IFN-Producing Killer Dendritic Cells Are Antigen-Presenting Cells Endowed with T-Cell Cross-Priming Capacity

Maria Pletneva, Hongi Fan, Jang-June Park, Vedran Radojicic, Chunfa Jie, Yanxing Yu, Camie Chan, Alec Redwood, Drew Pardoll, and Franck Housseau

Abstract

IFN-producing killer dendritic cells (IKDC) represent a recently discovered cell type in the immune system that possesses a number of functions contributing to innate and adaptive immunity, including production of type 1 and 2 IFNs, interleukin (IL)-12, natural killing, and ultimately antigen presentation to naive T cells. Here, we compared in vitro and in vivo responses of mouse IKDC, conventional dendritic cells (DC), and natural killer (NK) cells to murine cytomegalovirus infection and found distinct functions among these cell subsets. Upon recognition of infected fibroblasts, IKDC, as well as NK, produced high level of IFN-γ, but unlike NK, IKDC simultaneously produced IL-12p40 and up-regulated MHC class II (MHC-II) and costimulatory molecules. Using MHC-II molecule expression as a phenotypic marker to distinguish activated IKDC from activated NK, we further showed that highly purified MHC-II+ IKDC but not NK cross-present MHC class I–restricted antigens derived from MCMV-infected targets to CD8+ T cells in vitro and in vivo. Our findings emphasize the unique nature of IKDC as a killer antigen-presenting cell directly linking innate and adaptive immunity.

Introduction

We and others have recently described a new subset of antigen-presenting cells (APC), termed IFN-producing killer dendritic cells (IKDC) or natural killer (NK) dendritic cells (DC), that exhibit properties of both NK cells and DC (1–3). In-depth phenotypic analyses established splenic IKDC as a homogeneous CD11c+ B220–CD49b+ population, distinct from murine NK by their expression of MHC class II molecules (MHC-II) and from DC by their expression of NK receptors. Upon stimulation in vitro with CpG oligodeoxynucleotides or in vivo with Listeria monocytogenes splenic IKDC killed typical NK targets and showed the unique ability to produce IFN-γ, IFN-α, and interleukin (IL)-12 (1). Activated IKDC further differentiated into DC-type APC that accumulated in vivo in lymph nodes (LN) to activate CD4+ T cells. IKDC also mediated melanoma rejection via an IFN-γ and tumor necrosis factor (TNF)–related apoptosis-inducing ligand-dependent mechanism (2).

The functional relationship between IKDC and DC and their distinction from NK cells has been challenged in recent reports suggesting that the phenotypic definition of IKDC based on CD11c, B220, and CD49b expression could not clearly distinguish them from activated NK (4–7). However, a recent study showed that, although sharing a lymphoid origin, IKDC and NK derive from distinct precursors (8). We show here that IKDC, but not NK, cross-present mouse cytomegalovirus (MCMV)–encoded antigens, derived solely from MCMV-infected cells, in association with MHC-II and MHC-I to CD4+ and CD8+ T cells, respectively. This functional capacity of mature IKDC, which were previously shown to accumulate in the LN (1), correlates with expression of genes encoding the MHC-II processing machinery, costimulatory molecules, and coexpression of IL-12p40 and IFN-γ at the single cell level. The antigen cross-presentation functions, together with their natural killing activity against both virally infected and tumor cells (1), establish IKDC as a unique type of APC bridging innate and adaptive immunity.

Materials and Methods

Mice and reagents. BALB/c and C57BL/6 were purchased from National Cancer Institute and Harlan laboratories. IL-12p40−/− mice were purchased from Jackson Laboratories. Myd88−/− Trif−/− mice were obtained by crossing Trif−/−Myd88−/− mice (Bruce Beutler, Scripps Research Institute, La Jolla, CA) with Myd88−/− C57BL/6 mice (Maureen Horton, Johns Hopkins University, Baltimore, MD). NOD/severe combined immunodeficient/γc−/−Rag2−/−−/− OT-I, OT-II Clone-1, and 6.5 T-cell transgenic mice were maintained at Johns Hopkins University. Antibodies were purchased from BD Biosciences, except antibodies against NKG2D (CX5) and NKp46 (eBioscience). Intracellular staining (ICS) for cytokines was performed in presence of Brefeldin A using BD Biosciences Cytofix/Cytoperm reagent following manufacturer’s recommendations. Fluorescence-activated cell sorting (FACS) analysis was done on FACSCalibur or LSBR2 (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc). Sorting was performed on FACSARia (BD Biosciences). For some experiments, cells were activated with 6 μg/mL CpG1668 (MWG Biotech). For Western blot analysis, anti-invariant chain (IiCD74; clone OX6; Santa Cruz) and anti-Legumain (Clone 301417; R&D systems) monoclonal antibodies (mAb) were used as primary antibodies, and anti-tubulin mAb as a control. Secondary antibody was sheep anti-mouse IgG labeled with horseradish peroxidase (enhanced chemiluminescence; GE Healthcare Lifesciences). IL-12p40 and IL-12p70 were measured in supernatant using ELISA (Pierce Endogen).

Viruses. MCMV virus expressing green fluorescent protein (GFP; Dr. M. Messerle, Hannover Medical School, Hannover, Germany) and MCMV expressing HA (A/PR/8/34) or TfrOVA were previously described (9–11). Primary fibroblasts were infected at multiplicity of infection of 3 for 1.5 h. Cells were washed and cocultivated with splenocytes at a ratio 1 fibroblast: 10 splenocytes for 12 h. Infection was monitored by cytopathic effect and chemiluminescence; GE Healthcare Lifesciences). IL-12p40 and IL-12p70 were measured in supernatant using ELISA (Pierce Endogen).

Cell isolation. Cellular isolation was previously described (1). Briefly, spleens and livers were digested with DNase and collagenase (Liberase2 blendzyme; Roche Applied Science). Leukocytes were separated through Lymphocyte gradient (Accurate Chemical and Scientific, Inc.) and depleted for CD3+ T cells, CD19+ B cells, and granulocytes using purified anti-CD3.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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that, upon activation, NK or a subset among the total NK population will acquire properties and surface markers similar to IKDC. We cocultured the CD11c+ fraction containing naïve NK (CD11c+ B220+ CD49b+) or CD11c+ fraction containing IKDC (CD11cint B220+ CD49b+) and CDC (CD11ch/ B220+ CD49b+) in the presence of MCMV-infected or mock-treated primary fibroblasts. BALB/c IKDC, but not NK, strongly up-regulated MHC-II molecules when cultured in presence of MCMV-infected fibroblasts (41% versus 2.9%; Fig. 2C). Costimulatory molecules were also up-regulated on the surface of IKDC (data not shown; Fig. 3C). These conditions induced a robust IFN-γ production (Fig. 4) and up-regulation of CD11c and B220 by NK (Supplementary Fig. S1), indicating that the absence of MHC-II up-regulation did not reflect a failure of NK to recognize infected fibroblasts. It can be argued that NK did not up-regulate MHC-II or CD40 because of the absence of accessory cells in the CD11c+ fraction. We thus performed a mixing experiment, combining Ly5.1+ CD11c+ cells with Ly5.2+ CD11c+ cells and incubating them with uninfected or MCMV-infected fibroblasts. In this mix, activated Ly5.2+ NK did not up-regulate MHC-II above levels observed for NK derived from Ly5.2+ CD11c+ fraction cultured alone with MCMV-infected fibroblasts. Meanwhile, Ly5.1+ IKDC retained their ability to up-regulate MHC-II molecules (data not shown). We performed an adoptive transfer experiment in which freshly isolated naïve NK (CD11c+ B220+ NKp46+) and IKDC (CD11cint B220+ NKp46+) were FACs-sorted from BALB/c mice spleen to high purity and transferred into MCMV-infected NOB/severe combined immunodeficient (scid) mice. As shown in Fig. 28, MHC-II expression on NK before transfer is essentially negative, whereas expression is low on IKDC. Although transferred NK indeed up-regulated B220 and CD11c upon MCMV infection (37%), they failed to express any MHC-II (Fig. 2D). In contrast, 5% to 6% of transferred IKDC expressed intermediate to high MHC-II levels, at day 2 and 3 postinfection. Altogether, these results show that differential expression of CD11c, B220, and NK1.1/CD49b allows for the distinction between naïve IKDC, NK, and CDC, whereas surface MHC-II expression should be included to delineate activated IKDC from activated NK. Therefore, to perform antigen presentation assays on sorted cell populations, we define IKDC activated during incubation with MCMV-infected fibroblasts or in vivo after MCMV injection as CD11c+ CD49/NK1.1+ MHC-IIm+; whereas activated NK are defined as CD11c+ CD49/NK1.1+ MHC-I**.

IKDC, but not NK, presented antigens derived from MCMV-infected targets to CD4+ T cells. Highly purified MHC-IIhi IKDC from BALB/c mice infected with Listeria monocytogenes (Supplementary Fig. S2A), induced a robust proliferation of HA-specific 6.5 CD4+ T cells when loaded with the HA110-120 peptide (Supplementary Fig. S2B). To test the hypothesis that IKDC can present antigens derived from their killed targets, we cultured CD11c- and CD11c+ fractions of CD57BL/6 splenocytes with MCMV/GFP- or MCMV/OVA-infected fibroblasts (Fig. 3A). After 12 hours of incubation, activated IKDC (CD11c+ NK1.1+ MHC-IIhi) and CDC (CD11ch/ NK1.1+ MHC-IIhi) were sorted from the CD11c+ fraction. Among the CD11c+ fraction, NK up-regulated CD11c and B220 (Supplementary Fig. S1), but remained MHC-II- and, therefore, were sorted as CD11c+ NK1.1+ MHC-II-. IKDC derived from coculture with MCMV/OVA- but not MCMV/GFP-infected fibroblasts induced IL-2 secretion by OT-II CD4+ T cells (Fig. 3A). Treatment of IKDC with CpG1668 stimulated a higher level of IL-2 by OVA-specific OT-II cells, CDC, but not NK, cultured with MCMV/OVA-infected fibroblasts were potent in inducing OT-II stimulation. Although low, IL-2 induction by IKDC still ranged...
between one third and one half of that by CDC (Fig. 3A). We reproduced these findings using HA-specific 6.5 CD4+ T cells and MCMV/HA-infected fibroblasts (Fig. 3B). As a control, IKDC incubated with OVA323-339 or HA110-120 induced less IL-2 than CDC (Fig. 3A and B). NK, which lacked MHC-II, did not stimulate CD4+ T cells. BALB/c MHC-II hi IKDC used for the presentation assay expressed accessory molecules such as CD80, CD86, and CD40 (Fig. 3C). The high purity of each FACS-sorted population (>95%) ruled out the role of contaminating CDC in assays of T-cell activation by purified IKDC (Supplementary Fig. S3; Fig. 5). NK, which included similar percentages of contaminating CDC as did IKDC (1.2% for NK versus 1.0% for IKDC in Supplementary Fig. S3), did not stimulate T cells to produce IL-2. No IL-2 secretion was observed when sorted CD4+ T cells were incubated with peptide alone, ruling out their contamination with APC (data not shown). Reverse transcription-PCR performed on IKDC, NK, and CDC incubated with MCMV-GFP did not detect expression of viral genes ie1, m157, or GFP in IKDC (data not shown). Neither was free virus detected in the APC/fibroblast coculture in the timeframe of experiment (data not shown), indicating that IKDC and CDC rather ingested antigens from the infected target cells and presented the processed epitope in association with MHC-II.

Activating cytokine production by IKDC

Activated IKDC simultaneously produced IFN-γ and IL-12p40. We used ICS to examine the capacity of individual IKDC

Table 1. Fold changes of the mRNA microarray probe set signals for genes involved in MHC-II processing pathway

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<th>Probeset*</th>
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<th>SPL fold change</th>
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NOTE: Fold change, (−) to (+).

*Probeset from the Affymetrix murine genome GeneChip MOE430-2.

† Up-regulated and down-regulated genes are highlighted in green and red, respectively. The criterion of significance was set as the posterior probability of >0.5. Fold changes highlighted in gray are not statistically significant. Absolute value of each probeset is provided in Supplementary Table S1.
to secrete IFN-γ and IL-12p40 upon activation by MCMV-infected fibroblasts (Fig. 4A). Among CD11c+CD49b− NK1.1+IAbhi cells (CD11c+CD49b− NK1.1+IAbhi) secreted a large amount of IFN-γ. Notably, a significant fraction of IFN-γ− IKDC simultaneously produced IL-12p40. All CD11c+NK1.1+IAbhi cells (CD11c+CD49b− NK1.1+IAbhi) secreted significant quantities of IL-12p40 but not IFN-γ, whereas NK (CD11c+NK1.1$I^+$-Ab−) produced IFN-γ but not IL-12p40. Neither IFN-γ nor IL-12p40 was detected in response to exogenous IL-12/IL-23 signaling (Fig. 4B). As found with ICS, IKDC, but not CDC, simultaneously expressed IFN-γ mRNA. Furthermore, phenotyping of IKDC in spleen and LN highlighted concomitant expression of NK-associated receptors and APC markers (Supplementary Fig. S6).

Activated IKDC, but not NK, cross-primed CD8+ T-cells. Confocal microscopy using 16-D25-11 mAb showed that, unlike NK, CDC and IKDC generate H2-Kb/OVA"INFEKL" complexes when incubated in vitro with soluble OVA and IFN-α (Supplementary Fig. S7). We further sought to determine whether IKDC were able to cross-present MHC-I−restricted OVA"INFEKL" derived from MCMV/OVA-infected fibroblasts (18, 20) and stimulate OT-I Rag2−/−CD8+ T cells. IKDC, sorted as CD11c+CD49b+ NK1.1−MHC-II+ cells from the CD11c+ fraction, expressed I-A/E when activated in vivo by MCMV. FACS-sorted CD45.1+ BALB/c IKDC (NKp46+CD11c+CD49b+) and NK (NKp46−CD11c+CD49b−) were injected i.v. into CD45.2+ NOD/severe combined immunodeficient mice infected with MCMV. At days 1 and 3, transferred IKDC and NK were phenotyped for I-A/E when isolated from the recipient’s liver. Left, expression of CD11c and B220 on CD45.1+NKp46+ cells. Right, expression of I-A/E on IKDC (red), CD11c+ B220+ NK (black), and activated CD11c+ B220+ NK (green).

Figure 2. IKDC up-regulated MHC-II upon recognition of MCMV-infected targets. A, confocal microscopy of C57BL/6 IKDC (CD11c+CD49b+) and NK (CD11c+ B220− CD49b−), and CDC (CD11c+CD49b− CD49a−) labeled with anti-I-A/E (red) and anti-CD49b (green) antibodies. Blue, nuclei. B, IKDC, NK, and CDC shown in A were stained by ICS for I-A/E (see legend) or isotype-matched control (shaded histogram) and expression was assessed by FACS. C, BALB/c CD11c+ (including CDC and IKDC) and CD11c+ (including NK) splenocytes were incubated 12 h with MCMV-infected or mock-treated fibroblasts. MHC-II expression was assessed by FACS on NK (CD49b+ cells from the CD11c+ fraction), IKDC (CD49b+ from the CD11c− fraction), and CDC (CD11c+CD49b− from the CD11c+ fraction). Percentages of MHC-II expression when the cells were cultured in presence of uninfected fibroblasts (mock) and MCMV-infected fibroblasts (black) are indicated. D, IKDC but not NK expressed I-A/E when activated in vivo by MCMV.
proliferation of OT-I. The number of T cells after culture with NK stimulators was one tenth that in IKDC wells. Meanwhile, the number of T cells remaining in CDC wells was nine times that in IKDC wells. Whereas Fig. 5 shows proliferation at 1:3 APC/T cell ratio, CDC but not IKDC were still able to induce OT-I proliferation at 1:10 ratio, suggesting that IKDC are less efficient in stimulating T cells (data not shown). Cell subsets were highly purified via two rounds of sorting and doublet exclusion to assure the absence of NK/DC aggregates (Fig. 5A). NK included 2% CDC contamination (Fig. 5A), which, nonetheless, was not sufficient to trigger OT-I proliferation (Fig. 5C), indicating that T-cell proliferation cannot be attributed to the <0.7% contaminating CDC in the IKDC preparation. Homogeneous expression of CD49b and NK1.1 by sorted IKDC (>96%) excludes contamination by PDC. Figure 5B shows that MHC-IIhi IKDC, but not activated NK, also homogeneously up-regulated CD40 (85%; Fig. 5B), whereas both subsets expressed B220. The inability of activated NK to stimulate OT-I T cells does not reflect the lack of MHC-I expression because all three populations expressed comparable levels of H2-Kb (Fig. 5B). Perforin-1 IKDC did not activate OT-I cells indicating that killing is required for IKDC to uptake and cross-present antigen (data not shown).

Activated IKDC, CDC, and NK sorted from C57BL/6 mice infected with MCMV/OVA- or MCMV/GFP were used in direct ex vivo antigen detection assays (Supplementary Fig. S8B; Fig. 6). Although the proportion of MHC-IIhi IKDC was higher in LN than spleen of infected mice (Supplementary Fig. S4), sorting was performed on splenocytes to isolate higher number of activated IKDC. Each population was subjected to a stringent two round sorting and doublet exclusion to reach a 99% purity, especially avoiding contamination of IKDC with CDC (Fig. 6A). Sorted IKDC homogeneously expressed high levels of I-Ab, CD40, CD49b, and NKG2D (Fig. 6A–B), ruling out the presence of (Fig. 5A), which, nonetheless, was not sufficient to trigger OT-I proliferation (Fig. 5C), indicating that T-cell proliferation cannot be attributed to the <0.7% contaminating CDC in the IKDC preparation.

Figure 3. Upon recognition of MCMV-infected fibroblasts, IKDC differentiated into mature MHC-IIhi APC endowed with CD4+ T-lymphocyte stimulatory properties. A, C57BL/6 CD11c+ and CD11c- splenocytes were incubated with MCMV/GFP- or MCMV/OVA-infected fibroblasts. CDC (CD11c+CD11c+), IKDC (CD11c+CD11c+), and NK (CD11c+CD11c+) were FACs-sorted from gates 1, 2, and 3, respectively, and incubated with OVA-specific OT-II CD4+ T cells. IL-2 was measured by ELISA in day 3 culture supernatants. Oligodeoxynucleotides CpG 1668 was added or not to the culture to stimulate the APCs. APC were also incubated in presence of OVA323-339 to stimulate OT-II. B, similar presentation assay was performed with HA antigenic model. The purity of sorted populations used in this assay was shown in Supplementary Fig. S3. C, costimulatory molecules expression on CD11c+CD11c- IKDC (open) versus CD11c+CD11c- CDC (dark gray) in presence of infected fibroblasts.
putative NKG2D\(^+\) CD49b\(^+\) APC (7) and CD49b\(^+\) NKG2D\(^+\) PDC. Both NK and IKDC expressed KLRG1 (Fig. 6B). Sorted MHC-II\(^+\) IKDC and CDC, but not MHC-II\(^-\) NK, triggered OT-II T-cell proliferation when pulsed with OVA 323-339 (Supplementary Fig. S8C). Under these conditions, IKDC and CDC, but not NK, freshly sorted from MCMV/OVA-infected mice were able to induce proliferation of CFSE-labeled CD8\(^+\) OT-I at a ratio of 1 APC/3T cells. CFSE dilution was assessed by FACS after 4-d incubation. Percentages of proliferating T cells within gate are indicated for cultures containing stimulators previously incubated with MCMV/OVA-infected (black) or MCMV/GFP-infected (gray) fibroblasts.

**Discussion**

In this report, we further delineate the phenotypic and functional differences between NK and IKDC and provide evidence that, upon killing of their targets, IKDC, but not NK, can mature into fully competent APC that cross-prime naïve CD4\(^+\) and CD8\(^+\) T cells. First, our findings establish *in vitro* that cellular contact with MCMV-infected targets was mandatory for IKDC to up-regulate MHC-II, costimulatory molecules, and to secrete IFN-γ and IL-12p40. Second, activated IKDC, which remained MCMV-free, presented *in vivo* and *in vitro* antigens released from killed target cells to CD4\(^+\) and CD8\(^+\) T cells.

Although IKDC have been recently affiliated to an activated NK (4, 5, 7), the present report brings forward molecular (i.e., microarray analysis) and functional (i.e., T-cell proliferation assays) evidence clearly demonstrating that MHC-II expression by activated IKDC reflects a fully functional antigen processing...
machinery that facilitates efficient antigen cross-presentation. Highly purified activated IKDC (MHC-II$^{\text{hi}}$) isolated from cocultures of CD11c$^+$ splenocytes with MCMV/OVA-infected targets process OVA antigen to consequently stimulate specific CD4$^+$ and CD8$^+$ T cells. The striking discrepancy with the groups reporting a complete lack of T-cell activation by both NK and IKDC, even in presence of high doses of cognate peptide (5, 7), could be explained by the fact that we assessed highly purified FACS-sorted activated MHC-II$^{\text{hi}}$CD11c$^{\text{hi}}$B220$^-$CD49b$^+$ IKDC as APC in our antigen presentation assays because only MHC-II$^{\text{hi}}$ IKDC were found to behave as mature APC (1).

Similarly to our viral infection model, Anderson’s and Zitvogel’s groups showed that cellular contact with tumor cells triggered up-regulation of MHC-II and costimulatory molecules and licensed IKDC to cross-present antigen to CD4$^+$ and CD8$^+$ T cells (21, 22). Although activated by the tumor or MCMV, NK failed to function as an APC in both experimental situations. Although we established that killing of the target is mandatory for antigen processing and presentation by activated IKDC, the nature of the mechanism used by IKDC to capture OVA expressed by MCMV/ OVA-infected fibroblasts was not addressed. However, Zitvogel and colleagues found that “licensed” CD11b$^+$ IKDC engulfed soluble, OVA-infected fibroblasts was not addressed. However, Zitvogel and colleagues have shown that blockade of B7-H1 dramatically enhances the capacity of IKDC to stimulate effector functions of cognate T cells (22). This phenomenon suggests a putative role for IKDC in the regulation of T-cell function.

Activated human NK up-regulate MHC-II and stimulate antigen-specific CD4$^+$ T cells (23, 25, 33). Strikingly, our study showed that activated IKDC, unlike human NK, were additionally able to cross-present antigens to CD8$^+$ T cells. Therefore, we anticipate that putative activated human IKDC would be functionally distinct from activated NK. Recently, IFN-DC, generated in vitro by treatment of human monocytes with IFN-α, were shown to express CD56 and IFN-γ (38). These CD56$^+$ IFN-DC exhibited potent cytotoxic activity and seemed to stimulate in vivo antigen-specific CD8$^+$ T-cells. Another study described DC expressing perforin at the vicinity of tumor in vivo and showed their cytotoxic activity in vitro (39). These cell members represent potential human homologues of IKDC. However, only the identification of specific markers will allow the translation to human and the assessment of their therapeutic implications.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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