated cross-priming pathways were shown to be active in human myeloid cells [13]. In mice, intratumoral application of Curdlan was found to reprogram infiltrating DCs to become resistant to cancer-derived thymic stromal lymphopoietin (TSLP), thus favoring the generation of beneficial Th1-based immune responses [21]. In contrast, during microbial infections, Dectin-1 activation induced TSLP release by DCs, thus promoting

Th2-biased immunity [22]. However, the importance of DC-intrinsic TSLP production in the context of tumor immunity remains to be determined. Furthermore, Curdlan-induced Dectin-1 activation can block the immunosuppressive function of myeloid-derived suppressor cells and thereby reduce tumor burden [23]. These studies suggest that therapeutic targeting of Dectin-1 may allow to coordinate several antitumoral immune mechanisms.

Robust Dectin-1-induced Ag-specific antitumor immunity was mediated by relatively low frequencies of CTLs. These findings and a previous report that Dectin-1 signaling in myeloid APCs can induce NK-cell-mediated tumor cell killing prompted the question whether NK or other immune cells may play a role here [24]. However, targeted depletion demonstrated that antitumor immunity induced by Curdlan-based vaccination entirely relied on CD8⁺ cells and was independent of NK cells in our model. However, the efficacy of Ag-specific CTL induction by vaccination with either Curdlan or CpG was variable (our own data and [24]) and the frequency of circulating specific CTLs clearly correlated with individual tumor volume reduction and survival increase. We therefore propose that combinatorial treatment strategies that target costimulatory molecules such as CD137 (4-1BB) [25] or antagonize immune checkpoints [26] may potentially synergize with agonists of the Dectin-1/Syk/Card9 pathway and help to exploit the full potential of Card9-related targeted antitumor immunity.

Concluding remarks

Overall, our data establish Card9 as a master regulator of Dectin-1-induced CTL priming and specific antitumor immunity. Card9 signaling allows to coordinate Dectin-1-induced CTL priming with a potent helper T-cell response into broad, specific T-cell

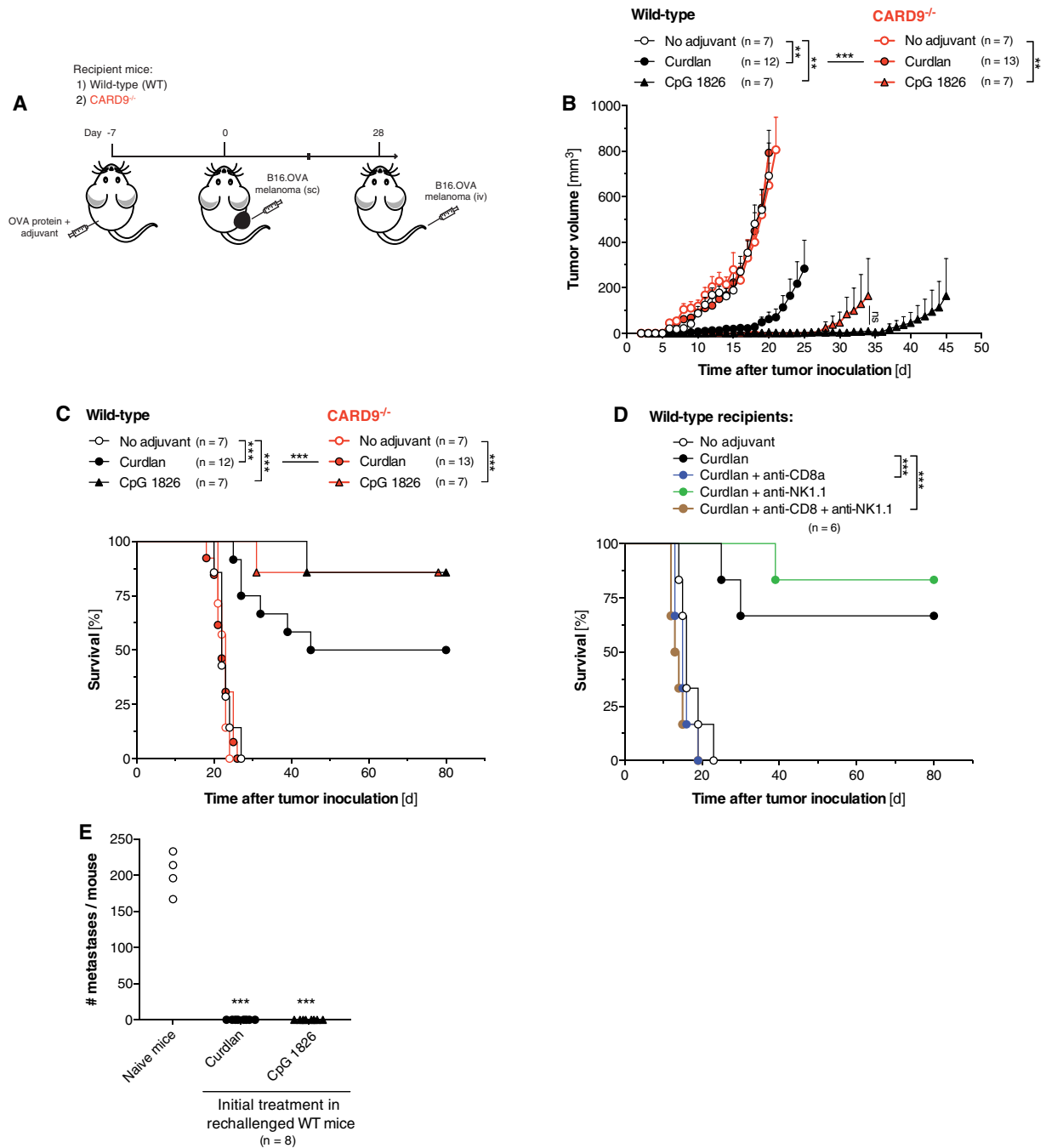


Figure 3. Curdlan-based vaccination results in potent $Card9$ -dependent antitumor immunity. (A) Treatment scheme: WT and $Card9^{-/-}$ mice were vaccinated s.c. with OVA in combination with either Curdlan or CpG. One week later, B16.OVA melanoma cells were injected into the contralateral flank. (B) Tumor growth of WT and $Card9^{-/-}$ recipient mice was monitored daily. Data show mean tumor growth \pm SEM from $n = 7$ –13 individual mice. Mean tumor growth analysis was discontinued when the first animal per group succumbed to tumor progression. Complete tumor growth curves of each individual mouse are shown in the Supporting Information Fig. 2A. Statistical significance was calculated using one-way ANOVA with Bonferroni posttests ($* p < 0.05$, $** p < 0.01$, $*** p < 0.001$) based on the tumor volume at day 20, if not stated otherwise. (C) Survival of tumor-bearing WT and $Card9^{-/-}$ mice. Survival was analyzed using the log-rank test ($*** p < 0.001$). (D) WT mice were treated as described above. Some mice ($n = 6$) were additionally injected with anti-CD8 α - and/or anti-NK1.1-depleting antibodies, beginning 2 days prior to Curdlan-based vaccination. $*** p < 0.001$; log-rank test. (E) Four weeks after initial tumor induction, long-term surviving animals were rechallenged by i.v. injection of B16.OVA cells. Pseudometastases in the lungs were counted on day 14. Treatment-naïve mice were used as controls for successful tumor induction. The graph shows mean number of pseudometastases of individual mice. Each circle represents a single replicate. Data are pooled from two independent experiments. $*** p < 0.001$; one-way ANOVA with Bonferroni posttests.

immunity. Combining Dectin-1-induced T-cell priming with recent immunomodulatory strategies such as checkpoint blockade seems a promising approach for future vaccine development to enhance cytotoxic immunity.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the guidelines of the German animal protection law (TierSchG). All procedures including housing, treatment, and sacrifice by cervical dislocation following isoflurane anesthesia were performed as authorized by and were reported to the responsible state office Regierung von Oberbayern (Munich, Germany) per standard legal procedure (references 55.2.1.54-2532-26-11 and 55.2-1-54-2532-111-14).

Mice

Female C57BL/6 mice were purchased from Harlan–Winkelmann and Janvier. Card9- (Card9^{-/-}) and ASC-deficient (ASC^{-/-}) mice have been described previously [7, 27]. All mice were at least 6 wk of age at the onset of experiments.

Media, reagents, and cell lines

RPMI-1640 medium (Invitrogen) and DMEM (Invitrogen) were supplemented with 10% v/v FCS (Hyclone), 3 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (all from Sigma-Aldrich). CpG 1826 and Curdlan were purchased from Invivogen and Wako Chemicals (Richmond, USA). The B16-F10 murine melanoma cell line expressing full length chicken OVA (here referred to as B16.OVA) was cultured in complete DMEM medium supplemented with 400 µg/mL G418 (from Sigma-Aldrich). CFSE and Celltracker Violet were purchased from Invitrogen.

Generation of BM-derived DCs and cell culture

BM cells were harvested from murine femur and tibia and erythrocytes were lysed with ammonium chloride buffer (BD Biosciences, Heidelberg, Germany). BMDCs were generated by culturing BM cells in complete RPMI medium supplemented with 20 ng/mL GM-CSF (from Immunotools, Friesoythe, Germany). On days 6 to 7, cells were harvested. CD8⁺ T cells from OT-I splenocytes were purified with magnetic beads according to the manufacturer's protocol (Miltenyi Biotech, Bergisch Gladbach). For in vitro stimulation assays, cells were cultured in complete RPMI. DCs were stimulated with Curdlan (50 µg/mL), CpG 1826 (0.075 µM), or Pam3CSK4 (1 µg/mL), if not stated otherwise. For coculture experiments,

10⁵ purified, CFSE-labeled CD8⁺ OT-I T cells were cultured in the presence of 25 000 stimulated DCs. After 48 h, T-cell proliferation and IFN-γ were analyzed.

Quantification of cytokines

Cell supernatants were analyzed for cytokine secretion by ELISA (BD, R&D Systems, or eBioscience) according to the manufacturer's protocol.

Flow cytometry

Cell suspensions were stained in PBS with 1% FCS. Fluorochrome-coupled antibodies were purchased from eBioscience or BioLegend. The Fixable Viability Dye eFluor 506 (eBioscience) was used to stain and exclude dead cells. The iTAg MHC-I murine tetramers detecting SIINFEKL-specific CD8⁺ T cells were from MBL (Woburn, MA). For intracellular cytokine staining the Foxp3 Transcription Factor Fixation/Permeabilization Kit (eBioscience) was used. Data were acquired on a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (TreeStar). Cells were gated as follows: DCs: singlets > Viability Dye⁻ > CD11c⁺ > CD86 (MFI). T cells: singlets > Viability Dye⁻ > CD3⁺ > CD8⁺ > CFSE^{low} (proliferating) + IFN-γ⁺.

In vivo cytotoxicity assay

In vivo cytotoxicity was evaluated 4 days after the final treatment as described previously [12]. In brief, splenocytes from naïve syngenic donor mice were pulsed with SIINFEKL peptide at different concentrations (0 and 200 nM) for 30–45 min at 37°C. These two populations were then stained with different concentrations of Celltracker violet (CTv, 0.1 and 5 µM), washed, mixed in a 1:1 ratio, and were injected i.v. The next day, animals were sacrificed and the target cell frequency in the LN draining the initial vaccination site was determined by flow cytometry as follows: singlets > Viability Dye⁻ > CTv^{low} versus CTv^{high}. Specific killing was calculated using the formula $100 - (1 - \%_{CTv\ peptide} / \%_{CTv\ no\ peptide})$ as described [12].

Tumor challenge and treatment

WT or Card9^{-/-} mice were injected s.c. in the right flank with 100 µg OVA together with 25 µg CpG 1826 or 100 µg Curdlan. One week later, the frequency of Ag-specific CD8⁺ T cells in the peripheral blood was analyzed with SIINFEKL-H2Kb tetramers. Subsequently, mice were injected s.c. with 1×10^5 B16.OVA cells in the left flank and tumor growth was monitored continuously. According to standard legal procedure (responsible state office Regierung von Oberbayern), mice were euthanized when the maximum tumor diameter exceeded 15 mm.

Treatment with anti-CD8 α (clone 2.43) or anti-NK1.1 (clone PK136, both from BioXCell) depleting antibodies was initiated 2 days prior to vaccination (100 μ g i.p.) and was repeated twice weekly (50 μ g i.p.). For rechallenge experiments, mice were injected i.v. with 5×10^5 B16.OVA cells on day 28 after initial tumor induction. Fourteen days later, mice were sacrificed and macroscopically visible, superficial pulmonary pseudometastases were counted.

Statistics

All data are presented as mean + SEM if not stated otherwise. For multiple statistical comparison of a data set, the one-way ANOVA test with Bonferroni posttest was used. Survival was analyzed using the log-rank test. Significance was set at p -values $p < 0.05$, $p < 0.01$, and $p < 0.001$ and was then indicated with asterisks (*, **, and ***). All statistical calculations were performed using Graphpad Prism (GraphPad Software, San Diego, USA).

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T.H., S.H., H.P., and J.R. designed the research, analyzed and interpreted the results. T.H., S.H., A.W. M.B, M.S., and S.B. did the experiments. S.H. and T.H. prepared the manuscript. C.P., J.C.F., G.E., and S.S. gave methodological support and conceptual advice. T.H., H.P., and J.R. guided the study.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: BMDCs: BM-derived DCs · CLR: C-type lectin receptor · CTL: cytotoxic T cell · IRF: IFN regulatory factor · MHC-I: MHC class I · Syk: Spleen tyrosine kinase · TSLP: thymic stromal lymphopoietin

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