Oncogenic Transformation of Dendritic Cells and Their Precursors Leads to Rapid Cancer Development in Mice

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Dendritic cells (DCs) are key players in the initiation and regulation of immune responses. They sense both infection and tissue damage and excel at processing and presenting Ags on MHC molecules and delivering accessory signals to T cells (1–3). DCs can additionally prime NK cells and other innate effector cells. Collectively, the actions of DCs act to promote T cell responses to pathogens and increase resistance to infection but are also thought to be operative in the induction of immune responses to cancer.

The DC lineage comprises several distinct subsets that differ to some degree in their functional capabilities and localization. Two main DC subsets can be identified in mice and humans. Plasmacytoid DCs are found primarily in blood and lymphoid tissues, where they produce high amounts of type I IFN in response to triggering of TLRs. The other subset is found in lymphoid and nonlymphoid organs and comprises conventional DCs (cDCs), which depend on signaling via Flt3 for their differentiation and survival (3–5). With the exception of the lung, cDCs located in peripheral and lymphoid organs possess a short half-life and are constantly replaced by new cells that differentiate locally from DC-restricted precursors (pre-DCs) that arrive through the blood (6–9). Pre-DCs and plasmacytoid DCs are thought to originate from common DC precursors (CDPs) within the bone marrow. We have recently found that the C-type lectin receptor DC, NK lectin group receptor-1 (DNGr-1) encoded by the C-type lectin domain family 9, member a (Clec9a) locus is expressed in pre-DCs in mice and additionally identifies within the CDP pool DC precursors with a cDC-restricted potential (1, 10, 11). We exploited this finding by generating a mouse line in which Cre recombinase was placed under control of the Clec9a promoter (Clec9a^Cre), which allowed for fate mapping of cDC precursors when the mice were crossed to Rosa26-stop-flxed enhanced yellow fluorescent protein (YFP) reporter mice (Clec9a^Cre Rosa26<sup>YFP/YFP</sup> mice) (11). In such mice, the history of DNGr-1 expression as assessed by YFP expression distinguishes cDCs from other cell lineages.
The propensity of DCs to elicit immunity could make transformed DCs highly susceptible to immune control (12, 13). This could help explain why hematological malignancies are relatively common but cancers of DC origin are only rarely observed in humans (11, 14–16). The only described DC-type cancer, Langhans cell (LC) histiocytosis (LCH), is a rare disease, which, despite its name, is likely to involve transformation of DC precursors rather than LCs (17, 18). Alternatively, Ag presentation by DCs need not always result in immunity and can lead to induction of immunological tolerance (19–21), for example, by T cell deletion (22, 23). Indeed, in some cancer vaccination trials, tolerance induction by DCs might have helped promote cancer progression (3, 24–26). As such, transformed DCs could be especially adept at escaping immunological control.

Two mouse models have been used to induce DC transformation and assess its consequences in vivo. In C11c:SV40Ltg mice, SV40 T oncogenes were transgenically expressed under the control of a minimal C11c promoter. The mice developed low-grade tumors that nonetheless resulted in progressive and systemic disease resembling the more aggressive, multisystemic subtype of LCH in humans (10, 11). Similar results were obtained in another model in which an oncogenic form of B-Raf was forcibly induced in C11c+ cells (1, 27). However, C11c is not uniquely expressed by DCs and is absent from CDPs (3, 28), which may impact the interpretation of those models. Interestingly, a much attenuated phenotype was obtained when the Langerin locus control region was used to restrict oncogenic B-Raf expression to differentiatated DC subtypes and LCs, which are now thought to constitute macrophages rather than DCs (6, 28). In the present study, we used the Clec9a driver to specifically test what happens when only mouse cDCs and their precursors become neo-class I mice were depleted of CD11c + cells (1, 27). However, CD11c is not uniquely expressed by DCs and is absent from CDPs (3, 28), which may impact the interpretation of those models. Interestingly, a much attenuated phenotype was obtained when the Langerin locus control region was used to restrict oncogenic B-Raf expression to differentiated DC subtypes and LCs, which are now thought to constitute macrophages rather than DCs (6, 28). In the present study, we used the Clec9a driver to specifically test what happens when only mouse cDCs and their precursors become neo-plastically transformed. We crossed the Clec9a+ mouse strain to Kirsten rat sarcoma viral oncogene homolog (Kras)G12D and transformation-related protein 53 (Trp53)fl/fl mice to drive oncogenic Ras expression and delete the p53 tumor suppressor in cells of the DC lineage. We show that these mice succumb to massive DC tumor development in lymphoid and nonlymphoid organs at an early age. Neoplastic transformation did not alter the phenotype or activation status of DCs and did not seem to evoke an immune response. Our data support and extend previous findings that DC cancer can occur in mice (1, 10, 29), indicating that the immunogenic potential of DCs does not by default result in anti-cancer immunity upon neoplastic transformation.

Materials and Methods

Mice

C57BL/6, B6.SJL, OT-I × Rag1−/−, OT-II, KrasG12D, Trp53−/−, Clec9a+/cre, Rosa26YPFP, Clec9a+/cre, Rosa26YPFP, Trp53−/−, KrasG12D, Rosa26YPFP, Clec9a+/cre; Rosa26YPFP, Trp53−/−, KrasG12D (Clec9aKrasG12D), and Clec9a+/cre; Rosa26YPFP, Trp53−/−, Clec9aKras-wt mice were bred at The Francis Crick Institute under specific pathogen-free conditions. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by the London Research Institute (now The Francis Crick Institute) Animal Ethics Committee and by the Home Office, U.K.

Generation of mixed bone marrow chimeras

C57BL/6 × B6.SJL mice (heterozygous for the congenic markers CD45.1 and CD45.2) were irradiated with two doses of 6.6 Gy separated by 4 h. Eight hours later, irradiated mice were injected i.v. with 3 × 10⁸ total bone marrow cells from B6.SJL mice (CD45.1) mixed with C11c+ bone marrow cells from either Clec9a+/cre; KrasG12D (CD45.2) or Clec9aKras-wt bone marrow cells. Prior to transfer, bone marrow cells from Clec9aKras-G12D mice and Clec9aKras-wt mice were depleted of CD11c+ cells by magnetic separation to prevent the transfer of differentiated DCs.

Adoptive transfer experiments

Splenid CD8+ YFP+ DCs from Clec9aKras-G12D mice were sorted by FACS. Cells (2 × 10⁶) were injected i.v. into the tail vein of C57BL/6 or Rag2−/− mice. Alternatively, Kras-induced cancer DC (KID) cells were harvested from in vitro cultures and washed three times in PBS. Cells (5 × 10⁶) were injected i.v. into the tail vein of C57BL/6 or Rag2−/− mice.

Survival analysis

Analysis of survival was done according to the United Kingdom animal welfare regulations. Mice that reached the endpoint of disease severity limits were sacrificed and considered as dead in the analysis. In the case of Clec9aKras-G12D × B6.SJL and Clec9aKras-wt × B6.SJL bone marrow chimeras, the day of bone marrow transfer was considered as the starting time point (day 0) of the analysis.

Isolation of cells

Except for bone marrow, organs were enzymatically digested with collagenase type 4 (200 U/ml, Worthington Biochemical) and DNase I (200 µg/ml, Roche). Leukocyte purification from lung and liver was performed by Percoll (GE Healthcare) gradient centrifugation as described (11, 30).

Cell culture

To generate KID cell lines, splenocytes from Clec9aKras-G12D mice were cultured in complete RPMI 1640 without additional growth factors. Cells were passed every 3–5 d and used for experiments after >10 passages.

Cytokine and chemokine production by DCs

Sorted splenic CD8a+ DCs were incubated at 37°C in the presence of CpG oligodeoxynucleotide 1668 (0.5 µg/ml), LPS (10 ng/ml), or polyinosinic-polycytidylic acid [poly(I:C)] (1 µg/ml) for 18 h. For some experiments, stimulation was performed in the absence of recombinant mouse IFN-γ (10 ng/ml). CD40L stimulation was done by culturing DCs on 2 × 10⁵ CD90.2-expressing fibroblasts or control fibroblasts as described previously (31). Detection of CXCL10 and IL-12p70 in supernatants from stimulated DCs was performed by ELISA (DuoSet ELISA kit, R&D Systems) according to the manufacturer’s instructions. Production of CCL3, CCL5, IL-6, TNF, and CXCL9 was assessed by cytometric bead array (BD Biosciences). Intracellular staining of cytokines was performed after 5 h of stimulation, with 5 µg/ml brefeldin A added during the last 4 h of stimulation.

T cell stimulation assays

CD8+ OT-I cells were isolated from spleen of OT-I × Rag1−/− mice using anti-CD8 microbeads (Miltenyi Biotec). CD4+ OT-II cells were isolated from spleen of OT-II mice using anti-CD4 microbeads (Miltenyi Biotec). T cells were labeled with 2 µM CFSE at 37°C for 15 min, washed three times in PBS, and incubated with sorted CD8a+ DCs fed with endotxin-free OVA (Hyglos; 100 µg/ml for OT-I assays and 20 µg/ml for OT-II assays). CpG oligodeoxynucleotide 1668 (0.5µg/ml) or LPS (10 ng/ml) were added to some groups as indicated. Five days later, CFSE dilution was assessed among live T cells (identified by CD90.2 staining) by flow cytometry.

Quantitative RT-PCR on genomic DNA

Genomic DNA isolated from tail tissue or sorted DCs was analyzed by quantitative real-time PCR for the presence of the stop cassette at the KrasG12D locus or exon 4 of the Trp53 gene (both sequences are located within the loxP-flanked regions of the respective genes) using the following primers: KrasG12D-genomic DNA forward, 5′-CACACCGCA-TAGACTTGTGCAACATACTTGTAATAGT-3′; reverse, 5′-CAAGCTACTCCAGATGCTGTAAGTCTC-3′; Trp53-exon 4–genomic DNA forward, 5′-GGCTTCTGACTTACTTGTGCTCTTA-3′, reverse, 5′-AGAC-CTCCGGTTGCTACAATA-3′. Amplification of a genomic segment of the Gapdh gene served as control for DNA input using the following primers: Gapdh-genomic DNA forward, 5′-CATATCCCGCAGACAGTGGTCC-3′, reverse, 5′-ATACGCGCAAATCTGAAAGAC-3′ (see Ref. 23). Real-time PCR was performed by the relative standard curve method on an ABI 7500 real-time PCR system (Applied Biosystems) using the Express SYBR Green ER Master mix (Invitrogen) according to the manufacturers’ instructions. PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 two-step cycles of 15 s at 95°C and 1 min at 60°C.
FIGURE 1. Cle9aKras-G12D mice spontaneously develop accumulation of DCs in lymphoid and peripheral organs. (A) Crossing scheme to illustrate the generation of Cle9aKras-G12D mice and Cle9aKras-wt mice. (B) Survival analysis of cohoused Cle9aKras-G12D mice \( (n = 9) \) and Cle9aKras-wt mice \( (n = 5) \) over time. Statistical analysis was performed using the log-rank (Mantel–Cox) test. (C) Representative images of spleen and inguinal lymph nodes from 7-wk-old Cle9aKras-G12D mice and Cle9aKras-wt mice. (D) Flow cytometry of splenocytes from 7-wk-old Cle9aKras-G12D mice and Cle9aKras-wt mice showing the frequency of CD11c+MHC II+ DCs \( \text{(left and middle panels)} \) and YFP expression in splenic CD11c+MHC II+ DCs from three mice of each group. \( \text{(E–J)} \) Cle9aKras-G12D mice and Cle9aKras-wt mice were sacrificed at indicated time points after birth and the absolute numbers of CD11c+MHC II+ DCs in (E) spleen, (F) inguinal lymph nodes, (G) blood, (H) bone marrow, (I) liver, and (J) lung were quantified by flow cytometry. In \( \text{(E–J)} \), each dot represents DCs from one individual mouse. Data are pooled from two to three independent experiments and shown as mean ± SEM. (K) Representative confocal immunofluorescence images of the spleen from 5.5-wk-old Cle9aKras-G12D mice and Cle9aKras-wt mice. Staining was done for YFP (green), B220 (white), and CD3e (blue). Scale bars, 200 μm. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
Flow cytometry and fluorescence-activated cell sorting

Flow cytometric analyses were performed with an LSRFortessa (BD Biosciences). Data were analyzed using FlowJo (Tree Star). Concatenation of flow cytometric data shown in Fig. 6A and 6C was done according to the FlowJo software guidelines. In brief, data from one experiment with several mice were pooled into one graph, using a numeral for each individual mouse (mouse ID) to separate CD11c+ splenocytes from each mouse on the x-axis. DAPI (0.5 µg/ml, Sigma-Aldrich) or a Live/Dead fixable near-IR dead cell stain kit (Invitrogen) was used to exclude dead cells in all experiments, and anti-CD11c/CD32 Ab (2.4G2) was used to block non-specific Ab binding via Fc receptors. The following Abs were purchased from BioLegend, BD Biosciences, or eBioscience: anti-CD3 (clone 17A2), anti-CD4 (RM4.5), anti-CD8α (53-6.7), anti-CD16/CD32 (2.4G2) anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD19 (1D3), anti-CD24 (M1/69), anti-CD25 (3C7), anti-CD40 (3/23), anti-CD43 (1B11), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD90.2 (30-H12),

FIGURE 2. DC cancer development from a minimal number of transformed cells. (A) Experimental setup for the generation of mixed bone marrow chimeras with a reduced frequency of Clec9a<sup>Kras-G12D</sup> or Clec9a<sup>Kras-wt</sup> bone marrow cells. Lethally irradiated C57BL/6 x B6.SJL mice (heterozygous for the congenic markers CD45.1 and CD45.2) were reconstituted with bone marrow cells from B6.SJL mice (CD45.1<sup>+</sup>) mixed with CD11c<sup>+</sup> bone marrow cells from Clec9a<sup>Kras-G12D</sup> mice (CD45.2<sup>+</sup>) at different ratios (50, 5, or 0.5% of Clec9a<sup>Kras-G12D</sup> bone marrow cells). Chimeras that received bone marrow cells from B6.SJL mice mixed with bone marrow cells of Clec9a<sup>Kras-wt</sup> mice (CD45.2<sup>+</sup>) served as control. (B) Analysis of survival of the different bone marrow chimeras after generation. Statistical analysis was performed using the log-rank (Mantel–Cox) test. Data are pooled from two independent experiments with a total of 16–18 mice in each group. Percentages stated in the legend denominate the fraction of CD45.2<sup>+</sup> bone marrow cells within the donor bone marrow for each group. (C–E) Flow cytometric quantification of donor bone marrow–derived CD45.2<sup>+</sup>/2 CD11c<sup>+</sup>MHC II<sup>+</sup> DCs and CD45.1<sup>+</sup>/1 CD11c<sup>+</sup>MHC II<sup>+</sup> DCs in (C) spleen, (D) liver, and (E) lung at the endpoint when mice had to be sacrificed due to disease severity limits. Each dot in (C–E) represents data for one individual mouse; data are representative for two independent experiments. Error bars depict SEM. ***p < 0.001.
anti-CD103 (2E7), anti-CD115 (AF884), anti-CD172a/Sirp1α (PS84), anti-CD205 (ND14-15), anti-CD220 (RA3-6B2), anti-NK1.1 (PK136), anti-Ter119 (TER119), anti-CD-1 (B20; 10F9; 9G2), anti-CD-L2 (TY25), anti-MHC class II (MHC II) 1-11-6 (M5/141,15,2), anti-DNGR-1 (1F6), anti-TNF (MP6-X220), and anti-IL-12 p40 (C17.8). Anti-CD207 (clone 92F01) was purchased from Dendritics. An Ab mixture consisting of anti-CD4, anti-CD8, anti-CD11b, anti-CD20, anti−MHC II, anti-NK-1.1, and anti-Ter119 was used for the exclusion of differentiated lineage (Lin) cells for the analysis of CDPs and pre-DCs in bone marrow and spleen. Bone marrow CDPs were identified as Lin−MHC II−CD11c+DNGR-1+ cells, bone marrow pre-DCs as Lin−MHC II+CD11c+DNGR-1− cells, and splenic pre-DCs as Lin−MHC II+CD11c+CD135+CD43+ cells.

Analysis of TCR VB families was done using a mouse TCR screening panel (BD Biosciences) according to the manufacturer's instructions. Quantification of total cell numbers by flow cytometry was done using fluorescent beads (Beckman Coulter). For intracellular staining of CD207 or the cytokines TNF and IL-12p40, cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Intracellular staining was performed in permeabilization buffer (eBioscience) for 30 min and cells were subsequently analyzed by flow cytometry.

For sorting of DCs, splenocytes were enriched for CD11c+ cells using anti-CD11c microbeads (Miltenyi Biotec) and CD11c-enriched splenocytes were then stained for CD8a, CD11b, CD11c, and Sirp1α. CD8α+CD11c+Sirp1α+CD11b+YFP+DC11c+ cells and CD8α−DCs (CD8α−Sirp1α−CD11b+YFP+DC11c− cells) were sorted using a BD FACSaria or a BD FACSaria Fusion.

**Immunofluorescence imaging**

Spleens, livers, thymi, and inguinal lymph nodes were harvested and fixed in paraformaldehyde/lysine/peroxidate−containing buffer as previously described (12, 13). Specimens were dehydrated in 30% sucrose prior to embedding in tissue Tek OCT freezing medium (Sakura Finetek) and stored at −80°C. Ten-micrometer sections were permeabilized, blocked, and stained in 0.1 M Tris (AppliChem) supplemented with 1% BSA, 0.3% Triton X-100 (Gerbu Biotechnik), 1% gelatin from cold water fish skin (Sigma-Aldrich), and normal mouse serum (Life Technologies). Staining was done with anti-mouse keratin 8 (clone Ks 8.7, Progen Biotechnik), anti-mouse keratin 14 (polyclonal, Covance), anti-mouse B220 (clone 145-2C11, eBioscience). To increase detection of YFP+ cells, sections were stained with a polyclonal anti-GFP rabbit IgG Alexa Fluor 488 Ab that is cross-reactive with TCR α. Stained sections were analyzed on LSM 710s or LSM 780 confocal microscopes (Zeiss) with ×20 and ×10 objectives. Images were analyzed using ImageJ or Imaris software.

**Statistical analysis**

Statistical significance was calculated using the unpaired, two-tailed Student's t test, ANOVA, or log-rank (Mantel–Cox) test. A p value <0.05 was considered significant.

**Results**

**Oncogenic transformation in Clec9aKras-G12D mice leads to massive accumulation of DCs in lymphoid and peripheral organs**

To develop a mouse model for oncogenic transformation of DCs, we sequentially crossed Clec9aKrasG12D/Rosa26YFP/YFP mice (11, 30) to Trp53-lox/lox (Trp53fl/fl) mice (17, 32) and “knock-in” Kras-stopflox G12D (Kras+/lsl-G12D) mice (19, 28, 33) to generate Clec9aKrasG12D/Rosa26YFP/YFP mice (hereafter referred to as Clec9aKrasG12D mice; Fig. 1A). In these mice, Cre recombinase activity induces both the expression of the oncogenic KrasG12D allele (KrasG12D) under its endogenous promoter and the deletion of the tumor suppressor p53 (encoded by the Trp53 gene). The combination of KrasG12D expression with p53 deficiency ensured that DCs did not undergo oncogene-induced senescence due to KrasG12D expression (15, 16, 22). Of note, this crossing strategy also generates Clec9aKrasG12D/Rosa26YFP/YFP mice (hereafter referred to as Clec9aKraswt mice) as appropriate littermate controls.

When analyzed for survival, Clec9aKrasG12D mice all succumbed within 9–12 wk after birth (Fig. 1B). In contrast, Clec9aKraswt mice were healthy over the same time period (Fig. 1B), despite the presumed deficiency of p53 within cells of the DC lineage. Furthermore, 7-wk-old Clec9aKrasG12D mice showed early onset of splenomegaly and lymphadenopathy (Fig. 1C), which was accompanied by a dramatic increase in the fraction of CD11c+MHC II+ DCs (Fig. 1D). Most splenic DCs in Clec9aKrasG12D mice were YFP+, indicating successful Cre recombinase activity at some point in their developmental history (Fig. 1D).

During ontogeny of Clec9aKrasG12D/Rosa26YFP/YFP mice, YFP+ DCs are not observed before 3−4 wk after birth (unpublished observations). Nevertheless, 4-wk-old Clec9aKrasG12D mice already showed a strong increase in the absolute number of splenic DCs compared with controls and this number continued to increase with age (Fig. 1E). A similar although less pronounced elevation in number of total DCs was observed in lymph nodes of Clec9aKrasG12D mice (Fig. 1F). The increase of DCs in Clec9aKrasG12D mice was not restricted to lymphoid organs, as we observed DC hyperplasia in all examined tissues, including blood (Fig. 1G), bone marrow (Fig. 1H), liver (Fig. 1I), and lung (Fig. 1J).

To assess whether the increase in numbers altered DC compartmentalization, we investigated the anatomical distribution of YFP+ cells within tissues using confocal immunofluorescence microscopy. In the spleen of Clec9aKrasG12D mice, YFP+ DCs did not show preferential localization compared with YFP−DCs in Clec9aKraswt mice, as they were highly enriched in both the T cell zone and the red pulp (Fig. 1K). Similarly, YFP+ DCs in lymph nodes of Clec9aKrasG12D mice were enriched but did not show altered distribution (Supplemental Fig. 1A). The same was observed in liver, where YFP+ DCs in Clec9aKrasG12D mice primarily accumulated at their normal anatomical niche, close to the periportal area (Supplemental Fig. 1B). However, in all these organs we observed YFP+ cell clusters, suggestive of local tumor formation. Taken together, these data demonstrate that oncogenic transformation of DCs in Clec9aKrasG12D mice leads to fast development of DC cancer in lymphoid and peripheral organs.

A minimum number of transformed cells is sufficient for rapid DC cancer development

The rapid growth of transformed DCs suggested that DC cancer development cannot be controlled in Clec9aKrasG12D mice. We hypothesized that the high precursor frequency of transformed cells might overwhelm the immune system, allowing DC tumor progression. Additionally, the dearth of untransformed DCs might hinder the initiation of an immune response against transformed DCs if the latter cannot prime T cells directly. To test these hypotheses, we restricted transformation to a reduced number of DCs by generating mixed bone marrow chimeras in which the fraction of Clec9aKrasG12D bone marrow cells versus normal marrow from B6, SJL mice was set to 50, 5, or 0.5% (Fig. 2A). To our surprise, all chimeras succumbed from DC cancer irrespective of the frequency of Clec9aKrasG12D bone marrow cells initially used for reconstitution (Fig. 2B). The only variation was seen in the time that it took for mice to reach the endpoint: groups reconstituted with only 0.5% of Clec9aKrasG12D bone marrow cells displayed marginally increased survival (median survival, 11.7 wk) compared with groups reconstituted with 5 and 50% (median survival, 9.8 and 7.9 wk, respectively) (Fig. 2B). At this endpoint, the absolute numbers of Clec9aKrasG12D-derived CD45.2+ DCs in all tested organs were similar across all chimeras groups (Fig. 2C–E, Supplemental Fig. 2). In some Clec9aKrasG12D/B6.SJL chimeric animals, there was a concomitant reduction in CD45.1+ DCs compared with Clec9aKraswt/B6.SJL reconstituted controls, which might have been due to competition by cancer DCs.
FIGURE 3. Cancer formation in Clec9α<sup>Kras-G12D</sup> mice is dominated by CD8α<sup>+</sup> DCs. (A and B) Bone marrow cells of Clec9α<sup>Kras-G12D</sup> mice and Clec9α<sup>Kras-wt</sup> mice were analyzed for absolute numbers of (A) CDPs (Lin<sup>-</sup> MHC II<sup>-</sup> CD11c<sup>-</sup> CD115<sup>-</sup> CD4<sup>-</sup>CD135<sup>-</sup>CD43<sup>-</sup> cells) or (B) pre-DCs (Lin<sup>-</sup> MHC II<sup>-</sup> CD11c<sup>+</sup> DNGR-1<sup>-</sup> cells). (C) Absolute numbers of splenic pre-DCs (Lin<sup>-</sup> MHC II<sup>-</sup> CD11c<sup>+</sup>CD135<sup>-</sup>CD43<sup>-</sup> cells). (D) Flow cytometric analysis and (E) absolute numbers of CD8α<sup>+</sup> and CD11b<sup>+</sup> DCs in spleens of from Clec9α<sup>Kras-G12D</sup> mice and Clec9α<sup>Kras-wt</sup> mice. Data are shown relative to control tissue (set as 100%). (F and G) Efficiency of Cre-mediated excision of the loxP-flanked stop codon at the (F) Kras<sub>G12D</sub> locus or (G) the loxP-flanked Trp53 locus in genomic DNA from tail tissue, FACS-sorted splenic CD8<sup>+</sup> DCs, or FACS-sorted CD8α<sup>+</sup>YFP<sup>+</sup> DCs from Clec9α<sup>Kras-G12D</sup> mice and Clec9α<sup>Kras-wt</sup> mice. (H) CD8α<sup>+</sup> DCs from Clec9α<sup>Kras-G12D</sup> mice and Clec9α<sup>Kras-wt</sup> mice were sorted and left unstimulated or stimulated ex vivo for 6 h with CpG (0.05, 0.5, and 5 μg/ml), LPS (1, 10, and 100 ng/ml), or poly(I:C) (0.1, 1, and 10 μg/ml). Surface expression of CD40, CD80, and CD86 was analyzed by flow cytometry. (I) FACS-sorted CD8α<sup>+</sup> DCs (5 × 10<sup>4</sup> cells/well) from Clec9α<sup>Kras-G12D</sup> mice and Clec9α<sup>Kras-wt</sup> mice were stimulated for 18 h as indicated. Quantification of IL-12p70, IL-6, CCL3, and CCL5 protein was done from cell culture supernatants by ELISA (for IL-12p70) or cytometric bead (Figure legend continues)
Cancer formation in Clec9a<sup>Kras-G12D</sup> mice is dominated by CD8α<sup+a</sup> DCs

Clec9a promoter activity early during DC ontogeny might lead to an increase in CDPs or pre-DCs. However, the number of CDPs and pre-DCs in bone marrow of Clec9a<sup>Kras-G12D</sup> mice was either reduced or unchanged compared with Clec9a<sup>Kras-wt</sup> mice (Fig. 3A, 3B). Additionally, we did not observe an elevated number of splenic pre-DCs in Clec9a<sup>Kras-G12D</sup> mice (Fig. 3C). Thus, the potential transformation of DC precursors did not seem to halt their differentiation into DCs. As pre-DCs give rise to two distinct subsets of classical DCs in lymphoid organs, CD8α<sup+a</sup> DCs and CD11b<sup+b</sup> DCs (1, 3, 14), both of these subsets could be responsible for the aberrant increase in DC numbers observed in Clec9a<sup>Kras-G12D</sup> mice. However, Clec9a<sup>Kras-G12D</sup> mice consistently displayed an elevated frequency of CD8α<sup+a</sup> DCs compared with CD11b<sup+b</sup> DCs (Fig. 3D, 3E). The total number of CD8α<sup+a</sup> DCs in Clec9a<sup>Kras-G12D</sup> mice continued to increase with age whereas the number of CD11b<sup+b</sup> DCs remained constant albeit elevated compared with controls (Fig. 3D, 3E). To address whether this subset difference correlated with a differential rate of oncogene activation, we performed recombination-sensitive quantitative real-time PCR on genomic DNA of CD8α<sup+a</sup> DCs and CD11b<sup+b</sup> DCs (identified by a CD8α<sup>+</sup> Sirp1α<sup+a</sup>YFP phenotype in this study). Compared to control genomic DNA from tail tissue, both CD8α<sup+a</sup> DCs and CD11b<sup+b</sup> DCs showed efficient excision of the stop cassette at the Kras<sup>G12D</sup> locus (Fig. 3F). However, whereas the Trp53-foxed gene was almost absent in CD8α<sup+a</sup> DCs, CD11b<sup+b</sup> DCs only showed a partial deficiency in the Trp53-foxed gene (Fig. 3G), despite their history of Cre activity indicated by YFP expression. Thus, the efficiency of Trp53 gene excision is most likely responsible for the time-dependent accumulation of CD8α<sup+a</sup> DCs but not CD11b<sup+b</sup> DCs in adult Clec9a<sup>Kras-G12D</sup> mice. This likely relates to the fact that the Clec9a locus continues to be expressed in CD8α<sup+a</sup> DCs, which permits longer Cre expression (11, 16) and might facilitate recombination at the Trp53 locus.

We next addressed whether oncogenic transformation would affect the phenotype or activation status of CD8α<sup+a</sup> DCs. When analyzing CD8α<sup+a</sup> DCs from spleen of 7-wk-old Clec9a<sup>Kras-G12D</sup> mice and Clec9a<sup>Kras-wt</sup> mice, we did not detect a difference in expression of YFP or lineage specific markers such as CD24, CD135, and only a slight elevation in CD103 expression (Supplemental Fig. 3A). At the steady-state, Clec9a<sup>Kras-G12D</sup> DCs and Clec9a<sup>Kras-wt</sup> DCs did not differ in their expression of the MHC class I molecule H2-K<sup>b</sup>, the costimulatory molecules CD40, CD80, and CD86, or the coinhibitory molecules PD-L1 and PD-L2 (Supplemental Fig. 3B). Upregulation of costimulatory molecules by Clec9a<sup>Kras-G12D</sup> DCs was observed within 6 h of stimulation by CpG, LPS, or poly(I:C) and might facilitate recombination by CpG, LPS, or poly(I:C) and was comparable to that in Clec9a<sup>Kras-wt</sup> DCs (Fig. 3). Clec9a<sup>Kras-G12D</sup> DCs were able to produce cytokines such as IL-12p70 and IL-6 and chemokines such as CCL3, CCL5, CXCL9, and CXCL10 after stimulation, but this did not occur when DCs were left unstimulated (Fig. 3I). In line with these results, Clec9a<sup>Kras-G12D</sup> DCs and Clec9a<sup>Kras-wt</sup> DCs were indistinguishable in their ability to induce proliferation of OT-I and OT-II T cells in an Ag-specific manner (Fig. 3J, Supplemental Fig. 3E). Of note, Clec9a<sup>Kras-G12D</sup> DCs could be serially passaged in vitro and retained their phenotype (Supplemental Fig. 3F), effectively allowing for the generation of immortalized cell lines (named KID and given a number suffix to designate different lines, for example, KID-1) akin to what has been reported for cells taken from CD11c:<sup>SV40LgT</sup> mice (27, 34). Together, these data demonstrate that CD8α<sup+a</sup> DCs of Clec9a<sup>Kras-G12D</sup> mice retain a normal phenotype and functional ability to respond to stimulation, despite their oncogenic transformation.

Early onset of DC cancer in the thymus leads to a loss of thymocytes in Clec9a<sup>Kras-G12D</sup> mice

In addition to their presence in secondary lymphoid organs, CD8α<sup+a</sup> DCs constitute most DCs within the thymus, where they contribute to negative selection of T cells (28). We observed an elevation of total DC numbers in thymi of Clec9a<sup>Kras-G12D</sup> mice that was evident from 4 to 7 wk of age (Fig. 4A). This increase in DC numbers was accounted for by accumulation of resident CD8α<sup+a</sup> DCs, as the number of Sirp1α<sup+a</sup> thymic migratory DCs was reduced or normal (Fig. 4B).

Thymic CD8α<sup+a</sup> DCs develop locally within the thymus and reside primarily within the thymic medulla (28). Indeed, most of the YFP<sup+a</sup> DCs in 4-wk-old Clec9a<sup>Kras-wt</sup> mice were found in the thymic medulla but not the cortex (Fig. 4C). Thymi of Clec9a<sup>Kras-G12D</sup> mice showed an increase of YFP<sup+a</sup> DCs in both medulla and cortex. As the total number of thymic Sirp1α<sup+a</sup> DCs remained unchanged in Clec9a<sup>Kras-G12D</sup> mice (see Fig. 4B), the increase in cortical DCs indicates that CD8α<sup+a</sup> DCs gained access to the thymic cortex of Clec9a<sup>Kras-G12D</sup> mice (Fig. 4C). Next, we addressed how these changes in DC numbers affected thymic T cell development. Clec9a<sup>Kras-G12D</sup> mice showed a dramatic reduction in thymocyte numbers that was apparent already in 4-wk-old mice (Fig. 4D). This reduction was observed at all stages of thymocyte development, but was most obvious in double-negative (DN) and double-positive thymocytes (Fig. 4E). Furthermore, DN thymocytes showed a complete loss of the DN2 (CD4<sup>+</sup>CD25<sup>+</sup>) and DN3 (CD4<sup>+</sup>CD25<sup>+</sup>) stages (Fig. 4E). These results demonstrate that T cell development is strongly affected in Clec9a<sup>Kras-G12D</sup> mice. However, this defect does not seem to rely on increased negative selection by transformed DCs, as it is observed in DN thymocytes and thus prior to the expression of a TCR.

Clec9a<sup>Kras-G12D</sup> mice develop a normal immune cell repertoire

We wondered how reduced thymocyte numbers in Clec9a<sup>Kras-G12D</sup> mice impacted the peripheral T cell repertoire. We first analyzed T cell numbers in spleen of Clec9a<sup>Kras-G12D</sup> and Clec9a<sup>Kras-wt</sup> mice. Strikingly, we did not observe alterations in the total number of splenic CD8<sup>+</sup> T cells (Fig. 5A) or CD4<sup>+</sup> T cells (Fig. 5B) at any time point. Furthermore, the frequencies of TCR Vβ families remained unchanged among CD8<sup>+</sup> T cells (Fig. 5C) and showed only minimal alterations among CD4<sup>+</sup> T cells (Fig. 5D). This indicated that the defect in thymic T cell development was compensated in Clec9a<sup>Kras-G12D</sup> mice, most likely by homeostatic proliferation. Consistent with that notion (29), we found that both the CD8<sup>+</sup> T cell pool and the CD4<sup>+</sup> T cell pool showed an elevated frequency of CD4<sup>+</sup>CD62L<sup>−</sup> T cells (Fig. 5E, 5F). These cells
lacked granzyme B (data not shown), indicating that they were not cytotoxic effector cells. Furthermore, Clec9a Kras-G12D mice did not show any alterations in the absolute number of NK cells, B cells, monocytes, macrophages, or neutrophils in spleen (data not shown).

Clec9a Kras-G12D DC cancers can be rejected following transplantation.

The rapid accumulation of cancer DCs suggested that DC transformation does not lead to anti-cancer immunity in Clec9a Kras-G12D mice despite a relatively intact immune system. We wondered...
whether transformed DCs could in principle be rejected by lymphocytes. To investigate this, we purified CD8αYFP+ DCs from Clec9aKras-G12D mice and transferred them into wild-type or T and B cell–deficient Rag22/2 mice. Twenty days later, YFP+ DCs were clearly visible and had undergone expansion in Rag22/2 mice whereas they could not be detected in wild-type mice (Fig. 6A, 6B). We obtained similar results after transfer of KID cell lines (Fig. 6C, 6D). This demonstrates that transformed DCs can in principle evoke an anti-cancer immune response that may result in their rejection, as reported recently (30). However, this does not happen during spontaneous DC cancer development in Clec9aKras-G12D mice.

Discussion
It has been hypothesized that the central role of DCs in priming the immune system might constitute a strong impediment to DC tumor development (13). However, the impact of neoplastic transformation on immunological properties of DCs and potential immune evasion mechanisms during DC cancer development have not received much experimental attention. In the present study, we report that DC cancer can develop with 100% penetrance in a genetically engineered mouse model in which neoplastic transformation is restricted to DC precursors and differentiated CD8α+ DCs. Mouse DC cancers are found in both nonlymphoid and lymphoid organs from an early age, grow aggressively, and result in death of the host within a short time frame. The rampant development of systemic DC cancer in the model described in the present study indicates that intrinsic DC immunogenicity is not necessarily a barrier to DC cancer development, leaving open the question as to the cause of its rarity among hematological malignancies.

Clec9aKras-G12D DCs, as well as cell lines derived from the same mice, can be rejected in wild-type but not Rag22/2 mice after adoptive transfer, as reported for DC lines derived from CD11c:SV40LgT mice (30). This finding indicates that DC cancers bear Ags that, in principle, can be targeted by lymphocytes to mediate cell rejection. We cannot exclude that some of these Ags might correspond to minor histocompatibility Ags despite extensive backcrossing of our mouse lines onto a C57BL/6 background. However, it is tempting to speculate that they might rather correspond to tumor Ags from mutations in self proteins that arise as a result of tumor genomic instability. Why these Ags do not cause rejection in the original host but do so after cell transplantation is at present unclear. It could reflect increased immunogenicity of
transferred DCs, which might result from technical aspects, for example, the process of cell sorting, which can promote so-called “spontaneous DC maturation” (32). Indeed, transformation by RasG12D does not appear to lead to DC activation, as transformed DCs do not spontaneously produce cytokines or display elevated levels of costimulatory molecules. The immune system might therefore fail to be appropriately primed by a continuous stream of DCs lacking a TCR, indicating that the impairment of development rather depends on mechanisms other than negative selection of certain TCR specificities. In line with this notion, the peripheral T cell repertoire seemed to be broadly unaffected in Clec9a<sup>Kras-G12D</sup> mice, probably due to compensatory homeostatic proliferation. More detailed analyses, for example, by TCR sequencing, are necessary to determine whether Clec9a<sup>Kras-G12D</sup> mice lack certain TCR specificities in their T cell repertoire.

In Clec9a<sup>Kras-G12D</sup> mice, expression of the oncogene Kras<sup>G12D</sup> in cells of the DC lineage occurs spontaneously during mouse development, which recapitulates many aspects of normal cancer development and allows us to extensively characterize the development of DC cancer over time and space. However, it also constitutes a supraphysiological cancer model, as neoplastic transformation in Clec9a<sup>Kras-G12D</sup> mice is predicted to constantly occur in most cells of the DC lineage. Strikingly, when we reduced the number of transformed DCs by generating mixed bone marrow chimeras, there was only marginally improved survival and no reduction in tumor incidence. This clearly indicates that a limited number of transformed cells is sufficient to generate DC tumors, even in the presence of a large majority of normal untransformed DCs. However, the onset of DC tumor formation cannot be controlled in Clec9aKras-G12D mice, which most likely precedes the development of a normal immune system. This issue should be taken into account in future studies by a further refinement of the model, for example by placement of a tamoxifen-inducible Cre (CreERT) under the Clec9a promoter to allow spatial and temporal control of oncogenic expression in DCs.

In humans, histiocytic disorders (a hyperplasia of DCs or macrophages) are very rare and sometimes suspected to reflect an immunological disorder rather than neoplastic transformation (15, 16). However, recent studies have demonstrated that pathological lesions from patients with LCH frequently display oncogenic mutations within genes encoding proteins in the Ras signaling pathway (1, 14). It was originally assumed that the neoplastic transformation responsible for cancer development in LCH occurs within CD1a<sup>+</sup>CD207<sup>+</sup> (Langerin<sup>+</sup>) LCs in the skin (16). However, oncogenic mutations are not only found in cells that phenotypically resemble LCs but are also detected in Langerin<sup>−</sup> cells within histiocytic lesions (34). Furthermore, Langerin is expressed on certain DC subsets, which puts in question the transformation of LCs as the underlying cause of LCH (35). Notably, multisystemic DC cancer does not occur when oncogene expression is restricted to Langerin<sup>+</sup> cells in a mouse model (1). In the same study, it was speculated that the development of multisystemic DC cancer might require the transformation of an early DC precursor rather than differentiated DCs. In line with this hypothesis, Clec9a<sup>Kras-G12D</sup> mice develop DC cancer in multiple organs, probably resulting from oncogene expression in DNGR-1<sup>−</sup> CDPs and pre-DCs (11). However, we did not observe elevated numbers of CDPs or pre-DCs in this mouse model, arguing that their transformation does not affect their further differentiation or pre-DC migration. As LCs do not express DNGR-1 and do not descend from DNGR-1<sup>+</sup> DC precursors (11), our data indicate that cells other than LCs can efficiently generate DC-like tumors upon neoplastic transformation. Whether neoplastic transformation of DNGR-1<sup>−</sup>BDCA3<sup>+</sup> DCs, the human equivalent of murine CD8α<sup>+</sup> DCs, could similarly be responsible for certain multisystemic histiocytic disorders in humans is a possibility that deserves to be investigated.

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Disclosures
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References
1. Berres, M.-L., K. P. H. Lim, T. Peters, J. Price, H. Takizawa, H. Salmon, J. Idoyaga, A. Ruzo, P. J. Lupo, M. J. Hicks, et al. 2014. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. J. Exp. Med. 211: 669–683.

2. Steinman, R. M., and J. Idoyaga. 2010. Features of the dendritic cell lineage. Immunol. Rev. 234: 5–17.

3. Merad, M., P. Sathe, J. Helft, J. Miller, and A. Mortha. 2013. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu. Rev. Immunol. 31: 563–604.

4. Karsunky, H., M. Merad, A. Cozio, L. L. Weissman, and M. G. Manz. 2003. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. J. Exp. Med. 198: 305–313.

5. Onai, N., O. Ohata-Onai, M. A. Schmidt, and M. G. Manz. 2007. Flt3 in regulation of type I interferon-producing cell and dendritic cell development. Ann. N. Y. Acad. Sci. 1106: 253–261.

6. Schulz, C., E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kiardorf, M. Prinz, B. Wu, S. E. W. Jacobsen, J. W. Pollard, et al. 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science 336: 86–90.

7. Naik, S. H., D. Metcalf, A. van Nieuwenhuijze, J. Wicks, L. Wu, M. O’Keeffe, and K. Shortman. 2006. Intraperitoneal steady-state dendritic cell precursors that are distinct from monocytes. Nat. Immunol. 7: 663–671.

8. Liu, K., C. Waskow, X. Liu, K. Yao, J. Hoh, and M. Nussenzweig. 2007. Origin of dendritic cells in peripheral lymphoid organs of mice. Nat. Immunol. 8: 578–583.

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

10. Stein, Q. G., L. A. Otten, M. J. Hicks, G. Kaya, F. Grosjean, E. Saeuberli, W., M. Brandes, Z. Wang, J. Herz, J. G. Egen, and R. N. Germain. 2003. Identification of a novel population of Langerin+ dendritic cells in the tumor microenvironment. Nat. Immunol. 14: 1014–1022.

11. Dholadkar, M. V., K. M. Dholadkar, and A. K. Palucka. 2008. Interactions of tumour cells with dendritic cells: balancing immunity and tolerance. Cell Death Differ. 15: 39–50.

12. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. Annu. Rev. Immunol. 18: 765–811.

13. Allen, C. E., L. Li, T. L. Peters, H. C. E. Leung, A. Yu, T. K. Man, S. Gurusiddappa, M. T. Phillips, M. J. Hicks, A. Gaikwad, et al. 2010. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. J. Immunol. 184: 4557–4567.

14. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. Annu. Rev. Immunol. 21: 685–711.

15. Allen, C. E., L. Li, T. L. Peters, H. C. E. Leung, A. Yu, T. K. Man, S. Gurusiddappa, M. T. Phillips, M. J. Hicks, A. Gaikwad, et al. 2010. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. J. Immunol. 184: 4557–4567.

16. Jackson, E. L., N. Willis, K. Mercer, R. T. Bronson, D. Crowley, R. Montoya, T. Jacks, and D. A. Tuveson. 2001. Analysis of lung tumor initiation and progression using conditional expression of oncogenic Kras. Genes Dev. 15: 3243–3248.

17. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. Annu. Rev. Immunol. 18: 765–811.

18. Steinman, R. M., C. E. Allen, L. Li, T. L. Peters, H. C. E. Leung, A. Yu, T. K. Man, S. Gurusiddappa, M. T. Phillips, M. J. Hicks, A. Gaikwad, et al. 2010. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. J. Immunol. 184: 4557–4567.

19. Jackson, E. L., N. Willis, K. Mercer, R. T. Bronson, D. Crowley, R. Montoya, T. Jacks, and D. A. Tuveson. 2001. Analysis of lung tumor initiation and progression using conditional expression of oncogenic Kras. Genes Dev. 15: 3243–3248.

20. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. Annu. Rev. Immunol. 21: 685–711.

21. Allen, C. E., L. Li, T. L. Peters, H. C. E. Leung, A. Yu, T. K. Man, S. Gurusiddappa, M. T. Phillips, M. J. Hicks, A. Gaikwad, et al. 2010. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. J. Immunol. 184: 4557–4567.

22. Jackson, E. L., N. Willis, K. Mercer, R. T. Bronson, D. Crowley, R. Montoya, T. Jacks, and D. A. Tuveson. 2001. Analysis of lung tumor initiation and progression using conditional expression of oncogenic Kras. Genes Dev. 15: 3243–3248.

23. Bursch, L. S., L. Wang, B. Igyarto, A. Kissenpfennig, B. Malissen, D. H. Kaplan, and K. A. Hogquist. 2007. Identification of a novel population of Langerin+ dendritic cells. J. Exp. Med. 204: 3147–3156.