Dectin 1 activation on macrophages by galectin 9 promotes pancreatic carcinoma and peritumoral immune tolerance

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The progression of pancreatic oncogenesis requires immune-suppressive inflammation in cooperation with oncogenic mutations. However, the drivers of intratumoral immune tolerance are uncertain. Dectin 1 is an innate immune receptor crucial for anti-fungal immunity, but its role in sterile inflammation and oncogenesis has not been well defined. Furthermore, non-pathogen-derived ligands for dectin 1 have not been characterized. We found that dectin 1 is highly expressed on macrophages in pancreatic ductal adenocarcinoma (PDA). Dectin 1 ligation accelerated the progression of PDA in mice, whereas deletion of Clec7a—the gene encoding dectin 1—or blockade of dectin 1 downstream signaling was protective. We found that dectin 1 can ligate the lectin galectin 9 in mouse and human PDA, which results in tolerogenic macrophage programming and adaptive immune suppression. Upon disruption of the dectin 1–galectin 9 axis, CD4+ and CD8+ T cells, which are dispensable for PDA progression in hosts with an intact signaling axis, become reprogrammed into indispensable mediators of anti-tumor immunity. These data suggest that targeting dectin 1 signaling is an attractive strategy for developing an immunotherapy for PDA.

PDA is a devastating disease with few long-term survivors. Inflammation is paramount in PDA progression; oncogenic mutations alone, in the absence of concomitant inflammation, are insufficient for tumorigenesis. Innate and adaptive immunity cooperate to promote tumor progression in PDA. In particular, specific innate immune subsets in the tumor microenvironment (TME) are apt at educating adaptive immune effector cells toward a tumor-permissive phenotype. Some antigen-presenting cell (APC) populations, including M2-polarized tumor-associated macrophages (TAMs) and myeloid dendritic cells (DCs), induce the generation of immune-suppressive type-2 helper T (Th2) cells rather than proinflammatory type-1 helper T (Th1) cells3,4. Similarly, we and others have shown that myeloid-derived suppressor cells (MDSCs) negate anti-tumor CD8+ cytotoxic T lymphocyte (CTL) responses in PDA and promote metastatic progression5–7. However, the drivers of monocytic cellular differentiation toward an immune-suppressive phenotype remain uncertain.

Dectin 1, encoded by CLEC7A, is a member of the C-type lectin family of pattern-recognition receptors and is expressed on the surface of macrophages and other cells of the myeloid-monocytic lineage. Dectin 1 is a crucial component of the innate immune system’s ability to recognize β-glucan polysaccharides derived from fungal cell walls. Ligation of dectin 1 by β-glucans phosphorylates spleen tyrosine kinase (Syk), recruits the CARD9 adaptor protein, and thereby initiates an anti-fungal immune response10,11. Our recent work suggested that chronic inflammatory injury upregulates dectin 1 expression in the liver12. Furthermore, we found that the PDA TME is rife with damage-associated molecular patterns (DAMPs), generated as by-products of inflammation and necrotic cell death3,13. However, unlike Toll-like receptors, which influence oncogenic progression when ligated by DAMPs3,14,15, the role of dectin 1 in non-pathogen-mediated inflammation or oncogenesis is not well defined, and sterile dectin 1 ligands have not been characterized. We discovered high dectin 1 expression and the presence of novel dectin 1 agonists within the PDA TME. We postulated that dectin 1 ligation in macrophages drives their immune-suppressive cellular differentiation in PDA and thereby governs the tolerogenic T cell program in the TME, which facilitates oncogenic progression.

RESULTS
High dectin 1 expression in mouse and human PDA
To test the relevance of dectin 1 signaling to PDA, we examined dectin 1 expression in two mouse models of PDA: a slowly progressive model, Ptf1aCre;Lox-Stop-Lox (LSL)-KrasG12D (KC), in which mice express oncogenic Kras in their pancreatic progenitor cells, and a more aggressive, orthotopic model of PDA using tumor cells
from Pdx1Cre;LSL-KrasG12D;Trp53R172H (KPC) mice, which express mutated Kras and Trp53. We also examined its relevance in human PDA. Immunohistochemical (IHC) analysis indicated high dectin 1 expression in leukocytes from KC mice pancreata and in transformed epithelial cells (Fig. 1a and Supplementary Fig. 1a,b). Pancreata from KC mice crossed with decid-1 double-negative (KC;Clec7a−/−) animals served as controls. Flow cytometry revealed ~2-fold higher dectin 1 expression in macrophages (46%), neutrophils and inflammatory monocytes (80%) and DCs (65%) within the KC TME as compared to their cellular counterparts in spleen (18%, 47% and 40%, respectively) (Fig. 1b). Similarly, in orthotopically implanted KPC tumors, decid-1 was highly expressed on leukocytes and in malignant epithelial cells (Fig. 1c and Supplementary Fig. 1c–e). Dectin 1 was also expressed at higher levels in leukocytes in PDA (44%) as compared to those in normal pancreas (9%) (Fig. 1d). Immunofluorescence microscopy of human PDA similarly indicated high dectin 1 expression in transformed epithelial cells and in tumor-infiltrating myeloid cells (Supplementary Fig. 1f). In parallel to those in mice, human PDA-infiltrating CD14+ and CD15+ monocytes and macrophages and CD11c+ DCs expressed higher levels of dectin 1 as compared to their cellular counterparts in peripheral blood mononuclear cells (PBMCs) (Fig. 1e).

To investigate the stimulus for the upregulation of dectin 1 expression in leukocytes in PDA, we co-cultured bone marrow–derived macrophages (BMDMs) with pancreatic tumor cells derived from KC mice. PDA cells and PDA cellular supernatant upregulated dectin 1 expression in BMDMs; however, select cytokines associated with PDA did not influence dectin 1 expression (Supplementary Fig. 1h). Collectively, these results suggest that dectin 1 expression is upregulated in the tumor and peritumoral inflammatory compartments in PDA.

Evidence of dectin 1 signaling and the presence of dectin 1 ligands in PDA

To investigate the relevance of dectin 1 signaling in pancreatic oncogenesis, we assayed 3-month-old and 6-month-old WT and KC mouse pancreata for the presence of activated signaling intermediates downstream of dectin 1 ligation. We found that, when compared to age-matched WT pancreata, KC pancreata expressed elevated levels of p-Syk and p-phospholipase C (PLC)-γ as well as high CARD9 expression and evidence of robust c-Jun N-terminal kinase (JNK) pathway activation (Supplementary Fig. 2a). IHC analysis confirmed the presence of high p-Syk expression in KC pancrea, whereas Syk signaling was reduced in PDA in the context of decid 1 deletion (Supplementary Fig. 2b). Similarly, flow cytometry showed elevated p-Syk expression in diverse myeloid cellular subsets in PDA, as compared to their counterparts in the spleen (Supplementary Fig. 2c).

To investigate whether decid-1 ligands were present in the pancreatic TME, we employed a human IgG Fc-conjugated decid-1 fusion protein. Whereas decid-1 ligands were absent in WT pancreata, we identified high levels of decid-1 ligands in the pancreata of KC mice, as measured by western blotting (Supplementary Fig. 2a) and immunofluorescence microscopy (Supplementary Fig. 2d). Flow cytometric analysis confirmed the expression of decid-1 ligands on tumor-infiltrating macrophages and DC in KC pancrea as compared to no expression in leukocytes in spleen (Supplementary Fig. 2e). The expression of decid-1 ligands was similarly elevated in APCs infiltrating orthotopic KPC tumors (Supplementary Fig. 2f). Furthermore, CD133+–transformed epithelial cells in KC tumors (Supplementary Fig. 2g) and KPC tumors (Supplementary Fig. 2h) expressed decid-1 ligands in vivo, as did KPC-derived tumor cells grown in culture (Supplementary Fig. 2l). Collectively, our data indicate high expression of the decid-1 receptor and decid-1 ligands in the epithelial and inflammatory compartments of PDA, along with upregulation of associated signaling intermediates.

Dectin 1 ligation accelerates pancreatic oncogenesis

Given that decid 1 and its cognate ligands are highly expressed in PDA, we postulated that decid 1 signaling might promote immune-suppressive inflammation, leading to accelerated tumorigenesis. To test this, we treated 6-week-old KC mice serially with the decid-1–specific agonists dezymosan (d-zymosan) or heat-killed Candida albicans (HKCA) and assessed tumor progression 8 weeks later by comparison to vehicle-treated animals. Ligation of decid 1 vigorously accelerated tumorigenesis (Fig. 1f–i). Whereas pancreata in vehicle-treated KC mice harbored large areas of residually normal acinar architecture, mice treated with decid 1 agonists exhibited near-complete effacement of their pancreatic acini with more advanced pancreatic intra-epithelial neoplasia (PanIN) lesions and numerous foci of invasive carcinoma embedded in dense fibroinflammatory stroma (Fig. 1f–i). Kras–transformed ductal epithelial cells in decid-1–agonist-treated KC mice also exhibited elevated proliferative rates, as measured by Ki67 staining (Supplementary Fig. 3a). Similarly, the administration of decid 1 agonists in vivo accelerated tumor growth in orthotypically implanted, KPC-derived tumors (Fig. 1i). These data suggest that decid 1 signaling promotes the progression of PDA.

Clec7a deletion is protective against PDA

To determine whether decid 1 signaling is required for the normal progression of pancreatic oncogenesis, we examined the tumor-phenotype in KC;Clec7a−/− mice over time. Dectin 1 deletion delayed malignant progression and stromal expansion. As compared to KC controls, age-matched KC;Clec7a−/− pancreata exhibited delayed development of pancreatic dysplasia and fibrosis (Fig. 2a and Supplementary Fig. 3b) and extended survival (Fig. 2b). To determine whether decid-1 deletion influences molecular oncogenesis, we probed pancreata from KC and KC;Clec7a−/− mice for select cell-cycle regulatory, oncogenic and tumor-suppressor genes. KC; Clec7a−/− pancreata exhibited higher expression of the tumor-modulatory genes encoding Bcl-xL, Rb, Smad4 and p16 but reduced p53 and c-Myc expression, which is indicative of a distinct oncogenic phenotype (Fig. 2c). Collectively, these data imply that decid 1 contributes to the normal progression of pancreatic neoplasia in the context of a driving Kras mutation.

Syk inhibition is protective against PDA

Given that decid 1 signals through Syk phosphorylation, and, as we showed, Syk activation is reduced in KC;Clec7a−/− pancreata, we postulated that Syk blockade would be protective against pancreatic oncogenesis. KC mice were treated from 6–14 weeks of life with piceatannol, a p-Syk inhibitor, and tested for tumor progression through comparison with vehicle-treated controls. We confirmed that piceatannol prevented Syk activation in vivo in PDA. Syk inhibition reduced pancreatic tumor weights and mitigated dysplastic changes in KC mice, but was not protective in KC;Clec7a−/− mice (Fig. 2d,e), which suggests that the blockade of signaling pathways downstream of decid 1 might be an attractive therapeutic strategy in pancreatic oncogenesis.

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High dectin 1 expression in mouse and human PDA and dectin 1 ligation accelerates PDA progression. (a) Frozen sections of 6-month-old KC;Clec7a+/+ and KC;Clec7a−/− pancreata were tested for the expression of dectin 1 by IHC. Scale bar, 100 µm. (b) PDA-infiltrating and splenic leukocytes from KC and aged-matched WT mice were tested for the expression of dectin 1 in CD11c−Gr1−CD11b+F4/80+ macrophages, Gr1+CD11b+ neutrophils and inflammatory monocytes and CD11c+MHCII+ dendritic cells. Representative contour plots and quantitative data from five patients with PDA. (c–i) 6-week-old KC mice were treated with the dectin 1 ligands d-zymosan, HKCA or PBS for 8 weeks before euthanization (n = 5/group). (f) Representative H&E-stained (scale bar, 100 µm) and Trichrome-stained (scale bar, 200 µm) sections. (g) The percentage of ducts exhibiting normal morphology, acinoductal metaplasia (ADM), graded PanIN lesions or foci of invasive cancer are shown. (h) The percentage of pancreatic area occupied by fibrotic tissues was calculated on the basis of trichrome staining. (i) WT mice were given orthotopic KPC-derived tumor cells and serially treated with d-zymosan, HKCA or vehicle control. Animals were killed at 3 weeks of age and pancreas weights measured. Representative gross images of pancreatic tumors and quantitative data are shown (n = 5/group; scale bar, 1 cm; data are means ± s.e.m.; "P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; n.s., not significant, by unpaired Student’s t-test).
Dectin 1 does not have direct protumorigenic effects on transformed pancreatic ductal epithelial cells

To determine whether dectin 1 ligation has direct mitogenic or activating effects on transformed pancreatic epithelial cells, we treated KPC-derived tumor cells in vitro with the dectin 1 agonist d-zymosan. Dectin 1 ligation failed to induce proliferation or cytokine production in PDA tumor cells (Supplementary Fig. 4a–c). HKCA similarly failed to induce proliferation or cytokine production in KPC cells (data not shown). To further test whether dectin 1 has direct oncogetic or proinflammatory effects in PDA cells, we silenced dectin 1 expression in KPC-derived tumor cells using shRNA (Supplementary Fig. 4d). However, knockdown of dectin 1 did not alter the growth rate of tumor cells in vitro (Supplementary Fig. 4e), which suggests that dectin 1 signaling in the transformed epithelial compartment is not crucial for the modulation of PDA. Similarly, transformed pancreatic ductal epithelial cells (PDECs) harvested from KC and KC;Clec7a–/– pancreata proliferated at equal rates in vitro (Supplementary Fig. 4f) and in vivo (Supplementary Fig. 4g).

Clec7a deletion in the extra-epithelial compartment alone is protective against oncogenesis

Given that dectin 1 ligation or knockdown does not influence the proliferative capacity of transformed pancreatic epithelial cells, we postulated that dectin 1 deletion in the extra-epithelial compartment alone would be protective against PDA. To investigate this, WT and Clec7a–/– mice were challenged with orthotopic injections of KPC-derived tumor cells with intact dectin 1 expression. Pancreatic tumors harvested at 3 weeks were markedly smaller in Clec7a–/– hosts,
which suggests that Clec7a deletion in the extra-tumoral compartment alone is protective against PDA (Supplementary Fig. 4h). Clec7a<sup>−/−</sup> mice also exhibited extended survival after orthotopic PDA tumor implantation, as compared to WT mice (Supplementary Fig. 4i). Similarly, KC mice made chimeric using Clec7a<sup>−/−</sup> bone marrow were protected against oncogenesis when compared with KC mice made chimeric using WT bone marrow, which confirms that the deletion of dectin 1 in leukocytes alone is protective (Supplementary Fig. 4j). Dectin 1 expression was not associated with adverse survival in the context of human PDA (Supplementary Fig. 4k); however, dectin 1 was a surrogate for total myeloid cell infiltration (Supplementary Fig. 4l).

Dectin 1 deletion induces immunogenic reprogramming of tumor-infiltrating macrophages

We speculated that the blockade of dectin 1 signaling in the stroma leads to protection against PDA by bolstering anti-tumor immunity. Specifically, we postulated that dectin 1 deletion leads to immunogenic reprogramming of macrophages, which results in reversal of the immune-suppressive phenotype of PDA-infiltrating CD11b<sup>+</sup> cells. To test this in vitro, we stimulated naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells using CD3/CD28 co-stimulation and measured the expression of inducible T cell costimulator (ICOS), CD44, IFN-γ and TNF-α, all of which were upregulated on activated T cells, and the expression of CD62L and IL-10, both of which were downregulated upon T cell activation. Clec7a<sup>+/+</sup> or Clec7a<sup>−/−</sup> CD11b<sup>+</sup> cells harvested from KPC-derived tumors were added to select wells. Whereas tumor-infiltrating WT myeloid cells abrogated the upregulation of ICOS expression in α-CD3/CD28-activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, PDA-infiltrating Clec7a<sup>−/−</sup> myeloid cells exhibited minimal inhibitory effects (Fig. 3a,b). Similarly, whereas WT CD11b<sup>+</sup> cells prevented CD4<sup>+</sup> and CD8<sup>+</sup> T cell adhesion of a CD44<sup>+</sup>CD62L<sup>+</sup> effector-memory phenotype in response to CD3/CD28 ligation, Clec7a<sup>−/−</sup> cells were noninhibitory (Fig. 3c,d). Moreover, in contrast to tumor-infiltrating WT CD11b<sup>+</sup> cells, which promoted IL-10 production from CD4<sup>+</sup> T cells (Fig. 3e) and negated IFN-γ and TNF-α expression in CD8<sup>+</sup> T cells (Fig. 3f), Clec7a<sup>−/−</sup> cells only minimally induced IL-10 production in CD4<sup>+</sup> T cells and were permissive of CD8<sup>+</sup> cytotoxic T cell activation (Fig. 3e,f). Similar differential effects on CD4<sup>+</sup> and CD8<sup>+</sup> T cell inhibition were observed when we used purified Gr1<sup>−/−</sup>CD11c<sup>−/−</sup>F4/80<sup>+</sup> PDA-associated macrophages (TAMs) harvested from WT hosts and compared them to TAMs harvested from Clec7a<sup>−/−</sup> hosts (Supplementary Fig. 5a,b). Conversely, the T cell inhibitory capacity of Gr1<sup>−/−</sup>CD11b<sup>−</sup> neutrophils and inflammatory monocytes was not diminished in the context of dectin 1 deletion, which suggests that whereas dectin 1 signaling in TAMs affects their capacity to influence T cell differentiation, dectin 1 signaling in neutrophils and inflammatory monocytes does not affect their capacity to suppress T cell function (Supplementary Fig. 5c).

To investigate whether dectin 1 signaling promotes macrophage-mediated immune-suppression in situ in PDA, we assessed macrophage recruitment and phenotype in both our slowly progressive (KC) and invasive (KPC) models of PDA in the contexts of either dectin 1 deletion or dectin 1 activation. KC:Clec7a<sup>−/−</sup> mice exhibited significantly reduced pancreatic infiltration with F4/80<sup>+</sup> and Arg1<sup>+</sup> TAMs on IHC analysis (Fig. 4a,b). Flow cytometry confirmed a ~50% reduction in the fraction of TAMs in KC:Clec7a<sup>−/−</sup> pancreata (Fig. 4c). We found a similar decrease in the fraction of TAMs in orthotopic KPC tumors in Clec7a<sup>−/−</sup> hosts as compared to the WT (Fig. 4d). Accordingly, analysis of orthotopic KPC tumors using a PCR array suggested reduced expression of diverse inflammatory mediators in tumors in Clec7a<sup>−/−</sup> hosts as compared to WT (Supplementary Fig. 5d). Moreover, cellular-phenotyping experiments indicated that dectin 1 deletion induced immunogenic reprogramming of TAMs toward M1-like differentiation. Specifically, TAMs infiltrating Clec7a<sup>−/−</sup> pancreata expressed elevated major histocompatibility complex (MHC) II, reduced levels of CD206 and higher levels of TNF-α and iNOS than Clec7a<sup>+/+</sup> hosts, which is suggestive of M1-like programming (Fig. 4e–g). By contrast, the prevalence and immune phenotype of...
Experiments were repeated more than three times with similar results. (l) Dectin 1 activation was confirmed by the upregulation of p-Syk expression in PDA-infiltrating macrophages in d-zymosan-treated, as compared to vehicle-treated, mice. (l) The fraction of tumor-infiltrating macrophages was determined by flow cytometry on day 21. (a) WT and Clec7a−/− mice were implanted with orthotopic KPC tumors and serially treated with d-zymosan or vehicle. (b) WT and Clec7a−/− mice were challenged with orthotopic KPC-derived PDA tumors: CD11c−Gr1−CD11b+F4/80+ macrophage infiltration was determined on day 21 by flow cytometry (n=5/group). Scale bar, 100 μm. (c) The fraction of CD11c−Gr1−CD11b+F4/80+ macrophages in the pancreata of 3-month-old KC;Clec7a+/+ and KC;Clec7a−/− mice was determined by flow cytometry (n=5/group). (d–g) WT and Clec7a−/− mice were challenged with orthotopic KPC-derived PDA tumors: CD11c−Gr1−CD11b+F4/80+ macrophage infiltration was determined on day 21 by flow cytometry (d), and PDA-infiltrating macrophages were gated and tested for the expression of MHC II (e), CD206 (f), TNF-α and iNOS (g) (n=5/group). (h–k) WT mice were implanted with orthotopic KPC tumors and serially treated with d-zymosan or vehicle. (h) Dectin 1 activation was confirmed by the upregulation of p-Syk expression in PDA-infiltrating macrophages in d-zymosan-treated, as compared to vehicle-treated, mice. (i) The fraction of tumor-infiltrating macrophages was determined by flow cytometry on day 21. (j,k) PDA-infiltrating macrophages were gated and tested for the expression of CD206 (j) and MHC II (k). Representative contour plots and quantitative data are shown (n=5 mice/group). Experiments were repeated more than three times with similar results. (l) Clec7a−/− mice were subcutaneously implanted with KPC-derived PDA tumor cells admixed with WT or Clec7a−/− macrophages. Tumor volume was recorded at serial intervals. This experiment was repeated twice with similar results (n=4/group; data are mean ± s.e.m.; *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant by unpaired Student’s t-test).
CD11c^+MHCII^+ DCs were similar in both WT and Clec7a^-/- PDA tumors (Supplementary Fig. 5e,f).

We confirmed that exogenous dectin 1 ligand administration using d-zymosan activates Syk signaling in the PDA TME (Fig. 4h). Accordingly, dectin 1 ligation in vivo increased the fraction of TAMs in orthotopic KPC tumors (Fig. 4i) and upregulated CD206 expression (Fig. 4j); however, MHC II expression was not significantly altered (Fig. 4k). Similar effects on macrophage programming were seen after treatment in vivo with HKCA (data not shown). Moreover, adoptive transfer of WT macrophages coincident with PDA tumor challenge in Clec7a^-/- hosts resulted in an accelerated tumor growth rate as compared to adoptive transfer of Clec7a^-/- macrophages (Fig. 4i).

Dectin 1 signaling suppresses T cell immunogenicity in a mouse model of PDA

On the basis of macrophage phenotyping and adoptive-transfer experiments, as well as our co-culture data from in vitro experiments, we postulated that the reprogramming of TAMs resulting from dectin 1 deletion in PDA leads to enhanced immunogenicity in tumor-infiltrated T cells. To test this, we interrogated the T cell phenotype in pancreas-draining lymph nodes in KC and KC;Clec7a^-/- mice, as well as in orthotopic KPC tumors in WT and Clec7a^-/- hosts. Consistent with our hypothesis, dectin 1 deletion in KC mice led to immunogenic reprogramming of tumor-draining CD4^+ and CD8^- T cells, which exhibited upregulated expression of CD44, OX40 and PD-1, indicative of cellular activation (Fig. 5a-c). Dectin 1 deletion also increased the CD8:CD4 ratio in tumor-draining lymph nodes (Fig. 5d). Similarly, dectin 1 deletion increased the CD8:CD4 ratio in tumor-infiltrating T cells in orthotopic KPC tumors (Fig. 5e). Furthermore, CD8^- T cells in KPC tumors in Clec7a^-/- hosts exhibited an activated phenotype with high expression of PD-1, TBX21 (T-bet), TNF^-alpha, CD107a, and granzyme B, which points to enhanced CD8^- T cell cytotoxic potential, as compared to that in orthotopic KPC tumors in WT hosts (Fig. 5f).

Accordingly, orthotopic administration of KC mice engineered to express ovalbumin (OVA) resulted in a markedly higher fraction of tumor-infiltrating, OVA-pentamer-positive cytotoxic T cells in Clec7a^-/- hosts as compared to WT (Supplementary Fig. 5g,h). Similarly, PDA-infiltrating CD4^- T cells in Clec7a^-/- hosts expressed higher levels of CD44, CD107a and ICOS and exhibited enhanced T\(_{H1}\) polarization, as evidenced by upregulated expression of T-bet and TNF^-alpha (Fig. 5g). Collectively, these data indicate enhanced T cell immunogenicity. Notably, in agreement with higher T cell activation and PD-1 expression detected in PDA tumors in Clec7a^-/- mice, dectin 1 deletion in combination with PD-1 blockade tended to offer synergetic protection and further enhanced intratumoral T\(_{H1}\) polarization, whereas PD-1 blockade had no efficacy in the absence of dectin 1 deletion (Fig. 5h,i).

We postulated that exogenous dectin 1 ligation would induce an immune-suppressive T\(_{H2}\) phenotype and reduce CD8^- T cell activation. Accordingly, CD4^- T cells harvested from d-zymosan-treated, KPC-tumor-bearing mice exhibited reduced T-bet and TNF^-alpha expression but higher IL-5, IL-10 and IL-13 expression, as compared to vehicle-treated, tumor-bearing mice (Supplementary Fig. 6a). Similarly, CD8^- T cells exhibited diminished T-bet and TNF^-alpha expression after dectin 1 ligation (Supplementary Fig. 6b). Furthermore, macrophage depletion in vivo activated PDA-infiltrating CD4^- and CD8^- T cells exclusively in WT hosts, and not in Clec7a^-/- hosts, which suggests that dectin-1-expressing macrophages drive T cell suppression in PDA (Supplementary Fig. 6c,d).

To test definitively whether tumor protection in the absence of dectin 1 signaling is contingent on immunogenic T cell reprogramming, we depleted T cells coincident with orthotopic KPC tumor administration in cohorts of WT and Clec7a^-/- animals. Pan^-T cell depletion did not affect PDA growth in WT mice; however, tumor protection was abrogated in Clec7a^-/- cohorts (Supplementary Fig. 6e). Similarly, CD4^- and CD8^- T cell depletion alone each reversed tumor protection in Clec7a^-/- mice (Supplementary Fig. 6f,g), but not in WT mice (data not shown). These data suggest that in PDA-bearing WT hosts, T cells are dispensable to outcome; conversely, in the absence of dectin 1 signaling, T cells are reprogrammed into indispensable mediators of tumor protection.

Galectin 9 ligates dectin 1 in PDA

Non-pathogen-derived dectin 1 ligands have not been well characterized. Therefore, we performed affinity purification–mass spectrometry, using the IgG Fc-conjugated dectin 1 fusion protein coupled to protein-G beads, to purify putative ligand(s) in KPC tumor extracts. The proteins co-purified with the dectin 1 fusion protein were contrasted with proteins purified with protein-G beads alone. Affinity purification coupled with mass spectrometry experiments were repeated twice, and only proteins that uniquely co-purified with the IgG Fc-conjugated dectin 1 fusion protein were considered as possible candidate dectin 1 ligands. A total of 19 proteins were identified.

Among the co-purified proteins was galectin 9 (Supplementary Table 1). Given that galectin 9 is a member of the β-galactoside-binding family of lectins, we hypothesized that galectin 9 is a sterile ligand for dectin 1. We assayed for the presence of galectin 9 in the mouse PDA TME and found robust expression of galectin 9 in diverse PDA-infiltrating myeloid cells and in cancer cells by flow cytometry (Fig. 6a,b). We also found modest expression of galectin 9 in both leukocytes (13%) and tumor cells (7%) in human PDA, whereas galectin 9 was minimally expressed in leukocytes in PBMCs (Fig. 6c). We further demonstrated the expression of galectin 9 in PDA-infiltrating leukocytes and cancer cells by confocal microscopy (Fig. 6d,e). To investigate whether dectin 1 ligates galectin 9, protein-G magnetic beads were loaded with the dectin-1 IgG Fc-conjugated fusion protein or control IgG Fc. Bead–IgG Fc complexes were incubated with recombinant galectin 9 and then labeled with a fluorescently conjugated α-galectin 9 monoclonal antibody (mAb) and tested for fluorescence by flow cytometry. We found that the IgG Fc-conjugated dectin 1 fusion protein avidly ligated galectin 9, whereas controls failed to elicit a positive signal (Fig. 6f). Furthermore, our data suggest that galectin 9 ligation of dectin 1 might be competitively inhibited by d-zymosan (Fig. 6f). Galectin 9 also bound mouse (Fig. 6g) and human (Fig. 6h) dectin 1 in a dose-dependent manner in an enzyme-linked immunosorbent assay (ELISA). By contrast, mouse dectin 1 did not avidly bind galectin 3 or galectin 4 (Fig. 6i). We further confirmed that galectin 9 binds dectin 1 in situ by precipitating dectin 1 ligands in pancreatic tissue extract from KC mice and then probing for galectin 9. Our results indicated dectin 1–galectin 9 complex formation (Fig. 6j). Given that galectin 9 binds to polyactosamine epitopes on glycoproteins, we assessed whether galectin 9 ligates dectin 1 through a glycan–galectin 9 interaction. To test this, we pretreated dectin 1 with PNGaseF, an enzyme that cleaves N-linked glycans, and tested whether this modulated the interaction. We observed no change in the binding affinity between the proteins, which suggested that galectin 9 ligation of dectin 1 is glycan independent (Fig. 6k). In addition, we pretreated galectin 9 with high concentrations of lactose, a known inhibitor of galectins, before incubation with dectin 1. Again, no alteration of
Figure 5  Dectin 1 signaling prevents immunogenic T cell differentiation in PDA. (a–d) The inferior pancreas-draining lymph node in 6-month-old KC:Clec7a+/- and KC:Clec7a−/− mice was assayed for CD44 (a), OX40 (b) and PD-1 (c) expression in CD4+ and CD8+ T cells, and the CD8+:CD4+ T cell ratio (d). Representative contour plots and quantitative data are shown (n = 5/group). (e–g) WT and Clec7a+/- mice were challenged with orthotopic KPC tumors. (e) The CD8+:CD4+ ratio was determined on day 21 by flow cytometry. (f) PDA-infiltrating CD8+ T cell expression of PD-1, T-bet, TNF-α, CD107a and granzyme B was determined by flow cytometry. (g) PDA-infiltrating CD4+ T cell expression of CD44, CD107a, ICOS, T-bet and TNF-α was determined by flow cytometry (n = 5 mice/group). (h,i) WT and Clec7a+/- mice were challenged with orthotopic KPC tumors. Cohorts were additionally treated with α-PD-1 or isotype control. Tumor weights were measured on day 21 (h), and the fraction of CD4+ T cells expressing IFN-γ was determined by flow cytometry (i); n = 5 mice/group. All experiments were repeated at least twice (data are mean ± s.e.m; *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant by unpaired Student’s t-test).
Galectin 9 is a novel dectin 1 ligand in PDA. (a) PDA-infiltrating and splenic Gr1+CD11b+ neutrophils and inflammatory monocytes, CD11c+Gr1+CD11b+F4/80+ macrophages and CD11c+MHCII+ DCs from mice harboring orthotopic KPC tumors were gated by flow cytometry and tested for the expression of galectin 9. Gates are based on respective isotype control (data not shown). Representative contour plots and quantitative data from five mice are shown. (b) CD45–CD133+ pancreatic cancer cells from orthotopic KPC tumors were gated by flow cytometry and tested for expression of galectin 9, as compared to isotype control. Representative data from more than three experiments is shown. (c) CD45+ and CD45– cells from human PDA tumor tissue were tested for expression of galectin 9, as compared to PBMCs. Representative data from one of three patients are shown. (d) Frozen sections of orthotopic KPC-derived pancreatic tumors were gated for CD45 and galectin 9 or isotype control and imaged by confocal microscopy. Representative images are shown. Scale bar, 50 μm. (e) Frozen sections of orthotopic KPC-derived pancreatic tumors were costained for cytkeratin 19 (CK19) and galectin 9 or isotype control and imaged by confocal microscopy. Representative images are shown. Scale bar, 50 μm. (f) Protein-G magnetic beads were loaded with the dectin 1 IgG Fc fusion protein. After blocking, bead–IgG Fc complexes were incubated with recombinant galectin 9 and then stained with fluorescently conjugated anti–galectin 9 and tested for fluorescence by flow cytometry. Controls included: unstained beads; bead–IgG Fc complexes + recombinant galectin 9 + fluorescently conjugated anti–galectin 9; and beads without dectin 1 IgG Fc incubated with recombinant galectin 9 + fluorescently conjugated anti–galectin 9. To test for competitive inhibition of galectin 9 binding with a well-characterized dectin 1 ligand, the bead–IgG Fc complexes were incubated with recombinant galectin 9 together with d-zymosan and then stained with fluorescently conjugated anti–galectin 9. This assay was repeated twice with similar results. (g) Galectin-9–coated ELISA plates were incubated with increasing doses of mouse (g) or human (h) dectin 1 IgG Fc or control IgG Fc. The galectin-9-bound dectin 1 IgG Fc was detected with anti-IgG–horseradish peroxidase (HRP). Average of triplicates is shown. ELISA results were repeated twice with similar results. (i) Galectin-3–coated, galectin-4–coated and galectin-9–coated ELISA plates were incubated with dectin 1 IgG Fc or control IgG Fc (2.5 μg/ml) in parallel. The galectin-bound dectin 1 IgG Fc was detected with anti-IgG–HRP. Averages of triplicates are shown. (j) We precipitated dectin 1 ligands in pancreatic tissue extracts from 6-month-old KC mice using the dectin 1 IgG Fc or control IgG Fc and then probed for galectin 9 by western blotting. Recombinant galectin 9 was used as a positive control. This assay was repeated twice with similar results. (k) WT and Clec7a−/− macrophages were treated with galectin 9 (10 μg/ml) for 3 h. Syk phosphorylation was determined by flow cytometry and compared with isotype control. Representative histogram overlays and quantitative data from five separate experiments are shown. (m) Dectin 1 reporter HEK293 cells were untreated or treated with low or high doses of galectin 9 or the well-characterized dectin 1 ligands curdian and d-zymosan. Dectin 1 activation was measured through the detection of secreted embryonic alkaline phosphatase. This assay was performed in triplicate (data are mean ± s.e.m.; *P < 0.05; **P < 0.01; ***P < 0.0001; n.s., not significant by unpaired Student’s t-test).
We found that serial treatment with a neutralizing galectin 9 mAb extended survival rates in mice harboring orthotopic KPC tumors (Supplementary Fig. 7a). Galectin 9 blockade also extended survival after mAb treatment was initiated in mice that harbored established orthotopic KPC tumors (Supplementary Fig. 7b). Similarly, elevated galectin 9 expression was associated with a trend toward reduced survival in the context of human PDA (Supplementary Fig. 7c).

To determine whether galectin 9 blockade induces tumor regression, WT mice were implanted subcutaneously with PDA cells, and treatment with α-galectin 9 or an isotype was commenced after tumor development. Galectin 9 blockade resulted in substantial tumor regression (Supplementary Fig. 7d). Furthermore, akin to dectin 1 deletion, combined blockade of galectin 9 and PD-1 tended to offer synergistic protection against orthotopic PDA (Supplementary Fig. 7e) and resulted in enhanced T cell activation (data not shown). Moreover, T cell deletion abrogated the protective effects of galectin 9 blockade (Supplementary Fig. 7f).

Interestingly, we found that, in addition to dectin 1 expression on antigen-presenting cells is well-described death receptor and ligates by-products of necroptotic cell death within the PDA TME, including Sin3A-associated prodeath receptor ligation does not directly influence tumor progression3,14. For example, CD8+ T cells and T H1-polarized CD4+ T cells mediate tumor protection in mouse models of PDA and are associated with prolonged survival in human disease20. Conversely, we and others reported that T H2-polarized CD4+ T cells promote the progression of PDA in mice, and that intratumoral CD4+ T H2 cells infiltrates are correlated with reduced survival rates in human disease3,20,21. Similarly, Foxp3+ regulatory T (T reg) cells facilitate tumor immune escape and shorten survival in the context of PDA22,23. Hence, T cell programming influences disease outcome in PDA. However, the mechanism of regulation of the balance between immunogenic and immune-suppressive T cell populations is uncertain. Our data suggest that dectin 1 signaling has a crucial role in the capacity of macrophages to educate CD4+ and CD8+ T cells toward immunogenic or tolerogenic phenotypes.

Dectin 1 is vital in the innate immune defense against fungal pathogens24. Patients with genetic deficiencies affecting dectin 1 are at risk for recurrent mucocutaneous fungal infections, such as vulvovaginal candidiasis or onychomycosis25. However, unlike their Toll-like receptor (TLR) cousins, a definitive role for dectin 1 in the promotion of non-pathogen-mediated inflammation or oncogenesis is lacking26. We previously reported that in the context of chronic liver disease in mice, dectin 1 signaling secondarily protects against the development of liver cancer by suppressing TLR4, which drives liver fibrosis and increases the associated risk of hepatocarcinogenesis development12. Additional evidence also suggests that, in select contexts, dectin 1 activation might bolster anti-tumor immunity. Chiba et al.27 showed that dectin 1 expression on antigen-presenting cells is crucial to natural killer (NK)-cell-mediated killing of tumor cells that overexpress N-glycan structures. Dectin 1 recognition of these tumor cells leads to activation of the interferon regulatory factor 5 (IRF5) transcription factor, as well as the gene induction necessary to initiate tumoricidal activity of NK cells. Orally administered β-glucans might confer protection against lung and mammary tumors in mouse models of these diseases by ligating dectin 1 and inducing tumoricidal responses28. By contrast, we demonstrate that dectin 1 crucially regulates the macrophage phenotype in the context of PDA, which dictates the immunogenic or tolerogenic properties of peritumoral T cells. Moreover, we show that whereas T cell deletion does not influence tumor growth and thus T cells are dispensable to PDA, dectin 1 deletion renders T cells indispensable to tumor protection. These data suggest that targeting dectin 1 might be an attractive strategy for PDA immunotherapy in experimental therapeutics. We reported recently that the activation of Mincle, another C-type lectin receptor, also has protumorigenic properties13. However, unlike dectin 1, Mincle is a well-described death receptor and ligates by-products of necroptotic cell death within the PDA TME, including Sin3A-associated protein 130 (SAP130), which leads to expansion of the T reg cell population13,19. Of note, we did not detect differential T reg cell expansion as a consequence of dectin 1 signaling in PDA (data not shown).

Interestingly, we found that, in addition to dectin 1 expression on peritumoral macrophages and myeloid cells, PDA cells also express dectin 1. However, the functional implications of the presence of dectin 1 in transformed epithelial cells seem to be inconsequential to tumorigenesis, because neither dectin 1 ligation nor dectin 1 knockdown in PDA cells influenced their proliferative or inflammatory properties, and neither condition affected tumor growth in vivo. Similarly, dectin 1 deletion in the inflammatory compartment alone is protective against PDA growth and extends mouse survival. These data were confirmed by our macrophage-adoptive-transfer experiments. This finding parallels our previous work investigating TLR4 or TLR9 signaling in PDA, which found that although these pattern-recognition receptors are highly expressed on tumor cells, receptor ligation does not directly influence tumor progression3,14.

Beyond elucidating a crucial role for dectin 1 signaling in macrophages that contributes to the modulation of T cell plasticity in the transformed pancreas, one of the most important observations in this study is the discovery that galectin 9, a lectin with affinity for β-galactosides,
is a functional ligand for dectin 1. Non-pathogen-derived ligands for dectin 1 have not been well described. Thus, these data might have far-reaching implications that suggest a broader role for dectin 1 in sterile inflammation and oncogenesis. Galectin 9 has been reputed to be an exhaustion ligand for the T cell immunoglobulin mucin receptor 3 (TIM3) checkpoint receptor on T cells. TIM3 is also expressed on macrophages and dendritic cells. However, the galectin 9–TIM3 relationship has recently been called into question.

Galectin 9 has also recently been shown to bind CD137, wherein it facilitates receptor–complex aggregation, signaling and functional activity in lymphocytes and myeloid cells. Hence, galectin 9 might have a broader role in immune regulation. However, we found that galectin 9 neutralization enhanced T cell activation only in Clec7a−/− hosts, which suggests that galectin 9 exerts primary immune-suppressive effects specific to dectin 1 signaling. Of note, Martinez-Bosch et al. reported recently that galectin 1 promotes PDA growth through stromal remodeling and Hedgehog-signaling activation, which suggests that other galectins also have a role in PDA progression. Additional reports have shown that galectin 1 contributes to tumor aggressiveness by promoting angiogenesis and T cell apoptosis, whereas the blockade of galectin 1 in vivo results in tumor rejection through the generation of tumor-specific, T cell–mediated responses. Conversely, galectin-3 ligation has been shown to activate tumor-infiltrating lymphocytes and promote anti-tumor immunity. We found that other galectins did not avidly bind dectin 1, which thus implicates alternative mechanisms.

Our data suggest that, akin to dectin 1, galectin 9 would be an attractive target for immunotherapy in PDA. In contrast to our findings, galectin 9 has been reported to prevent metastases in models of colon cancer and melanoma by inhibiting the binding of tumor cells to extracellular-matrix components, which leads to the prevention of tumor cell migration. There are also important limitations to targeting dectin 1 or galectin 9 to combat PDA because the tumors do eventually progress, according to our survival experiments in animal models. These data suggest that dectin-1-targeted or galectin-9-targeted immunotherapy would require additional treatments to achieve cure. Indeed, the complex network of immunosuppressive pathways present in tumors is unlikely to be overcome by intervention with a single immune-modulatory agent. Acquired resistance to checkpoint-based immunotherapy by patients with melanoma has been associated with defects in the pathways involved in interferon-receptor signaling and in antigen presentation. Relapse after immunotherapy has also been linked to upregulated expression of exhaustion ligands by tumors. Our comparison of day-21 and day-42 PDA tumors in cohorts of mice treated with α-galectin 9 suggests that, eventually, CD8+ T cell–population expansion and activation fails. It is conceivable that the CD8+ T cells activated by interrupting the dectin 1–galectin 9 axis eventually become exhausted, as suggested by reduced IFN-γ and T-bet expression in day-42 tumors. In fact, our data suggest that immunotherapy regimens targeting PD-1 combined with therapies targeting either dectin 1 or galectin 9 are likely to have synergistic efficacy, wherein dectin 1 or galectin 9 blockade enhances T cell activation, and PD-1 blockade prevents exhaustion through checkpoint-receptor ligation. In agreement with our findings, a recent report suggested that durable regression of established tumors in melanoma requires concurrent immunotherapy with four distinct agents that target complementary aspects of innate and adaptive immunity. Thus, the development of therapeutics targeting dectin 1 signaling will potentially produce immunotherapeutic options for human PDA.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

D.D. and V.R.M. (project leadership, data collection and analysis, manuscript preparation); N.M. (flow cytometry, western blotting, in vivo experiments and manuscript preparation); N.A. (IHC, IF, flow cytometry and in vivo experiments); A.O. (immunoprecipitation, binding assays, ELISA and western blotting); K.B.L., G.S.D.B.P., S.S. and R.R. (flow cytometry and in vivo experiments); D.W.H. (immunoprecipitation and biochemical analysis); S.N. (mass spectroscopy); D.W. (cell line transfection); G.W. (in vivo experiments); C.P.Z. (data collection and mouse breeding); R.M.B., A.T.-H., M.H., B.D. and B.A. (technical assistance in diverse in vivo and in vitro experiments), S.C. (data analysis), L.G. (cell-line transfection), L.K.M. (immunoprecipitation and biochemical analysis); B.U. (mass spectroscopy); G.M. (project design and leadership, data analysis and manuscript preparation).

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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1. Yadav, D. & Lowenfels, A.B. The epidemiology of pancreatitis and pancreatic cancer. Gastroenterology 144, 1252–1261 (2013).
2. Guerra, C. et al. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. Cancer Cell 11, 291–302 (2007).
3. Ochi, A. et al. MyD88 inhibition amplifies dendritic cell capacity to promote pancreatic carcinogenesis via Th2 cells. J. Exp. Med. 209, 1671–1687 (2012).
4. Zhu, Y. et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. Cancer Res. 74, 5057–5069 (2014).
5. Connolly, M.K. et al. Distinct populations of metastases-enabling myeloid cells expand in the liver of mice harboring invasive and preinvasive intra-abdominal tumor. J. Leukoc. Biol. 87, 713–725 (2010).
6. Pylayeva-Gupta, Y., Lee, K.E., Haidu, C.H., Miller, G. & Bar-Sagi, D. Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. Cancer Cell 21, 836–847 (2012).
7. Bayne, L.J. et al. Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. Cancer Cell 21, 827–835 (2012).
8. Goodridge, H.S. et al. Activation of the innate immune receptor Dectin-1 upon formation of a ‘phagocytic synapse’. Nature 472, 471–475 (2011).
9. Taylor, P.R. et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat. Immunol. 8, 31–38 (2007).
10. Strasser, D. et al. Syk kinase-coupled C-type lectin receptors engage protein kinase C-α to elicit Card9 adaptor-mediated innate immunity. Immunity 36, 32–42 (2012).
11. Gross, O. et al. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature 442, 651–656 (2006).
12. Seifert, L. et al. Dectin-1 regulates hepatic fibrosis and hepatocarcinogenesis by suppressing TLR4 signaling pathways. *Cell Rep.* 13, 1909–1921 (2015).
13. Seifert, L. et al. The necroosome promotes pancreatic oncogenesis via CXCL1 and Mincle-induced immune suppression. *Nature* 532, 245–249 (2016).
14. Zambrinis, C.P. et al. TLR9 ligation in pancreatic stellate cells promotes tumorigenesis. *Exp. Med.* 212, 2077–2094 (2015).
15. Ochi, A. et al. Toll-like receptor 2 regulates pancreatic carcinogenesis in mice and humans. *J. Clin. Invest.* 122, 4118–4129 (2012).
16. Walachowski, S., Tabouret, G. & Fourcas, G. Triggering dectin-1-pathway alone is not sufficient to induce cytokine production by murine macrophages. *PLoS One* 11, e0148464 (2016).
17. Zheng, L., Xue, J., Jaffee, E.M. & Habtezion, A. Role of immune cells and immune-based therapies in pancreatitis and pancreatic ductal adenocarcinoma. *Gastroenterology* 144, 1230–1240 (2013).
18. Clark, C.E. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res.* 67, 9518–9527 (2007).
19. Andrén-Sandberg, A., Dervenis, C. & Lowenfels, B. Etiologic links between chronic pancreatitis and pancreatic cancer. *Scand. J. Gastroenterol.* 32, 97–103 (1997).
20. Fukunaga, A. et al. CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. *Pancreas* 28, e26–e31 (2004).
21. De Monte, L. et al. Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. *J. Exp. Med.* 208, 469–478 (2011).
22. Hiroaka, N., Onozato, K., Kosuge, T. & Hirohashi, S. Prevalence of FOXP3+ lymphocyte density in pancreatic cancer correlates with tumor-immune privilege. *Cancer Res.* 75, 3418–3428 (2015).
23. Jiang, Y. FOXP3+ lymphocyte density in pancreatic cancer correlates with tumor-immune privilege. *Cancer Cell* 18, 241–251 (2010).
24. Hong, F. et al. Mechanism by which orally administered beta-1,3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. *J. Immunol.* 173, 797–806 (2004).
25. Yamashita, S. et al. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.* 9, 1179–1188 (2008).
26. Zhu, C. et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.* 6, 1245–1252 (2005).
27. Chiba, S. et al. Recognition of tumor cells by Dectin-1 orchestrates innate immune cells for anti-tumor responses. *eLife* 3, e04177 (2014).
28. Zaretsky, J.M. et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Cell Rep.* 11, e04177 (2014).
29. Martínez-Bosch, N. et al. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J. Exp. Med.* 211, 1433–1448 (2014).
30. Nobumoto, A. et al. The Tim-3 ligand galectin-9 mediates tumor growth and metastasis through stroma remodeling and Hedgehog signaling activation. *Cancer Res.* 74, 3512–3524 (2014).
31. Banh, A. et al. Tumor galectin-1 mediates tumor growth and metastasis through regulation of T-cell apoptosis. *Cancer Res.* 71, 4423–4431 (2011).
32. Banh, A. et al. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell* 5, 241–251 (2004).
33. Madireddi, S. et al. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J. Exp. Med.* 211, 1433–1448 (2014).
34. Rubinstein, N. et al. Targeted inhibition of galectin-1 gene expression in tumor cells corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. *Cancer Res.* 70, 7476–7488 (2010).
35. Demette, N. et al. A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. *Cancer Res.* 70, 7476–7488 (2010).
36. Yamasaki, S. et al. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell* 5, 241–251 (2004).
37. Moynihan, K.D. et al. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J. Exp. Med.* 211, 1433–1448 (2014).
38. Nobumoto, A. et al. Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices. *Glycobiology* 18, 735–744 (2008).
39. Zaretsky, J.M. et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. *Nat. Med.* 19, 819–829 (2016).
40. Banh, A. et al. Tumor galectin-1 mediates tumor growth and metastasis through regulation of T-cell apoptosis. *Cancer Res.* 71, 4423–4431 (2011).
41. Banh, A. et al. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell* 5, 241–251 (2004).
42. Banh, A. et al. Tumor galectin-1 mediates tumor growth and metastasis through regulation of T-cell apoptosis. *Cancer Res.* 71, 4423–4431 (2011).
43. Demette, N. et al. A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. *Cancer Res.* 70, 7476–7488 (2010).
44. Nobumoto, A. et al. Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices. *Glycobiology* 18, 735–744 (2008).
45. Moynihan, K.D. et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat. Med.* 22, 1402–1410 (2016).
46. Zaretsky, J.M. et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. *Nat. Med.* 19, 819–829 (2016).
47. Ansell, S.M. et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin’s lymphoma. *Nat. Med.* 22, 1402–1410 (2016).
Animals and in vivo models. C57BL/6 (H-2Kb) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in-house. Cleca7a−/− mice were a gift of G. Brown (University of Aberdeen, UK). KC mice (gift of D. Bar-Sagi) and KPC mice (gift of M. Philips, both New York University) develop pancreatic neoplasia endogenously by expressing mutant Kras alone or mutant Kras and Trp53, respectively, in the progenitor cells of the pancreas42,43. We previously detailed tumor progression and survival in control KC mice44. Cleca7a−/− mice were crossed with KC mice to generate KC/Cleca7a−/− animals. For orthotopic pancreatic tumor challenge, mice were administered intrapancreatic injections of either KrasG12D PDECs or FC1242 tumor cells derived from KPC mice. KrasG12D PDECs and FC1242 cells were generated as previously described46. In select experiments, we used KPC-derived tumor cells (1 × 106), which we engineered to express OVA using pcI-neo-cOVA (gift of M. Castro University of Michigan; Addgene plasmid #25097), as we have described44. Both male and female mice were used, but animals were sex- and age-matched in each experiment. For orthotopic tumor experiments, 8–10-week-old mice were used. No formal power analyses, randomization, exclusions or blinding were done. In preparation for intrapancreatic injection, cells were suspended in PBS with 50% Matrigel (BD Biosciences, Franklin Lakes, NJ) and 1 × 105 tumor cells were injected into the body of the pancreas through a laparotomy. Mice were killed 3 weeks later and tumor weights were recorded. In select experiments, KPC-derived tumor cells (5 × 106–107) total) were administered subcutaneously, alone or mixed with 2 × 105 macrophages. To study the effects of d-zymosan (500 µg/ml) or HKCA (5 × 106 cells; both Invivogen, San Diego, CA) by intraperitoneal (i.p.) injection five times weekly for 8 weeks in endogenous tumor models and for 3 weeks in the orthotopic tumor models. PDECs were harvested from the pancreata of KC mice and passed in vitro, as previously described48. DPEC proliferation was measured using the XTT assay, according to the manufacturer’s protocol (Roche, Nutley, NJ). In select experiments, cohorts of mice were treated five times weekly with the p-Syk inhibitor piceatannol (20 mg/kg; i.p.; Selleck Chemicals, Houston, TX). Pan-T cells (CD90, T24/31), CD4 T cells (GK1.5), CD8 T cells (53-6.72), and macrophages (F4/80, CL:A3-1, all BioXcell, West Lebanon, NH) were depleted by neutralizing mAbs using regimens that we have previously described45,46. In other experiments, animals were treated twice weekly with an i.p. injection of neutralizing mAbs directed against F4/80 (CL:A3-1, conc: 10 µg/ml), arginase 1 (Polyclonal, conc: 2 µg/ml), p-Syk (Polyclonal, conc: 10 µg/ml), Ki67 (Polyclonal, conc: 3 µg/ml) and dectin 1 (2A11, Conc: 5 µg/ml, all Abcam). For paraffin-embedded samples (F4/80, p-Syk, Ki67, arginase 1), samples were dewaxed in ethanol, followed by antigen retrieval with 0.01-M sodium citrate with 0.05% Tween. For frozen specimen (dectin 1), samples did not undergo antigen retrieval before incubation with the primary antibody. Immunofluorescence staining on frozen mouse tissues was performed using antibodies against dectin 1 (2A11; conc: 20 µg/ml, Abcam), CD45 (30-F11; conc: 6.25 µg/ml, BD Biosciences), CK19 (Troma-III; University of Iowa), CD68 (FA-11, conc: 5 µg/ml, Abcam), dectin 1 (Fc (m-ducela; InvivoGen), galectin 9 (Polyclonal; Conc: 20 µg/ml, Bioss) and DAPI (Vector Labs, Burlingame, CA). Immunofluorescence staining in human tissue was performed using antibodies against dectin 1 (Polyclonal; conc: 20 µg/ml, Abcam), CD11b (M1/70, conc: 5 µg/ml, Ep-CAM (G12; conc: 5 µg/ml, both BioLegend) and DAPI (Vector Labs, Burlingame, CA). Immunofluorescence images were acquired using the Zeiss LSM700 confocal microscope with ZEN 2010 software (Carl Zeiss, Thornwood, New York). All human tissues were collected using an Institutional Review Board–approved protocol, and donors of unidentified specimens gave informed consent. Sample sizes for human experiments were not determined on the basis of formal power calculations. Quantifications were performed by assessing ten high-power fields (HPF; 40×) per slide in a blinded manner.

Western blotting and RNA analysis. For protein extraction, tissues were homogenized in ice-cold RIPA buffer. Total protein was quantified using the DC Protein Assay according to the manufacturer’s instructions (BioRad, Hercules, CA). Western blotting was performed as previously described13, with minor modifications. Briefly, 10% Bis-Tris polyacrylamide gels (NuPage, Invitrogen) were equiloaded with 10–30 µg of protein, electrophoresed at 200 V and electrotransferred to PVDF membranes. After blocking with 5% BSA, membranes were probed with primary antibodies to β-actin (8H1D10), p53 (7F5), PLC-γ (polyclonal), p-PLC-γ (polyclonal), Bcl-XL (54H6; all Cell Signaling), JNK (2C6), p-JNK (G9), Smad4 (polyclonal), p16 (polyclonal), c-Myc (9E10), CARD9 (polyclonal), Syk (polyclonal), p-Syk (polyclonal), RB (C-15; all Cell Signaling), dectin 1 (polyclonal; Abcam), galectin 9 (polyclonal; Abcam), and dectin 1 Fc (m-ducela; InvivoGen). Blots were developed by ECL (Thermo Scientific, Asheville, NC). See Supplementary Figures 9 and 10 for full western blot results. RNA extraction was performed using the RNeasy Mini kit (Qiagen, Germantown, MD) as per manufacturer’s instructions. For Nanostring analysis, the nCounter mouse inflammation panel was employed using the nCounter Analysis System (both NanoString, Seattle, Washington).

Enrichment of endogenous dectin 1 ligands and mass spectrometry. Cell lysate of KPC-derived tumor cells were prepared and protein was quantified as above. Lysate (2 mg) was mixed overnight at 4 °C with a human IgG Fc-conjugated dectin 1 fusion protein (3 mg) and protein-G magnet beads were irradiating mice (9 Gy), followed by intravenous (i.v.) bone marrow transfer with neutralizing mAbs directed against PD-1 (29F.1A12, 6 mg/kg; both BioXcell), or twice weekly with an i.p. injection of neutralizing mAbs directed against Clec7a alone or mutant Clec7a in mice. Modifications. Briefly, pancreata were placed in cold RPMI 1640 with collagenase 1 ligation, mice were administered d-zymosan (500 µg/ml) and minced with scissors to submillimeter size. Animal Care and Use Committee.
and then alkylated with iodoacetamide at 37 °C in the dark for 45 min (200 µl of 50 mM in 100 mM ammonium bicarbonate). After alkylation, samples were loaded onto a NuPAGE 4–12% Bis-Tris Gel 1.0 mm (Life Technologies Corporation, Grand Island, NY) and run for 15 min at 200 V. The gel was stained using GelCode Blue Stain Reagent (Thermo Scientific, Rockford, IL). The short gel lane was cut into approximately 1-mm² pieces. The gel pieces were destained in 1:1 v/v solution of methanol and 100 mM ammonium bicarbonate at 4 °C with agitation. The destain solution was changed every 15 min at least five times and until pieces had no visibly blue stain left. Gel pieces were partially dehydrated with an acetonitrile rinse and further dried in a SpeedVac concentrator for 20 min. Sequencing grade-modified trypsin (300 ng; Promega, Madison, WI) was added to the dried gel pieces. After the trypsin was absorbed, 200 µl of 100 mM ammonium bicarbonate was added to cover the gel pieces, and digestion proceeded overnight on a shaker at 37 °C. Peptide extraction was performed by adding a slurry of R2 20-µm Poros beads (Life Technologies Corporation) in 5% formic acid; 0.2% trifluoroacetic acid (TFA) to each sample at an volume equal to that of the ammonium bicarbonate. Samples were incubated with agitation at 4 °C for 4 h. The beads were loaded onto equilibrated C18 ziptips (Millipore) using a microcentrifuge for 30 s at 6,000 r.p.m. Gel pieces were rinsed three times with 0.1% TFA, and each rinse was added to the corresponding ziptip followed by microcentrifugation. Extracted poros beads were further washed with 0.5% acetic acid. Peptides were eluted off the beads through the addition of 40% acetonitrile in 0.5% acetic acid, followed by the addition of 80% acetonitrile in 0.5% acetic acid. The organic solvent was removed using a SpeedVac concentrator, and the samples were reconstituted in 0.5% acetic acid. An aliquot of each sample was loaded onto the EASY spray 50-cm C18 analytical HPLC column with <2-µm bead size using the autosampler of an EASY-nLC 1,000 HPLC (Thermo Fisher) in solvent A (2% acetonitrile, 0.5% acetic acid). The peptides were gradient-eluted directly into a Q Exactive (Thermo Scientific) mass spectrometer using a 1-h gradient from 2% to 31% solvent B (95% acetonitrile, 0.5% acetic acid), followed by 10 min from 31% to 40% solvent B and 10 min from 40% to 100% solvent B. The Q Exactive mass spectrometer acquired high-resolution full MS spectra with a resolution of 17,500, AGC target of 5 × 10⁴, maximum ion time of 100 ms, and a maximum ion count of 100,000. Higher-energy collisional dissociation (HCD) MS/MS spectra were acquired using the following instrument parameters: resolution of 24,000, AGC target of 5 × 10⁵, maximum ion time of 100 ms, one microscan, 2 ms isolation window, fixed first mass of 150 m/z and normalized collision energy of 35 and dynamic exclusion of 30 s. The MS/MS spectra were searched against the Uniprot Mouse database combined with mammalian IgG database using Sequest within Proteome Discoverer (ThermoFisher). The results were filtered using a <1% false-discovery rate searched against a human IgG database using Sequest within Proteome Discoverer (ThermoFisher). The results were filtered using a <1% false-discovery rate searched against a human decoy database, and only proteins with two or more unique peptides were included. Proteins identified in the control were subtracted from the proteins identified in the lectin 1 affinity purification and a shortened list interrogated for potential lectin 1 ligands.

Analysis of lectin 1–galectin 9 interaction. To investigate whether galectin 9 can bind lectin 1, protein-G magnet beads (2 ml; Dynabeads) were loaded with lectin 1 IgG Fc (1 mg) or control IgG Fc before washing and blocking. Subsequently, beads were incubated with recombinant galectin 9 (2 mg; R&D Systems) for 30 min, washed and stained with PE-conjugated anti–galectin 9 (BioLegend, San Diego, CA). Galectin-9-specific staining was determined by flow cytometry. To investigate the interaction between galectin 9 and lectin 1 by ELISA, plates (Maxisorp, Nunc, St. Louis, MO) were coated with recombinant mouse galectin 9 (2 mg; R&D Systems, Minneapolis, MN), galectin 3 or galectin 4 (both 2 mg; Biologend) for 16 h at 4 °C, blocked with 1% BSA/PBS for 1 h, and incubated with increasing doses of lectin 1 IgG Fc or control IgG Fc for 2 h. The galectin-bound lectin 1 IgG Fc was detected with anti-IgG–HRP. For affinity-precipitation experiments, protein-G beads were loaded with lectin 1 IgG Fc and were then incubated with the extract of 6-month-old KC pancreata. Bead-bound precipitates were resuspended with loading buffer and resolved by SDS–PAGE under reduced conditions for western blotting using mAbs specific for galectin 9. To determine whether galectin 9 induces lectin 1 signaling, the lectin 1 reporter HEK293 cell line16 (Invivogen) was treated with recombinant galectin 9 (1–10 µg/ml; R&D Systems), d-zymosan (1–10 µg/ml) or curdian (10–100 µg/ml; both Invivogen). Lectin 1 signaling was measured by detection of secreted embryonic alkaline phosphatase. In other experiments, WT and Clec7a−/− splenic macrophages were treated in vitro with recombinant galectin 9 (10 µg/ml). Syk phosphorylation was measured by flow cytometry at 3 h after treatment.

Galectin 9 immunoprecipitation experiments with PNGase F or lactooligosaccharide treatment. Lectin 1 IgG Fc (8 µg; InvivoGen) was treated with PNGase F (20 µl, 10,000 units; New England BioLabs) following the company’s nonde- naturening protocol (37 °C, 24 h). PNGase-F-treated and untreated lectin 1 IgG Fc samples (4 µg; InvivoGen) were added to protein-G beads (25 µl, Dynabeads Protein G; novex) and the mixtures were incubated at room temperature for 10 min. The beads were washed 2× with PBS–T (pH 7.4 with 0.02% tween). Recombinant mouse galectin 9 (4 µg; R&D Systems) was resuspended in PBS–T or a solution of 100-mM lactose in PBS–T. Galectin 9 samples were then incubated with the Dynabead–lectin 1 IgG Fc complex for 20 min at room temperature. The Dynabeads were then washed three times with PBS–T and transferred to a clean tube. Galectin 9 and lectin 1 IgG Fc were eluted in SDS–PAGE buffer (PBS with 10% BME) by heating at 98 °C for 10 min. The samples were then analyzed by SDS–PAGE and stained with Coomassie blue.

Dectin 1 knockdown. Lentivirus was prepared by infecting HEK293T cells with either a scrambled or shDectin (NM_020008.1:2981-311) plasmid, Δ8.9CR2 plasmid, and vesicular stomatitis virus glycoprotein plasmid (3:1:4 ratio). Supernatant were collected for 3 d after infection. KPC cells were then infected with supernatant in the presence of polybrene (8 µg/ml) for 12 h × 2 and selected with puromycin (2 µg/ml). The efficacy of gene knockdown was confirmed by PCR, flow cytometry and western blotting.

Statistical analysis. Data is presented as means ± s.e.m. Human RNA-seq data and clinical correlations were performed using the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu)27. Survival was measured according to the Kaplan–Meier method. Statistical significance was determined by the Student’s t test (two-tailed) and the log–rank test using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). P values of <0.05 were considered to be significant.

Data availability. Data are available from the corresponding author upon reasonable request.

42. Hingorani, S.R. et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 4, 437–450 (2003).
43. Hingorani, S.R. et al. Tg53R127H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 7, 469–483 (2005).
44. Daley, D. et al. 8T cells support pancreatic oncogenesis by restraining αβ T cell activation. Cell 166, 1485–1499.e13 (2016).
45. Bedrosian, A.S. et al. Dendritic cells promote pancreatic viability in mice with acute pancreatitis. Gastroenterology 141, 1915–1926 (2011).
46. Greco, S.H. et al. Mincle suppresses Toll-like receptor 4 activation. J. Leukoc. Biol. 100, 185–194 (2016).
47. Hruban, R.H. et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. Am. J. Surg. Pathol. 25, 579–586 (2001).