

Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity

Camie W Chan¹, Emily Crafton¹, Hong-Ni Fan¹, James Flook¹, Kiyoshi Yoshimura¹, Mario Skarica¹, Dirk Brockstedt², Thomas W Dubensky², Monique F Stins³, Lewis L Lanier⁴, Drew M Pardoll¹ & Franck Housseau¹

Natural killer (NK) cells and dendritic cells (DCs) are, respectively, central components of innate and adaptive immune responses^{1,2}. We describe here a third DC lineage, termed interferon-producing killer DCs (IKDCs), distinct from conventional DCs and plasmacytoid DCs and with the molecular expression profile of both NK cells and DCs. They produce substantial amounts of type I interferons (IFN) and interleukin (IL)-12 or IFN- γ , depending on activation stimuli. Upon stimulation with CpG oligodeoxynucleotides, ligands for Toll-like receptor (TLR)-9, IKDCs kill typical NK target cells using NK-activating receptors. Their cytolytic capacity subsequently diminishes, associated with the loss of NKG2D receptor (also known as Klrk1) and its adaptors, Dap10 and Dap12. As cytotoxicity is lost, DC-like antigen-presenting activity is gained, associated with upregulation of surface major histocompatibility complex class II (MHC II) and costimulatory molecules, which formally distinguish them from classical NK cells. *In vivo*, splenic IKDCs preferentially show NK function and, upon systemic infection, migrate to lymph nodes, where they primarily show antigen-presenting cell activity. By virtue of their capacity to kill target cells, followed by antigen presentation, IKDCs provide a link between innate and adaptive immunity.

Ever since the original Janeway hypothesis that successful adaptive immunity is dependent on innate immunity³, numerous reports have documented DCs as a key component in bridging both arms of the immune system⁴. Before DCs activate naive T cells, they must complete a maturation program that is initiated either by direct exposure to pathogens or specific interactions with other innate immune cells⁴. To sense invading pathogens, DCs express an extensive panel of pattern-recognition receptors, including TLRs⁵. TLR signaling in DCs controls secretion of cytokines, migration, proliferation, survival and expression of MHC II and costimulatory molecules². Activation of DCs can also be enhanced by activated NK cells. A number of studies recently established that NK or NKT cells and DCs can mutually influence each other's respective activity, leading to

a finely orchestrated cellular crosstalk crucial in shaping the ensuing adaptive immune response⁶.

To study further mechanisms linking NK cells and DC-dependent T-cell activation, we investigated whether a single cell might be responsible for both NK-type killing of targets and subsequent processing and presentation to T cells of antigens derived from those killed targets. Here, we report a distinct population of cells, termed interferon-producing killer DCs (IKDCs), that expressed similar levels of CD11c, B220 and MHC II as plasmacytoid DCs (PDCs), but were Gr1⁻ (Fig. 1a,b). Notably, CD49b, which corresponds to VLA-2, an integrin commonly found on NK cells⁷, was also expressed on these cells. Expression of CD49b and expression of Gr1 were inversely correlated (Fig. 1c). None of the CD11c^{high} conventional DCs (cDCs) expressed CD49b. This cell subset has been identified in all mouse strains tested, including BALB/c, C57BL/6, DBA and C3H/HeJ (Supplementary Fig. 1 online). In C57BL/6 mice, which express NK1.1 (also known as NKR-P1c), all of the CD11c^{int}B220⁺Gr1⁻ cells stained brightly for NK1.1 (data not shown).

Comparison of differentially expressed genes between IKDCs and cDCs indicated selective expression of multiple NK-related genes in IKDCs (Supplementary Table 1 online). These included *Gzma*, *Gzmb*, *Gzmk*, *Gzmm*, *Prf1*, *Fasl*, *Klrc1*, *Klrk1*, *Klra* family genes, *Klrb1c*, *Nkg7*, *Ncr1* and *Mafa*. No NK-related genes were highly expressed in the PDCs. However, we found that B-cell lineage transcripts such as *Igj* and *Igh D-J-C* were equivalently expressed in IKDCs and PDCs (data not shown). Unlike PDCs, a proportion of IKDCs stained with monoclonal antibodies specific for Ly49 and NKG2A/C/E, and all expressed the NK-activating receptor NKG2D (Fig. 1d). IKDCs showed a high sensitivity to the Tlr-9 ligand, unmethylated CpG oligodeoxynucleotide (ODN) 1668, developing dendrites and veils (Supplementary Fig. 2 online) and upregulating MHC II molecules (Fig. 1e). Staining with an expanded panel of monoclonal antibodies showed the notable homogeneity of the IKDC population (Fig. 1f) and distinguished them from PDCs and NK cells. IKDCs were mPDCA-1⁻ and CD122⁺, whereas PDCs were mPDCA-1⁺ and CD122⁻. A similar cell subset was found in lymph nodes and had a

¹Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, CRB-440, 1650 Orleans Street, Baltimore, Maryland 21231, USA.

²Cerus Corporation, 2411 Stanwell Drive, Concord, California 94520, USA. ³Department of Pediatrics, Division of Infectious Diseases, Johns Hopkins University Medical School, 720 Rutland Avenue, Ross 1170, Baltimore, Maryland 21205, USA. ⁴Department of Microbiology and Immunology and the Cancer Research Institute, University of California San Francisco, 513 Parnassus Avenue, HSE 420, San Francisco, California 94143, USA. Correspondence should be addressed to F.H. (fhousse1@jhmi.edu) or D.M.P. (dmpardoll@jhmi.edu).

Received 28 July 2005; accepted 5 December 2005; published online 29 January 2006; doi:10.1038/nm1352

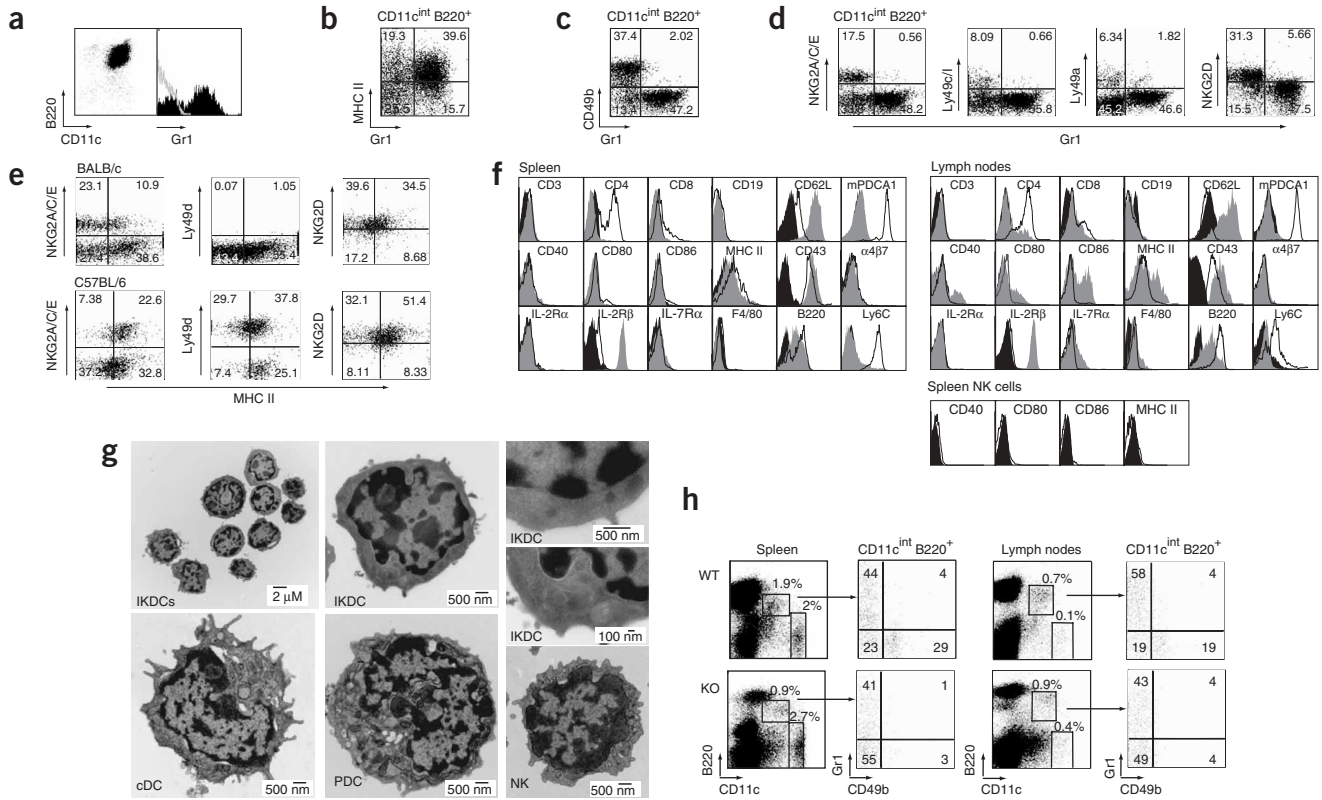


Figure 1 Comparative phenotypic and morphologic characterization of IKDCs, PDCs and NK cells. **(a)** We purified BALB/c splenic CD11c^{int}B220⁺ cells (left panel) and stained them for surface expression of Gr1 (right panel). Isotype control, open histogram. **(b)** MHC II expression on both CD11c^{int}B220⁺Gr1⁻ and Gr1⁺ cells. **(c)** We used Gr1 and CD49b-specific monoclonal antibodies to stain CD11c^{int}B220⁺ cells. IKDCs are CD11c^{int}B220⁺Gr1⁻ and CD49b⁺. **(d)** We stained CD11c^{int}B220⁺ cells for the expression of Gr1 versus NK markers. **(e)** BALB/c and C57BL/6 splenic CD11c^{int}B220⁺Gr1⁻ IKDCs co-express NK markers and MHC II. FACS plots in panels **a–e** are representative of at least three experiments. **(f)** Representative FACS staining of splenic and lymph node IKDCs (gray histogram), PDCs (open histograms) and splenic NK cells. Isotype control, black histogram. **(g)** Representative ultrastructure details of freshly sorted splenic IKDCs, cDCs, PDCs and NK cells using transmission electron microscopy. **(h)** Splenic (four left panels) and lymph node (four right panels) CD11c^{int}B220⁺CD49b⁺ IKDCs were virtually absent in *Il2rb*^{-/-} (KO) mice (bottom panels) compared to wild-type (WT) C57BL/6 (top panels) whereas CD11c^{int}B220⁺ cDCs and CD11c^{int}B220⁺Gr1⁺ PDCs developed normally. Representative staining from three experiments is shown.

virtually identical pattern of cell-surface marker expression with the exception of MHC II, CD86 and CD40, which were expressed at high levels on roughly half of the lymph node IKDCs. To distinguish IKDCs from NK cells, we showed that classical NK cells did not express any MHC II, CD80 and CD86 costimulatory molecules (Fig. 1f). These results suggest that IKDCs represent a cell lineage related to DCs. Notably, morphologic analysis using transmission electron microscopy clearly distinguished IKDCs from cDCs, PDCs or NK cells (Fig. 1g). IKDCs showed a relatively smooth plasma membrane with few small pseudopodia. The cytoplasm contained few organelles (mitochondria) and had a dense and ‘negative-staining’ appearance. We also noted marked differences in the shape of the nucleus and the appearance of the chromatin, which unlike NK cells, PDCs and cDCs, was smooth in the nuclei of IKDCs.

The substantial difference in the expression of CD122 between IKDCs and PDCs or cDCs led us to ask whether the IL-2–IL-15 receptor beta chain (CD122) was necessary for the development of IKDCs. Indeed, although relatively normal numbers of PDCs and cDCs could be found in IL-2–IL-15 receptor beta chain-deficient mice (*Il2rb*^{-/-}), IKDCs were virtually absent (Fig. 1h), which established this subset as a distinct DC lineage. IKDCs were detected in most organs tested (Supplementary Fig. 3 online). Larger proportions of these cells expressed high levels of surface MHC II

molecules in the peripheral lymphoid organs including peripheral lymph nodes, mesenteric lymph nodes and Peyer’s patches, as compared with spleen, liver and lung. Together, these phenotypic, morphologic and developmental studies provide evidence that IKDCs represent a unique and homogeneous cell subset distinct from cDCs, PDCs and NK cells.

Functional characterization showed that whereas freshly FACS-sorted BALB/c splenic IKDCs did not kill YAC-1 cells, CpG ODN-activated IKDCs lysed YAC-1 cells with efficiency comparable to that of IL-2-activated splenic NK cells (Fig. 2a). In contrast, neither PDCs nor cDCs were able to kill YAC-1 cells, even after activation with CpG ODN. YAC-1 killing by IKDCs was completely blocked by NKG2D-specific monoclonal antibody CX5, and lysis of Ba/F3 pro-B cells was increased by the expression of Rae-1 ϵ , a ligand for NKG2D (Fig. 2b). C57BL/6 NK cells and IKDCs, which were Ly49h⁺, efficiently killed Ba/F3 cells expressing the murine cytomegalovirus protein m157, compared with parental Ba/F3 cells (Fig. 2c). Cytotoxicity was blocked by Ly49h-specific monoclonal antibody 1F8, thus showing direct involvement of Ly49h in recognition of Ba/F3-m157 cells. Notably, lymph node IKDCs never killed YAC-1 cells, even after activation with CpG ODN (data not shown).

We also evaluated the ability of IKDCs to produce cytokines. IKDCs secreted IL-12 and IFN- α upon activation with CpG ODN or

CD40-specific monoclonal antibody (Fig. 2d). These cells were notably more responsive to CpG ODN than to the Tlr-4 agonist lipopolysaccharide. IKDCs also produced substantial amounts of IFN- α in response to influenza virus infection, though at levels lower than those produced by PDCs (Fig. 2e). Intracellular staining confirmed the secretion of IFN- α by FACS-sorted PDCs (CD49b⁻) and IKDCs (CD49b⁺; Fig. 2f). Neither CD11c^{high} cDCs nor CD11c⁻ NK cells produced IFN- α under these conditions. The kinetic analysis of *Ifna4* transcript expression highlighted a delayed (beginning 4 h) and transitory production of IFN- α by IKDCs, whereas PDCs showed a rapid (2 h after influenza virus infection) and sustained (beyond 20 h) production of *Ifna4* mRNA (Fig. 2g). It is possible that the type I IFN produced by IKDCs is crucial for autocrine-paracrine regulation of their maturation as well as for a direct antiviral activity^{8,9}. Notably and similar to NK cells, IKDCs produced IFN- γ when activated by IL-15 and IL-12 and to a much larger extent than with IL-15 plus activation with CpG ODN 1668 (Fig. 2h). Under these conditions,

IFN- α was never detected. Thus, IKDCs can produce high levels of either type I or type II IFNs, depending on the stimulus.

Whereas IKDC killing of YAC-1 cells was highly efficient 14 h after activation with CpG ODN, their lytic activity diminished substantially by 45 h after activation (Fig. 3a). Quantitative RT-PCR for the long (*Klrk1-L*) and short (*Klrk1-S*) isoforms of *Klrk1* mRNA, which are the products of differential splicing that encode isoforms of NKG2D¹⁰, showed an initial increase followed by a continuous drop in expression of both isoforms (Fig. 3b). Expression of *Klrk1-S* mRNA, the only isoform that associates with Dap12 (encoded by *Tyrobp*)¹¹, peaked at 15 h after stimulation with CpG ODN followed by a rapid decline. By 36 h, *Klrk1-L* and *Klrk1-S* mRNA levels were extremely low. Similarly, levels of *Tyrobp* mRNA also decreased gradually from high at 15 h after activation to very low at 36 h (Fig. 3b). Cell-surface expression of NKG2D decreased substantially between 14 h and 36 h after stimulation on cells that upregulated expression of MHC II but maintained expression of CD49b and B220 (Fig. 3c). This gradual loss of surface

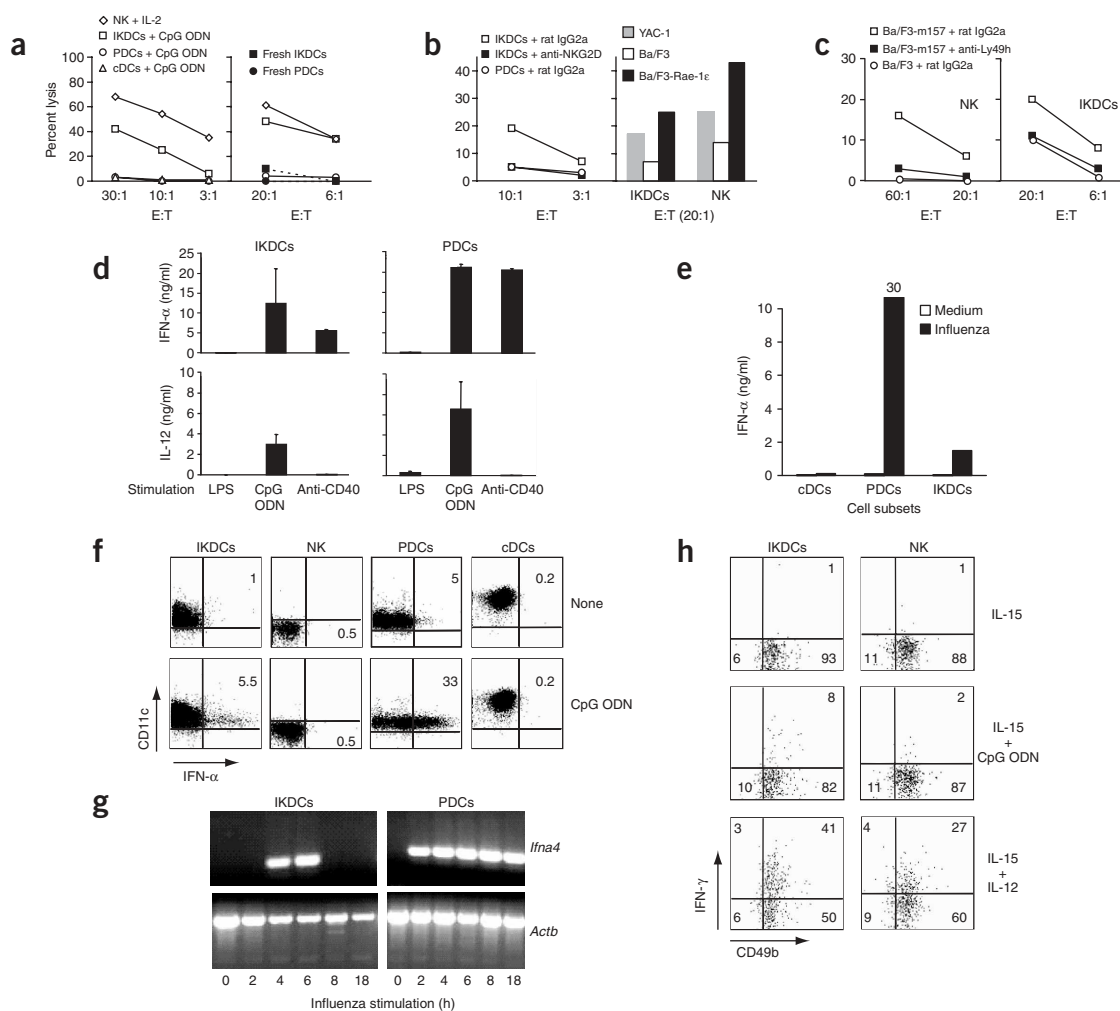


Figure 2 Cytotoxic activity and cytokines production by activated splenic IKDCs. (a–c) Cytotoxic activity of splenic IKDCs was tested using ⁵¹Cr-release assay using different effector/target ratio (E:T). Representative results are shown. CpG ODN-activated but not resting IKDCs killed YAC-1 (a) or Ba/F3-Rae-1 ϵ (b, right panel). Cytotoxicity toward YAC-1 was blocked by NKG2D-specific monoclonal antibody (b, left panel). (c) Cytotoxicity of C57BL/6 IKDCs toward Ba/F3-m157 was blocked by Ly49h-specific monoclonal antibody. (d, e) Representative ELISA showing production of IL-12 and IFN- α by IKDCs, PDCs or cDCs after 20 h activation with CpG ODN. (f) Representative FACS plots of IFN- α production by IKDCs, NK, PDCs and cDCs stimulated or unstimulated for 9 h with CpG ODN. CD11c⁺IFN- α ⁺ IKDCs remained CD49b⁺. (g) Representative RT-PCR assessing production of *Ifna4* versus *Actb* mRNA at indicated time points by IKDCs and PDCs upon stimulation with influenza virus. Sorted populations were 99% pure. (h) Representative FACS plots of IFN- γ production versus CD49b expression by activated IKDCs and NK.

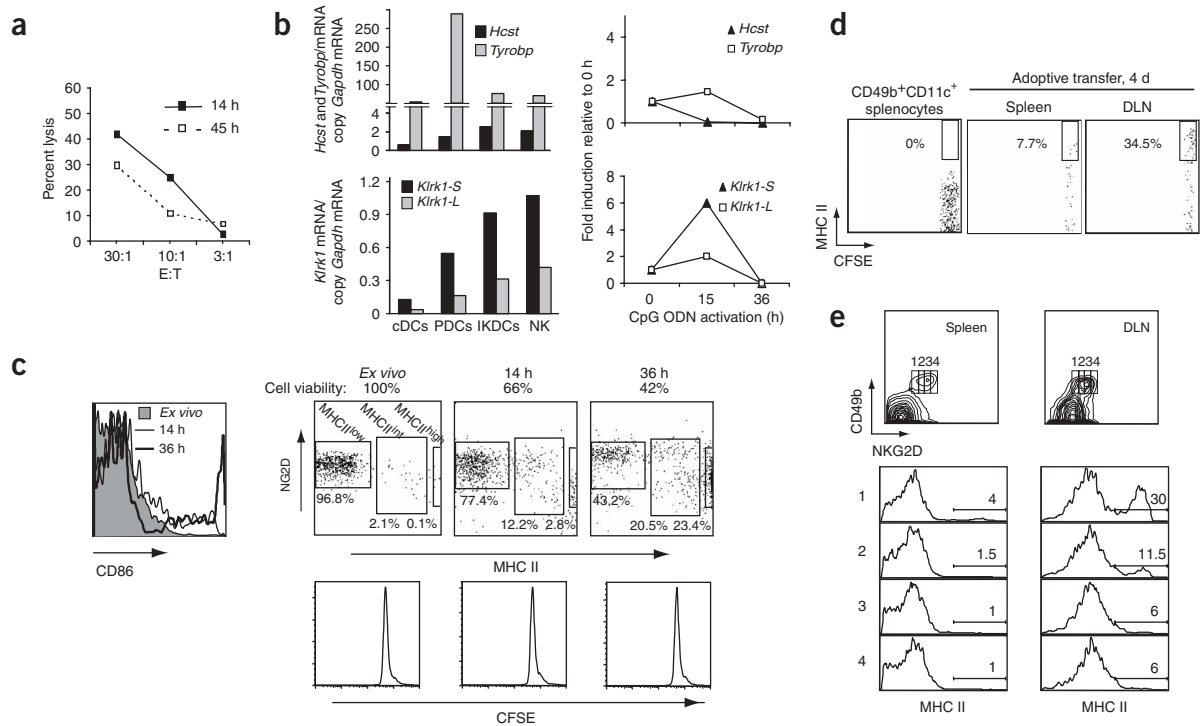


Figure 3 The transition of splenic IKDCs from cytotoxic to APCs upon activation with CpG ODN. **(a)** Representative ^{51}Cr assay showing killing of YAC-1 by IKDCs activated for 14 h and 45 h with CpG ODN. **(b)** Real-time PCR quantification of *Klrk1*, *Hcst* and *Tyrobp* transcripts encoding NKG2D, Dap10 and Dap12, respectively. In freshly sorted cells (two left panels) mRNA copy numbers were normalized to *Gapdh* mRNA. In activated IKDCs (two right panels) results are presented as fold induction, compared to 0 h. Representative plots of three independent experiments. **(c)** CD86 expression (left panel) and MHC II versus NKG2D expression (three top panels) on FACS-sorted IKDCs upon activation. The plots represent CD49b⁺ gated cells. Viability calculated as percentage of starting cell number and CFSE labeling of IKDCs (three bottom panels), showed that IKDCs did not proliferate upon activation. Data are shown as a representative plot of three experiments. **(d)** CFSE-labeled BALB/c splenic IKDCs transferred into spleens of *L. monocytogenes*-infected recipients matured (MHC II^{high}) and migrated to liver DLNs. IKDCs were gated as CFSE⁺MHC II⁺CD49b⁺NKG2D⁺ cells. **(e)** FACS staining showing the inverse correlation of MHC II and NKG2D expression on draining lymph node IKDCs from *L. monocytogenes*-infected mice. Data shown in **d** and **e** are representative plots of two experiments.

NGK2D on the cells that were in the process of upregulating MHC II suggests that extended activation with CpG ODN effects a true phenotypic transition at the population level (Fig. 3c). Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling studies indicated that the MHC II^{high}NKG2D^{low} cells generated after culture in the presence of CpG ODN had not in fact proliferated at all. As shown, the total cell yield after 36 h culture represented slightly less than half the starting cell number. These results indicate that the MHC II^{high}NKG2D^{low} cells that developed after 36 h stimulation did not represent outgrowth of a small contaminating population.

Finally, we sought to determine whether the transition from NK-like to DC-like phenotype occurred *in vivo*. We injected CFSE-labeled enriched IKDCs into the spleens of naive mice and infected them systemically with *Listeria monocytogenes*. Immunofluorescence analysis of *L. monocytogenes*-infected mice showed CFSE-labeled IKDCs predominantly in T-cell zones of both spleen and lymph nodes (Supplementary Fig. 4 online). Although the injected IKDCs were all MHC II^{low}, a substantial number of undivided IKDCs migrated from spleen to draining lymph nodes (DLNs) and roughly 35% became MHC II^{high} (Fig. 3d). NKG2D and MHC II levels on lymph node IKDCs were inversely correlated (Fig. 3e). NKG2D^{high} IKDCs expressed low levels of MHC II and IKDC fractions with successively lower NKG2D levels contained progressively higher proportions of MHC II^{high} cells. This result is in concordance with the phenotypic analysis of lymph

node versus splenic IKDCs and supports the idea that activated lymph node IKDCs encompass a later activation-induced maturation stage. To determine whether the increasing expression of MHC II and costimulatory molecules observed on maturing IKDCs reflected their capacity to present antigen to naive T cells, we evaluated their capacity to present ovalbumin (OVA) to MHC II-restricted and OVA-specific naive DO11.10 transgenic T cells *in vitro*. IKDCs were capable of inducing proliferative responses among naive DO11.10 cells to a greater level than PDCs and somewhat less efficiently than cDCs (Fig. 4a). Analysis of DO11.10 proliferative responses by CFSE dilution shows that by later times after stimulation with CpG ODN, both IKDCs and PDCs given either peptide or whole-protein antigen were capable of stimulating T-cell proliferation (as measured by CFSE dilution) similarly to cDCs (Fig. 4b). These analyses indicate the capacity of IKDCs to process antigen and show DC-like stimulatory activity for naive T cells. We next investigated the ability of lymph node and splenic IKDCs to process and present antigen *in vivo* using an assay of direct *ex vivo* antigen detection (DEAD assay). We infected BALB/c mice with recombinant *L. monocytogenes* actA⁻ (LM actA⁻) or *L. monocytogenes* actA⁻ expressing OVA (LM actA⁻ova). IKDCs and cDCs were then sorted by FACS from either spleen or lymph node 5–6 d after infection and were cultured with CFSE-labeled naive DO11.10 CD4⁺ T cells. IKDCs from lymph nodes but not spleens induced proliferative responses (Fig. 4c). In contrast,

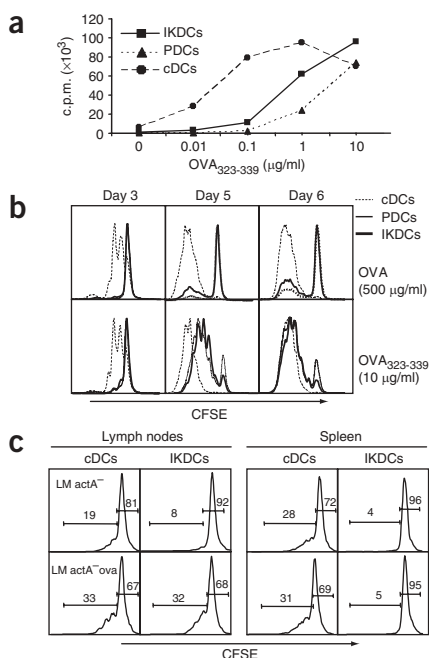


Figure 4 *In vitro* and *in vivo* antigen presentation by IKDCs. **(a,b)** Representative proliferation of OVA-specific DO11.10 CD4⁺ T cells when incubated with CpG ODN-activated APCs and with indicated concentrations of OVA₃₂₃₋₃₃₉ peptide or OVA protein. Proliferation was determined as (a) ³H-thymidine incorporation at day 3 and (b) CFSE dilution of labeled T cells at days 3, 5 and 6. **(c)** Spleen and lymph node IKDCs (MHCII⁺CD49b⁺CD11c⁺) and cDCs (MHCII⁺CD49b⁻CD11c⁺) from LM actA^{ova} or control LM actA⁻-injected mice were sorted by FACS and cultured with CFSE-labeled DO11.10 CD4⁺ T cells. Representative results of CFSE dilution of OVA-specific T cells at day 4 are shown.

ance¹⁷. IKDCs seem to be fully equipped with NK-activating receptors such as Ly49h, which recognizes MCMV m157 protein¹⁸ and NKG2D, which recognizes the stress-induced proteins Rae-1 and H60 (ref. 19). As IKDCs express other NK-activating receptors (**Supplementary Table 1** online) and a full complement of inhibitory receptors, it will be important to determine their relative roles in target recognition and killing. IKDCs are also clearly distinct from classical NK cells by virtue of their sensitivity to CpG ODN, which induce upregulation of MHC II and allow presentation of antigen to T cells. Our demonstration that lymph node but not splenic IKDCs can present antigen to naive T cells after *in vivo* infection with a recombinant *L. monocytogenes* represents direct evidence for *in vivo* APC function by a cell type in addition to cDCs. Our results support the notion that IKDCs lose NK activity as they migrate to draining lymph nodes, and then develop APC activity. This makes functional sense because lymph nodes are the primary site of T-cell priming by DCs. Our hypothesis is bolstered by the kinetics of killing activity by splenic IKDCs after *in vitro* activation with CpG ODN, which showed that upregulation of MHC II on IKDCs is correlated with the downregulation of NKG2D and its adaptors Dap10 or Dap12, resulting in loss of cytotoxic activity.

A few recent reports have described shared functional properties between certain NK cells and DC populations. But none of the studies have clearly defined a phenotypically, functionally and developmentally distinct homogeneous cell lineage as we have with IKDCs. Unlike mouse NK cells, activated human NK cells express MHC II and costimulatory molecules, enabling them to present antigen to T cells^{20,21}. Rat cDCs have also been reported to mediate NK-like cytolytic function²². One study recently isolated a subset of CD11c⁺B220⁺CD49b⁺ cells with their mouse blood DC precursors²³ and another study described, in bone marrow, a PDC subset characterized by the absence of Ly-6C and expression of CD49b²⁴. Functional analyses of these subsets have not yet been pursued. Treatment of mice with CD40L-specific monoclonal antibodies elicited a CD11c⁺B220⁻CD49b⁺ population, termed a 'bitypic NK/DC,' which was believed to suppress diabetes in nonobese diabetic mice²⁵. Recently, one group described CD11c⁺NK1.1⁺ NKDCs in C57BL/6 mice²⁶. This population killed NK targets, presented antigens and secreted IFN- γ upon activation with CpG ODN. Although these activities share similarities to those of the IKDC population, data described in these reports were derived from heterogeneous populations, possibly including classical CD11c⁺ NK cells^{27,28}.

Whether IKDCs serve a unique or redundant role in linking the innate and adaptive immunity remains to be addressed. But the finding that IKDCs were activated by Tlr-9 ligands, which did not directly activate NK cells, suggests that this cell population has a distinct role in the context of certain TLR ligand-expressing pathogens. We propose that IKDCs extend the DC family, representing a cell type possessing dual innate effector functions and antigen-presenting capacity. Just as classical cells of the innate immune system diversify into multiple subtypes based on their different effector mechanisms,

virtually no proliferation was induced by IKDCs isolated from control LM actA⁻-infected mice. IKDCs from lymph nodes showed roughly equivalent APC activity to cDCs from lymph nodes in the DEAD assay, although there was a higher background proliferation induced by cDCs from lymph nodes of control LM actA⁻-infected mice. No OVA-specific proliferative responses were observed in DO11.10 T cells incubated with splenic IKDCs or cDCs. Analysis of IL-2 in supernatants from these cocultures indicated that DO11.10 T cells incubated with lymph node IKDCs from LM actA^{ova}-infected mice produced roughly fourfold more IL-2 than T cells incubated with lymph node IKDCs from control LM actA⁻-infected mice (data not shown). Compatible with these findings, IKDCs from lymph nodes contained a notable proportion of CD86^{high}MHC II^{high} cells, whereas splenic IKDCs did not (data not shown). These results indicate that IKDCs are capable of presenting antigen *in vivo* and are consistent with the idea that IKDCs in lymph nodes represent, at least in part, a mature form of IKDC that has lost NK activity and developed DC-like APC activity.

Together, the morphologic, phenotypic and developmental features that we have defined for IKDCs represent the hallmarks of a unique homogeneous population clearly distinct from classical NK cells, cDCs and PDCs. The phenotypic and developmental similarities of IKDCs and NK cells are correlated with functional similarities such as their ability to kill NK targets, and to secrete IFN- γ in the presence of IL-15 or IL-2 and IL-12. The developmental biology of IKDCs presents an interesting set of questions because NK cells and DCs are typically thought to represent separate lineages that arise from distinct precursors whose differentiation is dependent on various cytokines, signaling pathways and transcription factors^{12,13}. We identified the IL-2-IL-15 receptor beta chain, previously known to be involved in NK development¹⁴, as crucial for development of IKDCs but not PDCs or cDCs. Notably, Ly49 family members are differentially expressed on IKDCs between BALB/c and C57BL/6 mice, as found for classical NK cells^{15,16}. These findings suggest that IKDCs may undergo a similar process of selection and education as NK cells, through interactions between inhibitory receptors and MHC Ia molecules, to reach a functional competence and allow 'self toler-

the DC family seems to segregate into distinct subtypes characterized by analogous effector mechanisms. A population of tumor necrosis factor (TNF)- α - and inducible nitric oxide synthase (iNOS)-producing DCs (TIP-DCs) is detected in mice infected with *L. monocytogenes*²⁹. By virtue of multiple cell-surface markers, TIP-DCs seem to be a distinct cell population from IKDCs. Likewise, the dual killing and antigen-presenting activities of IKDCs make them an interesting potential player in antitumor immunity. A recent study described a DC subset in C57BL/6 mice that is phenotypically and morphologically identical to ours (CD11c^{int}B220⁺CD49b⁺MHC II⁺) and which secretes high levels of IFN- γ when encountering tumor cells in the presence of IL-2 (ref. 30). In fact, although *in vitro* stimulation with CpG ODN did not evoke production of IFN- γ by IKDCs, treatment with IL-15 and IL-12 induced secretion of IFN- γ . IKDCs described by our group and others indicate the functional flexibility of DCs, which are endowed with the ability to differentiate into distinct 'effector DCs' according to the nature of the aggressor (pathogens versus tumor) and to subsequently efficiently translate the innate immune response into an adaptive immune response by presenting antigens captured from their targets to T cells.

METHODS

A detailed description of methods and reagents used is in **Supplementary Methods** online.

Mice. We purchased BALB/c and C57BL/6 and DBA mice (6–10 weeks old) from the US National Cancer Institute and the Harlan Laboratories. C3H/HeJ, DO11.10 T-cell receptor transgenic mice and *Il2rb*^{-/-} mice (B6.129P2-*Il2rb*^{tm1Mak}) were purchased from the Jackson Laboratory. Mouse care and experimental procedures were performed under approval from the Animal Care Committee of Johns Hopkins University.

Preparation of DC subpopulations and NK cells. We prepared DC subpopulations from tissues including spleen, liver, lung, thymus, lymph node, Peyer's patches, gut intraepithelial and lamina propria lymphocytes (**Supplementary Methods** online). We obtained DC-enriched populations by depleting CD3⁺ and CD19⁺ cells and positively selecting CD11c⁺ cells (CD11c⁺ beads, Miltenyi Biotec). We stained the CD11c⁺ fraction with FITC-conjugated CD11c (HL3)-, CyChrome-conjugated B220 (RA3-6B2)-, and PE-conjugated Gr1 (RB6-8C5)- or PE-conjugated CD49b (DX5)-specific monoclonal antibodies (BD Biosciences). We sorted CD11c^{high}B220⁻ cDCs, CD11c^{int}B220⁺Gr1⁺ cells (PDCs) and CD11c^{int}B220⁺CD49b⁺ cells (IKDCs) from spleens and lymph nodes using a FACS Vantage instrument (BD Biosciences). We performed routine reanalysis to ensure that the purity was >95%. We cultured DC populations in RPMI-1640 supplemented with 10% FCS, L-glutamine, sodium pyruvate, HEPES, nonessential amino acids and β -mercaptoethanol (complete medium) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 1,000 U/ml; R&D Systems) and stimulated 14 h or 36 h with CpG ODN (ODN 1668, 6 μ g/ml). PDCs and IKDCs share similar viability in culture with GM-CSF, with improved viability after activation with CpG ODN. We prepared NK cells from splenocytes using a NK cell-negative isolation kit (Miltenyi Biotec). In some cases, pure NK cells were prepared by sorting by FACS (purity, >95%). We cultured NK cells in complete medium with 4,000 IU/ml or 400 IU/ml of human recombinant IL-2 (Chiron) or 50 ng/ml mouse recombinant IL-15 (R&D Systems), in the presence or absence of 1 ng/ml of mouse recombinant IL-12.

***In vitro* analysis of DC and NK function.** We assessed NK cells and DC subpopulations for their cytotoxicity in a standard 4-h ⁵¹Cr-release assay using YAC-1 cells (ATCC) or Ba/F3 transfectants as targets. We used freshly or CpG ODN-activated FACS-sorted DC subpopulations as effectors. Spontaneous release did not exceed 15%. For blocking experiments, we preincubated effectors for 30 min with 20 μ g/ml NKG2D-specific monoclonal antibody (CX5), 50 μ g/ml Ly49h-specific monoclonal antibody (IF8, provided by M. Bennett from Southwestern University of Texas) or rat IgG2a before adding

the ⁵¹Cr-labeled target cells. We performed *in vitro* antigen-presentation assays by incubating purified DO11.10 OVA-specific T-cell receptor transgenic CD4⁺ T cells (1–2 \times 10⁵) in the presence of APCs (3–5 \times 10⁴) and OVA_{323–339} peptide or OVA protein at various concentrations. We collected cell-free supernatants at 12 and 24 h and tested for IL-2 using ELISA kits (Pierce Endogen). In some experiments, we labeled CD4⁺ cells with CFSE (5 μ mol/ml; Molecular Probes) before incubation with APCs, and T-cell proliferation was analyzed by CFSE dilution. Alternatively, we assessed T-cell proliferation by ³H-thymidine incorporation.

TaqMan quantitative RT-PCR. We examined the expression of *Klrk1-S* and *Klrk1-L* isoforms, *Hcst* and *Tyrobp* transcripts by TaqMan real-time PCR. Briefly, we extracted total RNA from both freshly and CpG ODN-activated FACS-sorted cells (15 h and 36 h) using the Trizol method, followed by DNase treatment. We generated cDNA using Superscript II reverse transcriptase (Invitrogen) with random hexamers (specific primer sequences used are listed in **Supplementary Methods** online). We expressed transcript levels in freshly isolated samples as copy number of mRNA of interest per copy of *Gapdh* mRNA. We assessed variation of expression of the gene of interest upon activation with CpG ODN using the ribosomal *18S* as internal control and results were expressed as fold induction compared to the 0 h time point.

Intrasplenic injection of IKDCs followed by activation with *L. monocytogenes*. We prepared CD11c⁺ cell-enriched populations from BALB/c spleens as described above. We injected the CFSE-labeled cells (3 \times 10⁶) into BALB/c spleens. Before injection, we divided the spleen into hemi-spleens using titanium clips, leaving the vascular pedicles intact. We then injected the cells into one of the hemi-spleens. The injected hemi-spleen was then surgically removed, leaving a functional hemi-spleen. We intraperitoneally injected one-tenth of 50% lethal dose (LD₅₀) of LM act⁻ into the mice and 3 d after *L. monocytogenes* infection, spleens and liver DLNs were collected and the expressions of NKG2D and MHC II on CFSE-positive cells were analyzed using FACS analysis. In some experiments, we injected CFSE-labeled cells intravenously into the tail vein and visualized the spleens and lymph node frozen sections by immunofluorescence (**Supplementary Methods** online).

Direct *ex vivo* antigen-detection assay. We intraperitoneally injected BALB/c mice with one-tenth of the LD₅₀ of LM act⁻ or LM act⁺ova. We then sorted the activated cDCs (CD11c^{high}CD49b⁻MHC II⁺) and IKDCs (CD11c^{int}CD49b⁺MHC II⁺) from lymph node and spleen CD11c⁺ cell-enriched fractions at day 4 after infection and tested for their ability to activate OVA-specific CFSE-labeled DO11.10 CD4⁺ cells (10⁵ splenic APCs/well or 10⁴ lymph node APCs/well; 10⁵ T cells/well), as described above. CD4⁺ T cells were subjected to cell sorting to reach purity superior or equal to 98%.

Accession codes. Gene Expression Omnibus: GSE3691.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank L. Zitvogel's group for communicating its data on IKDCs; K. Palucka and J. Banchereau for discussions. We also thank M. Delannoy for his technical assistance with electronic microscopy. We thank C. Chia and L.S. Laird for their help on the adoptive transfer experiments, and M. Jones for her technical assistance in immunofluorescence techniques. We thank F. Murillo for his technical assistance with microarray techniques. C.W.C. is supported by a Cancer Research Institute postdoctoral fellowship. This work was supported by grants from the US National Institutes of Health (NIH), the Janney Fund and Seraph Foundation, and gifts from Bill and Betty Topecer and Dorothy Needle. L.L.L. is an American Cancer Society Research Professor and is funded by NIH grants CA89189 and CA89294.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at <http://www.nature.com/naturemedicine/>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

1. Cerwenka, A. & Lanier, L.L. Natural killer cells, viruses and cancer. *Nat. Rev. Immunol.* **1**, 41–49 (2001).

2. Steinman, R.M. Some interfaces of dendritic cell biology. *APMIS* **111**, 675–697 (2003).
3. Janeway, C.A., Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13 (1989).
4. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
5. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **5**, 987–995 (2004).
6. Walzer, T., Dalod, M., Robbins, S.H., Zitvogel, L. & Vivier, E. Natural killer cells and dendritic cells: “l’union fait la force.” *Blood* (2005).
7. Arase, H., Saito, T., Phillips, J.H. & Lanier, L.L. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). *J. Immunol.* **167**, 1141–1144 (2001).
8. Honda, K. *et al.* Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. *Proc. Natl. Acad. Sci. USA* **100**, 10872–10877 (2003).
9. Asselin-Paturel, C. *et al.* Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *J. Exp. Med.* **201**, 1157–1167 (2005).
10. Diefenbach, A. *et al.* Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat. Immunol.* **3**, 1142–1149 (2002).
11. Gilfillan, S., Ho, E.L., Cella, M., Yokoyama, W.M. & Colonna, M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat. Immunol.* **3**, 1150–1155 (2002).
12. Yokoyama, W.M., Kim, S. & French, A.R. The dynamic life of natural killer cells. *Annu. Rev. Immunol.* **22**, 405–429 (2004).
13. Ardavin, C. Origin, precursors and differentiation of mouse dendritic cells. *Nat. Rev. Immunol.* **3**, 582–590 (2003).
14. Suzuki, H., Duncan, G.S., Takimoto, H. & Mak, T.W. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. *J. Exp. Med.* **185**, 499–505 (1997).
15. Ortaldo, J.R. *et al.* Ly-49 receptor expression and functional analysis in multiple mouse strains. *J. Leukoc. Biol.* **66**, 512–520 (1999).
16. Anderson, S.K., Ortaldo, J.R. & McVicar, D.W. The ever-expanding Ly49 gene family: repertoire and signaling. *Immunol. Rev.* **181**, 79–89 (2001).
17. Kim, S. *et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436**, 709–713 (2005).
18. Arase, H., Mocarski, E.S., Campbell, A.E., Hill, A.B. & Lanier, L.L. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* **296**, 1323–1326 (2002).
19. Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N. & Raulet, D.H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol.* **1**, 119–126 (2000).
20. Zingoni, A. *et al.* Cross-Talk between activated human NK cells and CD4+ T cells via OX40–OX40 ligand interactions. *J. Immunol.* **173**, 3716–3724 (2004).
21. Hanna, J. *et al.* Novel APC-like properties of human NK cells directly regulate T cell activation. *J. Clin. Invest.* **114**, 1612–1623 (2004).
22. Josien, R., Heslan, M., Souillou, J.P. & Cuturi, M.C. Rat spleen dendritic cells express natural killer cell receptor protein 1 (NKR-P1) and have cytotoxic activity to select targets via a Ca²⁺-dependent mechanism. *J. Exp. Med.* **186**, 467–472 (1997).
23. del Hoyo, G.M. *et al.* Characterization of a common precursor population for dendritic cells. *Nature* **415**, 1043–1047 (2002).
24. Pelayo, R. *et al.* Derivation of 2 categories of plasmacytoid dendritic cells in murine bone marrow. *Blood* **105**, 4407–4415 (2005).
25. Homann, D. *et al.* CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. *Immunity* **16**, 403–415 (2002).
26. Pillarisetty, V.G., Katz, S.C., Bleier, J.I., Shah, A.B. & Dematteo, R.P. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12. *J. Immunol.* **174**, 2612–2618 (2005).
27. Laouar, Y., Sutterwala, F.S., Gorelik, L. & Flavell, R.A. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat. Immunol.* **6**, 600–607 (2005).
28. Kamath, A.T., Sheasby, C.E. & Tough, D.F. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. *J. Immunol.* **174**, 767–776 (2005).
29. Serbina, N.V., Salazar-Mather, T.P., Biron, C.A., Kuziel, W.A. & Pamer, E.G. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* **19**, 59–70 (2003).
30. Taieb, J. *et al.* A novel dendritic cell subset involved in tumor surveillance. *Nat. Med.* **12**, 214–219 (2006).