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The Dendritic Cell–like Functions of IFN-Producing Killer Dendritic Cells Reside in the CD11b$^+$ Subset and Are Licensed by Tumor Cells

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Abstract

IFN producing killer dendritic cells (IKDC) were originally defined as CD11c^{int} B220$^+$NK1.1$^+$ (or CD49b$^+$) cells that exert a potent tumoricidal activity in animals lacking B, T, and conventional natural killer effectors. MHC class II expression on tumor infiltrating IKDC prompted us to investigate their putative antigen presenting function. Here, we show that tumor cells license IKDC to acquire the properties of antigen presenting cells, i.e., expression of MHC class II and costimulatory CD86 molecules. We show that the CD11b$^+$ subset of IKDC are able to prime naïve CD4$^+$ T cells and cross-prime naïve CD8$^+$ T lymphocytes. Licensing of IKDC by tumor cells was mandatory for the full differentiation of T cells into polarized effectors. IKDC could engulf and process soluble Ova protein in a CD206-dependent manner. Finally, we show that CD11b$^+$IKDC is selectively endowed with CTLA4Ig-inhibitable antigen presenting capacities and that targeting this subset with the detoxified adenylate cyclase toxin of *Bordetella pertussis* fused to antigen resulted in efficient cross-presentation of antigen by IKDC to specific TCR transgenic CD8$^+$T cells *in vivo*. Collectively, our data indicate that upon exposure to tumor cells, IKDC subserve DC-like functions. [Cancer Res 2009;69(16):6590–7]

Introduction

Tumors can regress as a result of autonomous cell death pathways and/or invading myeloid and lymphoid effectors that act in concert to kill cancer cells. Although myeloid cells are widely recognized as antigen-presenting cells and lymphoid cells as classic effectors, the functional concept of natural killer (NK) myeloid dendritic cells (DC; refs. 1, 2) is emerging in murine (3, 4) and human (5–7) *in vitro* studies but lacks *in vivo* relevance. Conversely, a subset of non-T, non-B cells called IFN producing killer DC (IKDC) was recently identified as potentially endowed with the dual potential of killing and antigen presentation (8–10).

IKDCs were originally defined as CD11c^{int}B220$^+$NK1.1$^+$ (or CD49b$^+$) cells lacking the expression of CD3, CD19, and Gr-1 molecules (8, 10). The nomenclature "IKDC" relied upon the presence of functional MHC class II (MHCII) molecules harbored on IKDC in lymph nodes (8) and tumor beds (9, 10) together with their capacity to produce IFNα(after viral infection or TLR9 triggering; ref. 8) or IFNγ [after exposure to tumor cells or interleukin (IL)-12+IL-18] and to kill a variety of cancer cell types (9, 10). However, several observations suggested that IKDC could represent a subset of NK cells. First, IKDC express receptors that are typically present on NK cells such as CD122, NKG2D, and Ly49 molecules (8–10). Second, IKDC rely on the *trans*-presentation of IL-15 by IL-15Rα for their homeostatic (9, 11) and inflammation-driven proliferation (9, 12). Based on the facts that IKDC express the NK-specific marker NKp46 (11), which B220 and CD11c molecules can be acquired upon NK cell proliferation (11), and the poor antigen-presenting capabilities of IKDC (13, 14), three independent teams of investigators have challenged the view that IKDC belong to a separate DC lineage.

Considering that MHCII expression by IKDC was up-regulated in tumor beds (10) and down-regulated by *trans*-presentation of IL-15 by IL-15Rα *in vitro* (9), we studied the antigen-presenting capacities of resting IKDC freshly isolated from splenocytes. Our data indicate that the CD11b$^+$ subset of IKDC selectively exerts DC-like functions, i.e., priming of naïve CD4$^+$ and CD8$^+$T lymphocytes. IKDC required prior exposure to tumor cells to become fully competent at polarizing Th1 and Tc1 lymphocytes *in vivo*. CD11b$^+$IKDC cross-prime to CD8$^+$T cells in a B7-dependent manner *in vitro* and *in vivo*. Moreover, CD11b$^+$IKDC engulf soluble Ova protein in a CD206-dependent manner. Finally, cross-presentation to CD8$^+$T cells of antigens fused to the detoxified adenylate cyclase of *Bordetella pertussis* toxin capable of binding to the $\alpha_M\beta2$ integrin was mediated by CD11b$^+$ IKDC *in vivo*. Collectively, these data support the concept that IKDC can acquire DC-like capacities in a tumor environment.

Materials and Methods

Cell lines and mouse strains. C57Bl/6 wild-type (WT) and H-2K^b/H-2D^b/β2 microglobulin knockout mice were obtained from the Centre d'Elevage Janvier and from Charles River Laboratories and used at 7 to 10 wk of age. C57Bl/6*Rag*$^{-/-}$TCR transgenic OT-II mice (specific for the Ova$_{257\text{-}264}$ peptide) were kindly provided by Dr. O. Lantz (Institut Curie, Paris, France), C57Bl/6*Rag*2$^{-/-}$TCR (Vα2, Vβ5) transgenic mice (OT-I) by Dr. A. Boissonas (Institut Curie), CD11c-GFP/DTR mice (15) by P. Aucouturier (Institut National de la Sante et de la Recherche Medicale, St. Antoine Hospital, Paris, France), CD83$^{-/-}$mice (16) by T. Tedder

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

M. Terme, G. Mignot, E. Ullrich, N. Chaput, and L. Zitvogel contributed equally to this work.

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(Department of Immunology, Duke University Medical Center, Durham, NC). Animals were all maintained according to the Animal Experimental Ethics Committee Guidelines. B16-Rae1 and B16-Ova were stably transfected with Rae1 or Ova encoding cDNA, respectively (kindly provided by E. Tomasello, CIML, Marseille, France and C. Théry, Institut Curie, Paris, respectively).

Antibodies and flow cytometry analyses. Fluorescence-activated cell sorting (FACS) analyses were performed using monoclonal antibody (mAb) against CD11c (HL3), NK1.1 (PK136), B220 (RA3-6B2), CD3 (17A2), CD19 (1D3), MHCII (AF6-120.1), IA/IE (NIMR-4), CD40 (3/23), CD80 (16.10A1), CD86 (GL1), NKG2D (CX5), CD69 (H1.2F3), or CD27 (LG.3A10). Abs were purchased from Pharmingen or eBioscience. Immediately before FACS analysis, 4′,6-diamidino-2-phenylindole was added. FACS analysis was performed by LSRII using FACS Diva Software and CellQuestPro Software (Becton Dickinson) or Flowjo (Treestar). Neutralizing Abs were used in some experiments: neutralizing anti-IFNγ (Pharmingen), anti–tumor necrosis factor-related (TRAIL) apoptosis-inducing ligand Ab (kindly provided by H. Yagita, Department of Immunology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo, Japan), anti-CD206 mAb targeting the mannose receptors (MMR, clone 310301; R&D Systems), CTLA4Ig (Roche), or anti-CD40L Ab (Clone MR-1; Pharmingen).

Sorting and purification of IKDC, B220$^-$NK, and cDC. The procedures were previously described (9). Briefly, IKDC and B220$^-$NK cells were sorted from spleens of naïve C57Bl/6 mice after enrichment of NK cells (negative selection; Miltenyi Biotec). IKDC were sorted as CD3$^-$CD19$^-$CD11c^{int}B220$^+$NK1.1$^+$ cells, B220$^-$NK cells as CD3$^-$CD19$^-$CD11c$^-$B220$^-$NK1.1$^+$ cells. We sorted separately CD11b$^+$IKDC and CD11b$^-$IKDC. cDC were sorted as CD11c$^+$I-Ab$^+$ cells. Cell separation was performed on a Mo-Flo instrument (DAKO). The purity of cell separation exceeded 98%.

Licensing experiments and *in vitro* T-cell priming. BM-DC were generated as previously described (17). For licensing experiments, 1.5×10^5 FACS-sorted IKDC, NK, or cDC from spleens of naïve C57Bl/6 mice were cocultured with B16 tumor cells at the 10:1 ratio in presence of 1 mg/mL of Ova protein for 12 h. Then, either FACS was performed to analyse MHCII or costimulatory molecules expression, or cocultures were washed extensively (to remove resting traces of Ova protein) to proceed to test T-cell priming *in vitro*. Resting T lymphocytes purified from naïve OT-I or OT-II mice (negative selection; Miltenyi Biotec) were incubated at the 1/1 effector/T-cell ratio with different APCs. After a 24-h incubation period, IFNγ and IL-2 secretion were measured by ELISA (Pharmingen), and T cells were stained with anti-CD3, anti-CD4 or anti-CD8, anti-Vα2, and anti-CD69 Ab.

***In vivo* priming studies.** Resting CFSE-labeled OT-I (10^6), OT-II, or GFP/OT-I lymphocytes purified from naïve OT-I or OT-II mice were injected i.v. into naïve C57Bl/6 mice 18 h before footpad inoculation of the IKDC, NK, or DC (see experimental settings in figure insets). FACS-sorted IKDC or NK cells (1.5×10^5) or 3×10^5 DC were pretreated for 12 h as follows: (*a*) unpulsed or pulsed with 1 mg/mL of Ova protein, (*b*) cocultured with B16 (B16-Rae1 or B16-Ova) tumor cells at a 10:1 E/T ratio in the presence (or not) of 1 mg/mL of Ova holoprotein. In Fig. 4*C*, After an extensive washing, IKDC, NK, or DC were injected into the footpad of mice. After 3 to 5 d, popliteal lymph node cells were harvested and examined to define the activation, proliferation, and polarization status of adoptively transferred transgenic T cells. (*a*) DLN cells were stained with anti-CD69 Ab or proliferation of CFSE-labeled T cells was analyzed by FACS by gating on OT-I or OT-II cells. (*b*) DLN cells (10^5 cells per well) were restimulated or not with 1 mg/mL of Ova protein or SIINFEKL peptide *in vitro*. Supernatants were harvested 72 h later, IFN-γ secretion was assessed by ELISA. (*c*) Alternatively, DLN cells were restimulated with 1 mg/mL of Ova protein for 12 h (with addition of Golgi-Stop; Pharmingen) and stimulated

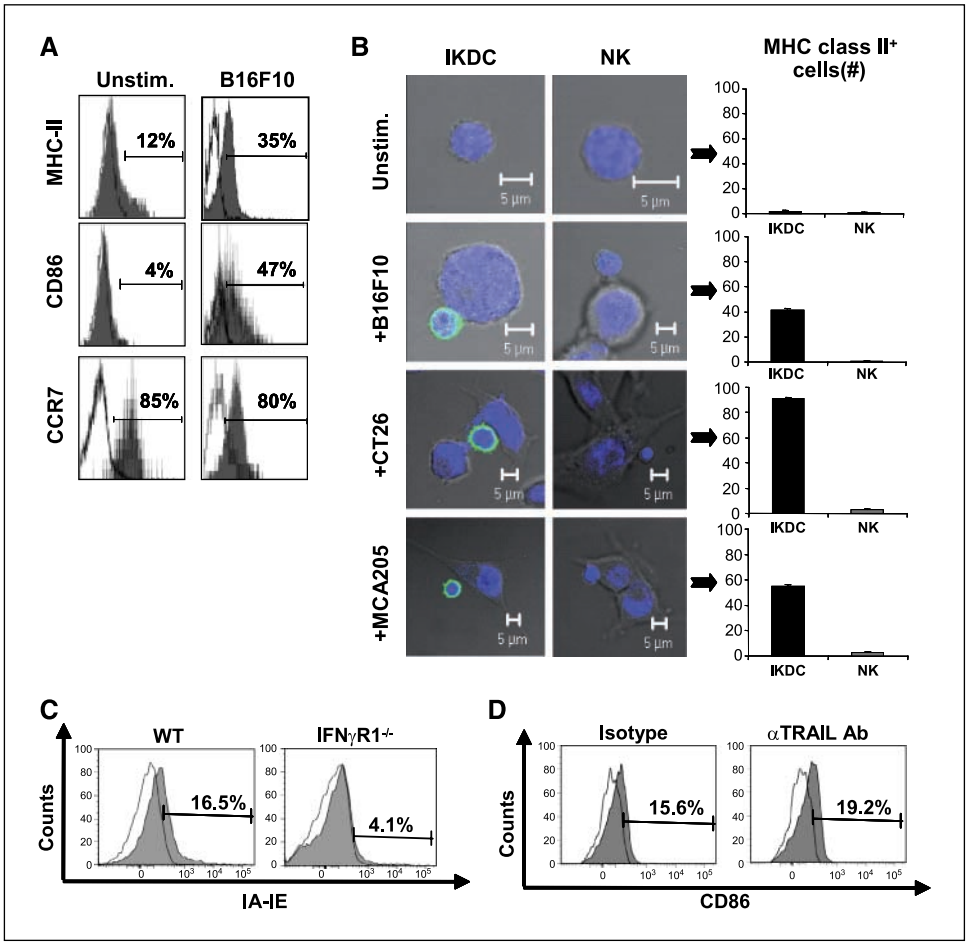

Figure 1. Tumor cells license IKDC to express MHCII and CD86 molecules. *A* and *B*, tumor cells license IKDC for MHCII and CD86 expression. B16F10 tumor cells were incubated for 24 h with spleen-derived IKDC sorted from naïve C57Bl/6 animals, at a 10:1 E/T ratio. Flow cytometry analyses (*A*) of effector cells were performed gating on IKDC cells, respectively, after staining with anti–I-A^b/I-E^b Ab or anti-CD86 or anti-CCR7 mAb. A representative experiment of three is depicted. Confocal microscopy allowed to visualize MHCII expression in green and cell-cell contacts between a variety of tumor cells and IKDC or B220$^-$NK cells (*B, left*) and to enumerate the MHCII expressing effectors (*B, right*). The photographs depict typical cocultures and the results are presented as means ± SE of fluorescent cells examined in 100 cells. *C* and *D*, IFNγR-dependent tumor licensing of IKDC. Identical setting as in *A*, but IKDC were sorted from spleens of naïve WT and IFNγR$^{-/-}$ mice (*C*) or incubated in the presence of N2B2 (anti-TRAIL neutralizing Ab; *D*). The experiment was performed twice with identical results.

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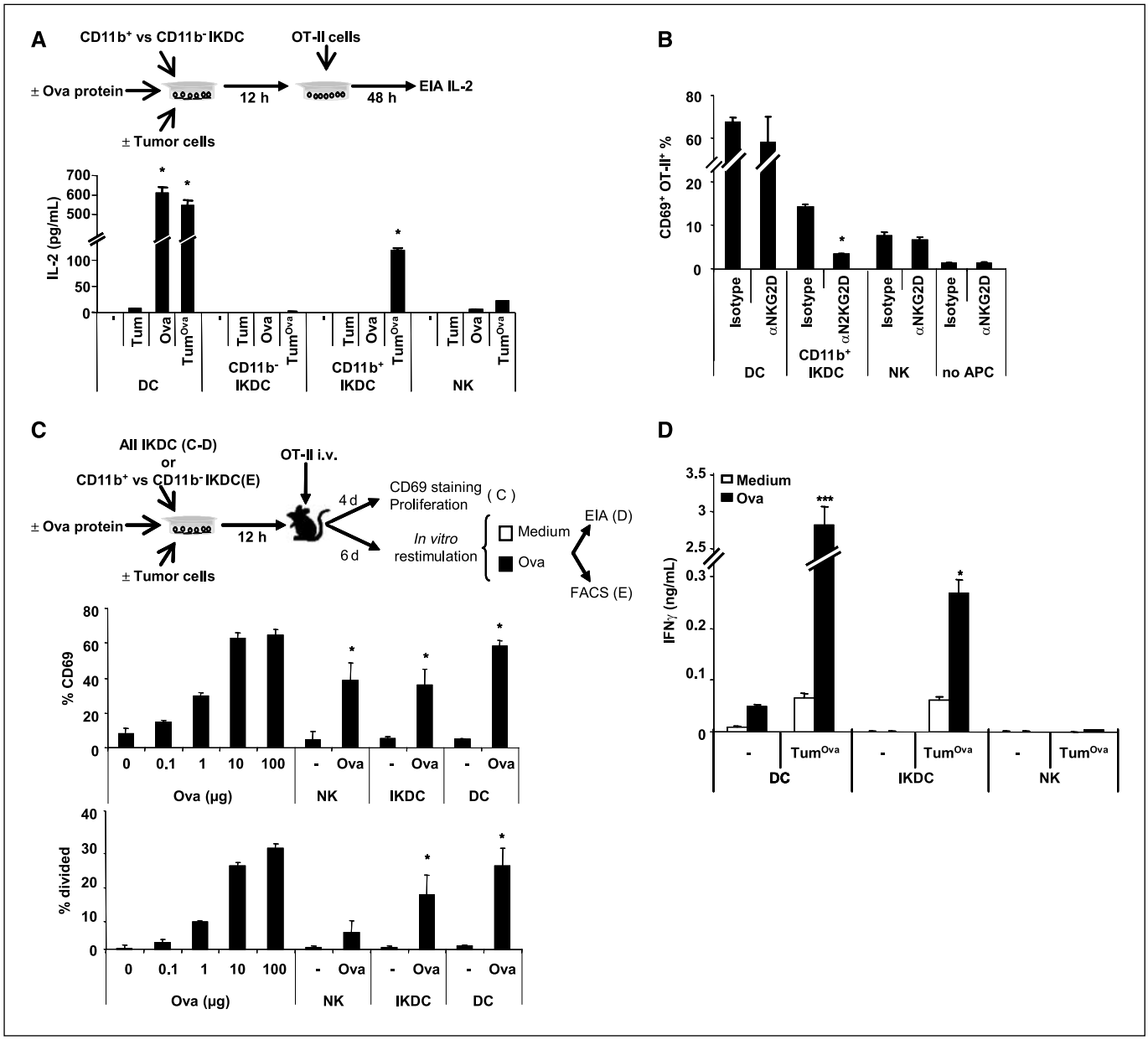

Figure 2. IKDC can prime naïve OT-II cells after tumor licensing. *A,* CD11b+IKDC can prime naïve OTII cells after tumor licensing *in vitro.* IKDC (CD11b$^+$ or CD11b$^-$) or B220$^-$NK cells or BM-DC (DC) were first incubated for 12 h in the presence of B16-Rae1 tumor cells (*Tum*) at a 10:1 E/T ratio or soluble Ova protein at 1 mg/mL (*Ova*) or B16-Rae1 tumor cells and soluble Ova protein at 1 mg/mL (*Tum^{Ova}*). The coculture was washed before addition of naïve OT-II cells at a 1:1 APC/OT-II ratio for 18 h. Supernatants were analyzed by commercial mIL-2 ELISA (see experimental setting in the inset of *A*). *B,* Id, as in *A*, but anti-NKG2D neutralizing or isotype control Abs were added during the licensing period. OTII cells were next analyzed in flow cytometry for CD69 expression. *C* and *D,* IKDC can prime naïve OT-II cells after tumor licensing *in vivo.* CFSE-labeled OT-II (10^6) were inoculated i.v., 150 to 250 $\times$ 10^3 live IKDC (or B220$^-$NK cells), pulsed with soluble Ova protein (*Ova*) and licensed [or not (*C*)] with tumor cells (*Tum^{Ova}*), were inoculated in the footpad of C57Bl/6 mice 18 h later. Positive controls included footpad inoculation of 300 $\times$ 10^3 live BM-DC (DC) cultured in similar conditions or increasing dosages of Ova (see experimental setting in the inset of *C*). After 4 d, DLN were harvested, stained with anti-CD69 mAb, and analyzed in flow cytometry analysis. The means ± SE of percentages of CD69 expressing CFSE$^+$ cells (*C, top*) and the percentages of OT-II dividing cells (*C, bottom*) in three independent experiments containing two mice per group. APC could be licensed by B16-Ova tumor cells during the pulsing period with Ova before inoculation (*D*). In another group of mice, day 6 DLN were restimulated with PBS or Ova protein and the production of IFNγ was measured by ELISA (*D*). *, $P < 0.05$; ***, $P < 0.001$; *ND,* not done.

for 2 additional h with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL), followed by staining with Abs specific for Vα2, CD3, CD4 or CD8, anti–IL-2, and anti-IFN–γ mAbs.

Statistical analysis. All results are expressed as means ± SE or as ranges when appropriate. Aberrant values were excluded using Dixon's test. Normality of distributions was assessed using the Shapiro-Wilk's test. Normal distributions were compared by the Student's *t* test; non-normal samplings were compared using the Mann-Whitney's test. ANOVAs were performed with the Kruskal-Wallis test. Statistical analyses were performed using Prism 5 software (GraphPad).

Results

Licensing of IKDC by tumor cells for MHCII and CD86 expression *in vitro*. We previously reported that a high proportion of CD11c^{int}B220$^+$NK1.1$^+$ IKDC infiltrated regressing (but not

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progressive) tumors following therapy of established lung B16F10 metastases with imatinib mesylate and IL-2 (10, 12). IKDC possessed TRAIL-dependent killing activity and controlled tumor progression in the absence of other lymphoid effectors (10). IKDC produced IFNγ in contact with tumor cells (10, 18) and expressed MHCII molecules in regressing tumor beds (10). Here, we addressed whether tumor cells could dictate the DC-like phenotype of IKDC. Splenic IKDC were obtained from C57Bl/6 mice by a combination of positive and negative selection processes, as $CD3^-CD19^-CD11c^{int}B220^+NK1.1^+$ cells. Splenic IKDC freshly isolated from naïve mice express low level of MHCII antigen (Fig. 1*A*). Upon contact with a variety of distinct tumor cell lines (B16F10 melanoma, CT26 colon cancer, MCA205 sarcoma), IKDC could up-regulate MHCII molecules and CD86 on the cell surface, as shown by flow cytometry or confocal microscopy (Fig. 1*A* and *B*). In identical conditions, freshly isolated $B220^-$NK cells did not express MHCII (Fig. 1*B*). The expression of MHCII molecules by IKDC depended upon the autocrine production of IFNγ because IKDC sorted from $IFN\gamma R^{-/-}$ mice failed to express MHCII in coculture with B16F10, although they produced IFNγ (data not shown; Fig. 1*C*). Similar results were achieved using neutralizing anti-IFNγ Ab (data not shown). Beyond the induction of MHCII molecules, tumor cells stimulated the expression of the costimulatory molecule CD86 on IKDC (Fig. 1*A*). Although TRAIL expression reportedly can be up-regulated by IFNγ (19), blocking TRAIL failed to inhibit the up-regulation of CD86 molecules on IKDC (Fig. 1*D*).

In conclusion, IKDC (but not $B220^-$NK cells) can be licensed by tumor cells to exhibit a DC-like phenotype *in vitro*.

$CD11b^+$ IKDC elicit the differentiation of Th1 cells *in vivo*. $CD11c^{int}B220^+NK1.1^+$ cells could be subdivided into $CD11b^+$ and $CD11b^-$ subsets (Supplementary Fig. S1). After coculture with B16F10 tumor cells, both subsets of IKDC indistinguishably acquired MHCII expression, a phenomenon that we refer to as "licensing." We assessed the functionality of MHCII molecules expressed by tumor-licensed $CD11b^+$ versus $CD11b^-$IKDC *in vitro*. Freshly isolated splenic IKDC were cocultured for 12 hours with B16F10 cells overexpressing the NKG2D ligand Rae1 (B16-Rae1) in the presence of soluble Ova protein (licensing incubation), the coculture was then washed and IKDC exposed to naive TCR-transgenic OT-II cells. Only Ova-pulsed $CD11b^+$ IKDC licensed by tumor cells could trigger IL-2 production from naïve OT-II cells *in vitro*. Thus, in contrast to cDC, IKDC required licensing by tumor cells to acquire antigen presenting cells function (APC; Fig. 2*A*). Recognition and/or killing of B16-Rae1 through NKG2D receptors may play an important role in the IKDC-mediated activation of OTII cells *in vitro* (Fig. 2*B*).

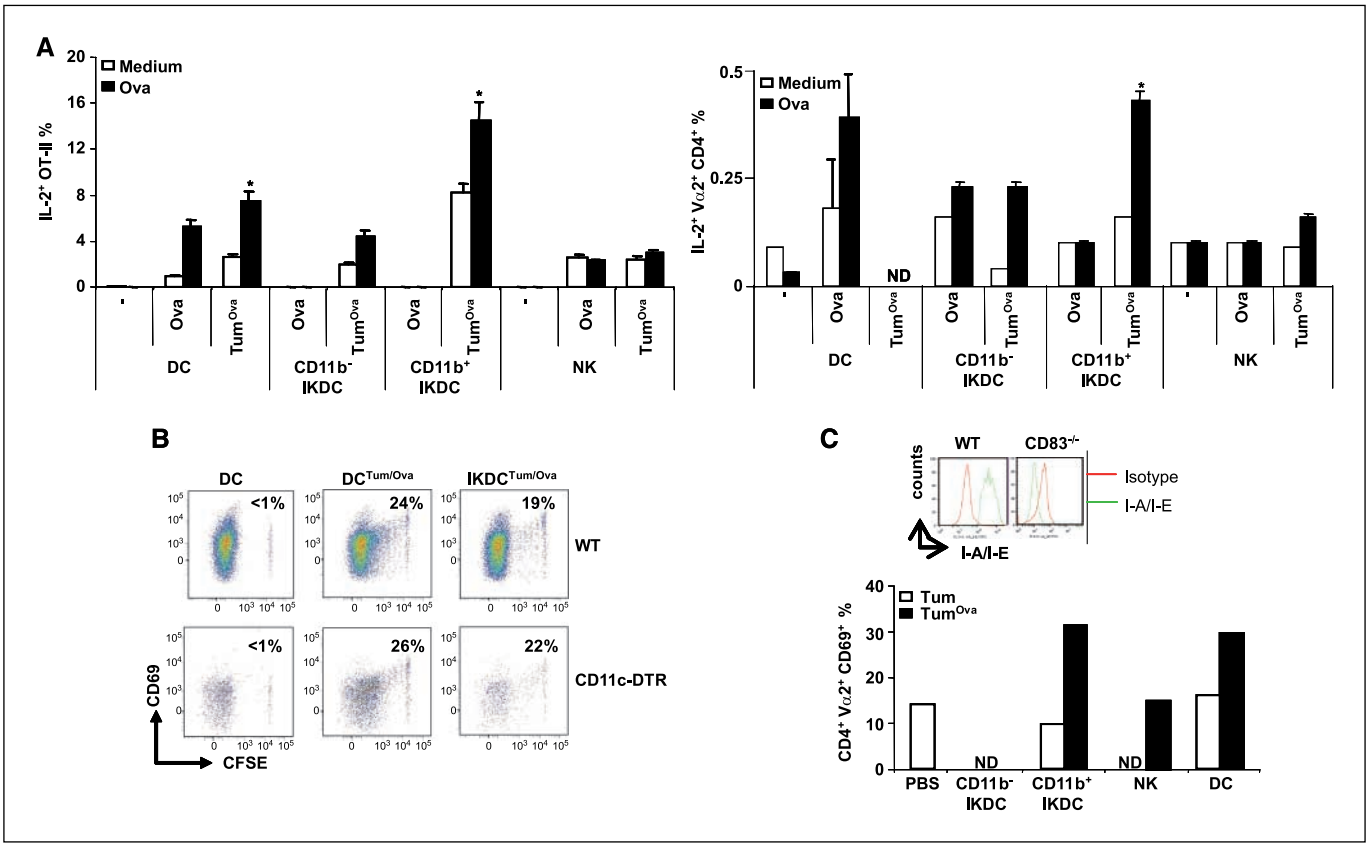

Figure 3. $CD11b^+$IKDC can directly prime naïve OT-II cells after tumor licensing. *A,* $CD11b^+$IKDC can prime naïve OT-II cells after tumor licensing *in vivo.* CFSE-labeled OT-II (10^6) were inoculated i.v., 150 to 250 × 10^3 live $CD11b^+$ or $CD11b^-$IKDC (or $B220^-$NK cells), pulsed with soluble Ova protein (*Ova*), and licensed with tumor cells (Tum^{Ova}), were inoculated in the footpad of C57Bl/6 mice 18 h later. At day 6, DLN were restimulated with PBS or Ova protein and intracellular staining of IL-2 after Ova restimulation was monitored (as described in Materials and Methods) gating on GFP-labeled OTII cells (*top*) or all Vα2 unlabeled $CD4^+$ T cells (*bottom*; *E*). *B,* ablation of host cDC did not compromise IKDC-mediated OT-II stimulation. WT or CD11c-DTR transgenic mice were treated with diphtheria toxin 1 d before OT-II injection. At day 2, IKDC pulsed with Ova were injected. The graph outlines a representative staining of CD69 expressing $CFSE^+$ cells of two independent experiments containing two mice per group. Flow cytometry analyses of $CD11c^{high}$/MHCII expressing cDC revealed their ablation in control animals (data not shown). *C,* IKDC are competent APC in CD83-deficient mice. Surface MHCII expression was checked by flow cytometry on gangliocytes obtained from $CD83^{-/-}$ and WT mice (*top*). $CD11b^+$ IKDC, $B220^-$ $CD11c^-$ NK cells, cDC licensed by tumor cells and pulsed with Ova protein were injected into $CD83^{-/-}$ mice, 1 d after OT-II cells transfer. Eighteen hours later, CD69 expression was monitored on OT-II cells defined as $CD3^+CD4^+V\alpha2^+$ cells (*bottom*). *, $P < 0.05$; ***, $P < 0.001$; *ND,* not done.

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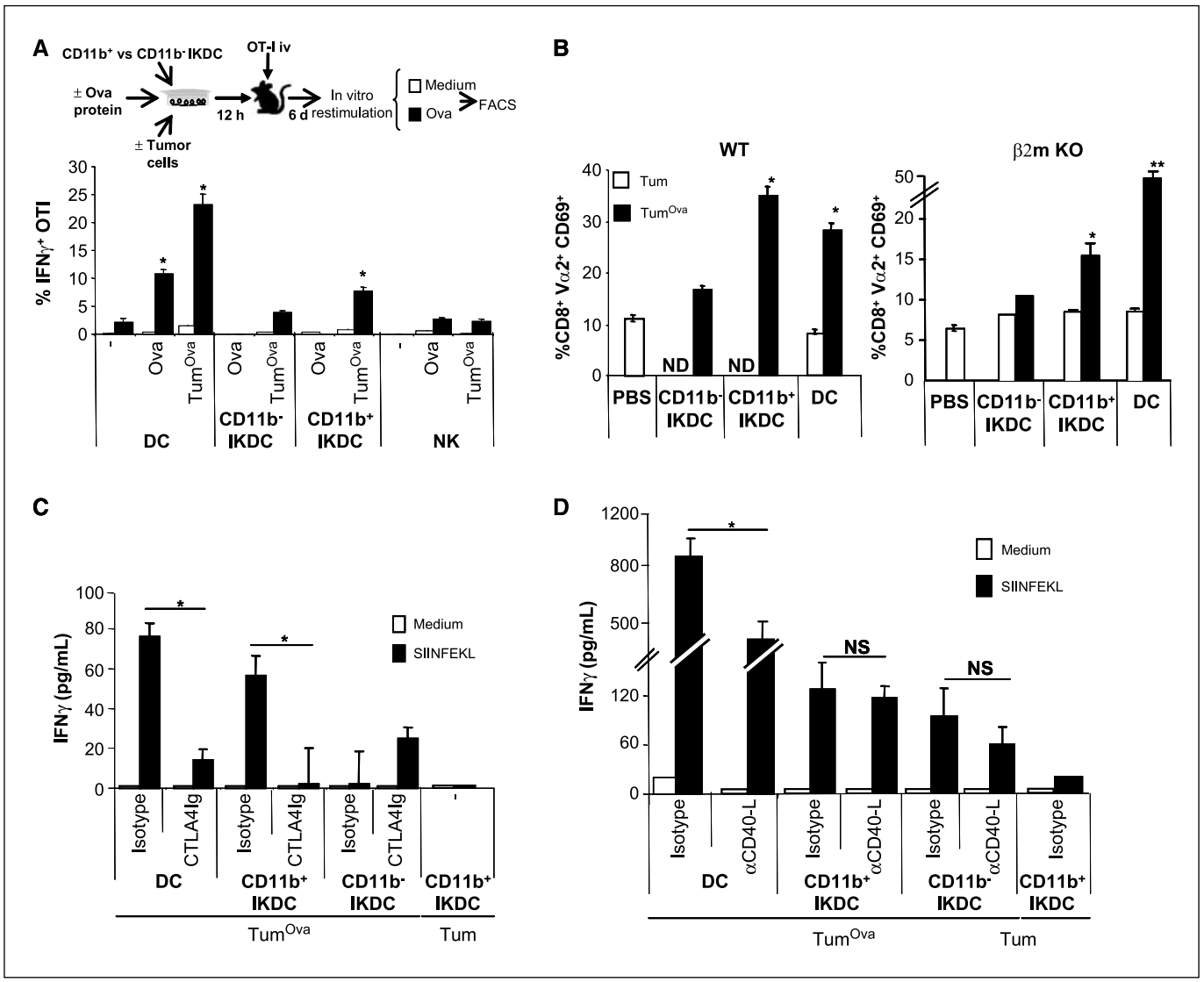

Figure 4. Licensed CD11b$^+$IKDC cross-present soluble antigens to naïve CD8$^+$T cells. *A,* CD11b$^+$IKDC prime naïve OT-I cells after tumor licensing. Identical settings as in Fig. 3*A* but adoptive transfer of naïve OT-I-GFP lymphocytes was performed and the intracellular staining identified IFNγ producing OT-I. A representative experiment out of two yielding similar conclusions, showing the proportion of IFNγ producing OT-I. *B,* CD11b$^+$IKDC are competent APC in H-2D^b/H-2K^b/β2 microglobuline knockout mice. OT-I cells defined as CD3$^+$CD8$^+$Vα2$^+$ T lymphocytes were monitored for CD69 expression in the DLN of WT and β2 microglobuline knockout host after transfer (18 h later) of WT DC or IKDC licensed with tumor cells and Ova. A representative experiment containing three mice per group is shown. *C* and *D,* 10^6 OT-I CD8+ T cells were inoculated i.v. Eighteen hours later, IKDC or DC were injected in footpads of C57Bl/6 mice after licensing with B16-Rae and Ova (Tum^{Ova}). Neutralizing CTLA4Ig fusion proteins (*C*) or anti-CD40L Ab (*D*) were coinjected together with the APC. At day 6, DLN were restimulated with the SIINFEKL peptide (10 μg/mL). Cytokine production was assessed at 48 h using IFNγ ELISA. A representative experiment containing three mice per group is depicted. *, $P < 0.05$; *ND,* not done.

Next, we addressed which minimal signals are required for IKDC to trigger naïve Ova-specific TCR-transgenic CD4$^+$T cells to become activated, to proliferate, and to differentiate into Th1 cells *in vivo.* Pulsing IKDC with soluble Ova protein was sufficient to induce CD69 expression and proliferation in T cells. However, for promoting the differentiation of OT-II cells into Th1 cells, IKDC had to be exposed to both soluble Ova protein and tumor cells (Fig. 2*A* and *D*). Finally, CD11b$^+$-licensed IKDC pulsed with soluble Ova promoted the priming and differentiation of Ova-specific CD4$^+$T lymphocytes *in vivo*, whereas CD11b−licensed IKDC or B220$^-$NK cells failed to do so (Fig. 3*A*). In this setting, Ova-pulsed B16-Rae1 failed to stimulate T-cell responses *in vitro* or *in vivo* (data not shown). To boost the antigen presentation capacity of CD11b$^+$IKDC, we exploited the fact that IKDC expressed B7-H1/CD274 (20), an immunosuppressive molecule involved in T-cell apoptosis or anergy. Blocking B7-H1/CD274 on CD11b+ IKDC by specific neutralizing mAb during licensing with tumor cells and pulsing with Ova augmented the priming capacity of IKDC on OTII cells, but could not turn CD11b- IKDC cells into functional APCs *in vivo* (Supplementary Fig. S2).

To exclude an antigen transfer to host cDC, we took advantage of two model systems, i.e., mice expressing the diphtheria toxin receptor under the control of the CD11c promoter (15) or CD83$^{-/-}$mice, which lack cell surface expression of MHCII (16, 21). In both circumstances, Ova-pulsed CD11b$^+$IKDC licensed by tumor cells maintained their ability to induce to activate CD4+ T cells (Fig. 3*B* and *C*).

Altogether, these data illustrate the potential of CD11b$^+$IKDC to behave as DC-like cells that display functional MHCII/peptide complexes after contact with cancer cells and soluble antigen.

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B7-dependent priming of naïve CD8$^+$ T lymphocytes by CD11b$^+$ IKDC. We investigated *in vivo* cross-presentation by incubating IKDC with soluble Ova in the presence or absence of B16-Rae1 tumor cells (at a 10:1 E/T ratio), and followed by adoptive transfer of these IKDC (or cDC or NK cells as controls) with freshly isolated naive SIINFEKL-specific TCR transgenic OT-I lymphocytes. Loading of IKDC (but not cDC) with soluble Ova was not sufficient to promote significant differentiation of IFNγ producing OT-I cells in DLN (Fig. 4*A*). IKDC (but not cDC) required licensing by tumor cells to become cDC-like APCs endowed with the capacity to present soluble Ova (Fig. 4*A*). Of note, CD11b$^+$IKDC (but not CD11b$^-$IKDC or NK cells) were electively competent in cross-presenting soluble Ova *in vivo* (Fig. 4*A*). To reinforce that IKDC themselves (rather than host-derived DC) could function as APC, we analyzed OT-I activation triggered by WT IKDC injected into H-2D^b/H2-K^b/β2-microglobuline$^{-/-}$ mice. The APC function of CD11b$^+$IKDC was intact in hosts compromised for MHC class I presentation (Fig. 4*B*), indicating that CD11b$^+$IKDC themselves act as APC.

Priming of naïve OT-I by CD11b$^+$IKDC required B7 molecules because a CTLA4Ig fusion protein abrogated the activation of OT-I by IKDC (as well as by cDC) *in vitro* (Fig. 4*C*) and *in vivo*, in a setting in which neutralizing CD40L with a specific Ab had no inhibitory effect (Fig. 4*D*).

Altogether, CD11b$^+$IKDC can cross-present class I–restricted antigens, but only when they are licensed by tumor cells.

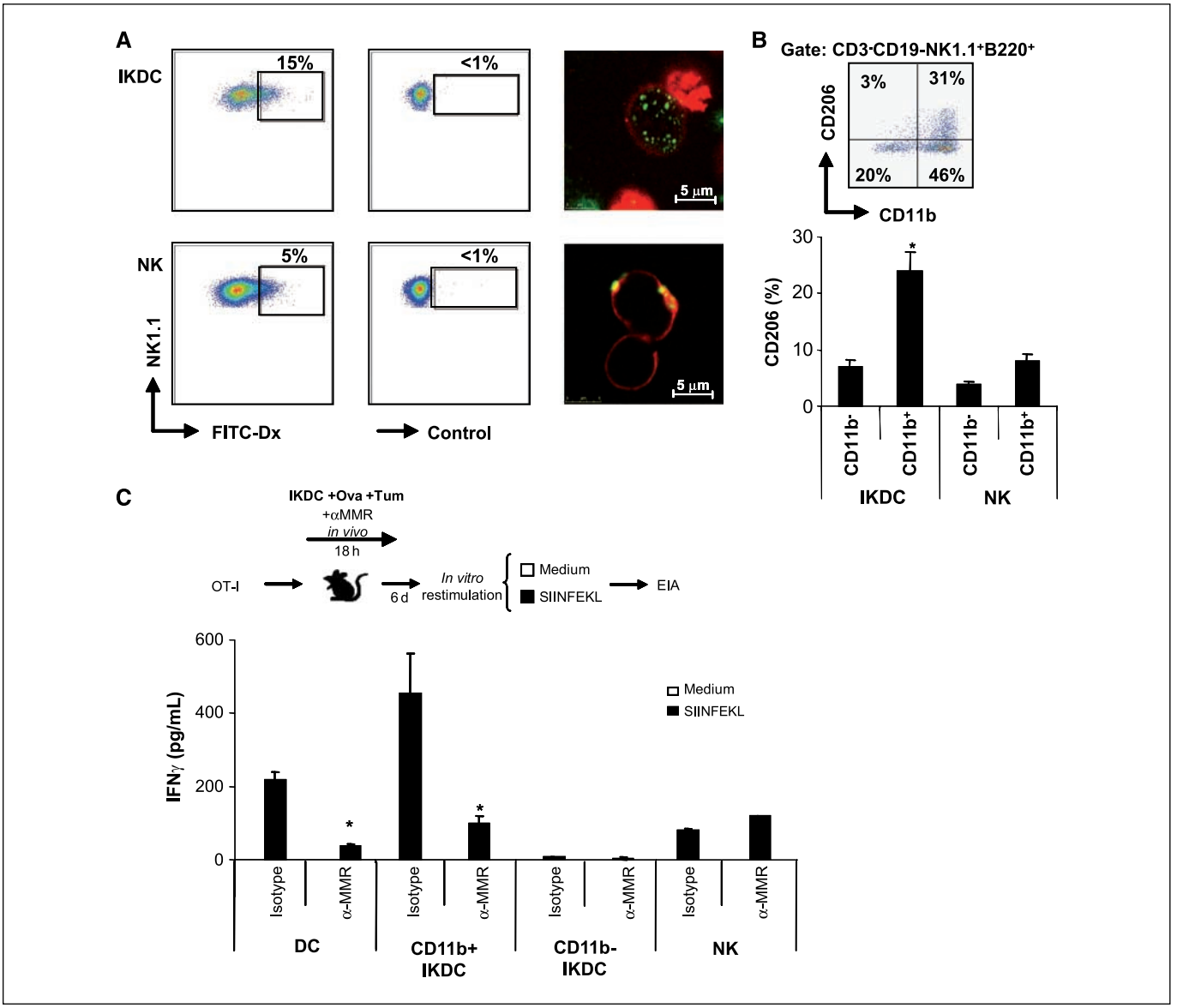

Figure 5. CD206-dependent T-cell priming by CD11b$^+$IKDC. *A* and *B,* IKDC can internalize fluid phase markers. IKDC and B220$^-$NK cells (*A*) were incubated or not with FITC-Dextran at 37°C. At 0°C, the specific staining was not observed (data not shown). Cells were analyzed at 4 to 6 h for FITC staining in flow cytometry and confocal microscopy after membrane labeling with wheat germ agglutinin A647 fluorescent dye. A representative picture is shown (*A, right*). The uptake was performed twice with identical results. *B,* mannose receptor expression on NK or IKDC. Naïve spleens were stained intracellularly with anti-CD206 mAb in addition to the classic six colors staining allowing the identification of IKDC and B220$^-$NK cells in flow cytometry analyses. The dot plots (*top*) are a representative staining of IKDC. *Columns,* mean three independent experiments; *bars,* SD. *C,* mannose receptor-dependent activation of OT-I by CD11b$^+$IKDC *in vivo*. Same experimental setting as in Fig. 4*C* and *D* but using anti-MMR neutralizing or isotype control Ab during the licensing phase. DLN were restimulated with the H-2^b–restricted Ova peptides (SIINFEKL at 10 μg/mL) for 24 h, and IFNγ levels were measured using ELISA. The mean values of triplicate wells are depicted in a representative experiment containing three mice per group. *, $P < 0.05$.

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IKDC engulf soluble antigen: a role for mannose receptors. Because IKDC apparently could process soluble Ova for antigen presentation (Fig. 2), we investigated their endocytic capacity, by incubating freshly isolated splenic IKDC (including CD11b$^+$ and CD11b$^-$) with the fluid phase marker dextran conjugated with fluoresceine isothiocyanate (FITC-DX). A significant fraction of IKDC was indeed labeled with FITC-DX at 37°C (but not at 0°C; data not shown), as shown by flow cytometry (Fig. 5*A, left*) and corroborated by confocal fluorescence microcopy (Fig. 5*A, right*). Importantly, CD11b$^+$IKDC contained higher levels of mannose receptors (CD206, MMR) than any other NK cell subset present in resting spleens (Fig. 5*B*). Next, we added a neutralizing anti-MMR Ab to IKDC (or other APC) when they were licensed by tumor cells and loaded with Ova. MMR neutralization abolished the activation of OT-I cells by cDC and CD11b$^+$IKDC *in vivo* (Fig. 5*C*). Hence, such as cDC (22–24), CD11b$^+$IKDC have the potential to internalize fluid phase proteins and/or to take up antigens via mannose receptors.

Targeting of IKDC *in vivo*. To further explore targeting and antigen delivery to IKDC *in vivo*, we used the adenylate cyclase (CyaA) of *Bordetella pertussis*. CyaA binds specifically to the $\alpha_M\beta_2$ integrin (CD11b/CD18; ref. 25) and delivers its catalytic domain into the cytosol of CD11b$^+$ cells (26, 27). Therefore, CD8$^+$T cell epitopes fused with the catalytic site of CyaA are processed and presented by MHC class I molecules at the surface of CD11b^{high}cDC (28). We injected the CyaA-Ova peptide (29) i.v. and monitored the capacity of FACS-sorted CD11b$^+$ or CD11b$^-$IKDC or cDC to activate OT-I lymphocytes *ex vivo*. CD11b$^+$IKDC (but not CD11b$^-$ IKDC), as well as CD11b$^+$cDC, mediated OT-I activation (CD69 expression and IFNγ secretion), provided that the animals had been injected with a CyaA-Ova vaccine (but not with an irrelevant CyaA-E7 fusion protein as control; Fig. 6*A* and *B*). In the presence of TLR9 agonists coinoculated with CyaA, CD11b$^+$IKDC (but not CD11b$^-$IKDC), as well as CD11b$^+$cDC, promoted the production of IL-2 by OT-I cells *ex vivo* (Fig. 6*C*). These results underscore the antigen-presenting capacity of CD11b$^+$IKDC, as evaluated in an *in vivo/ex vivo* system.

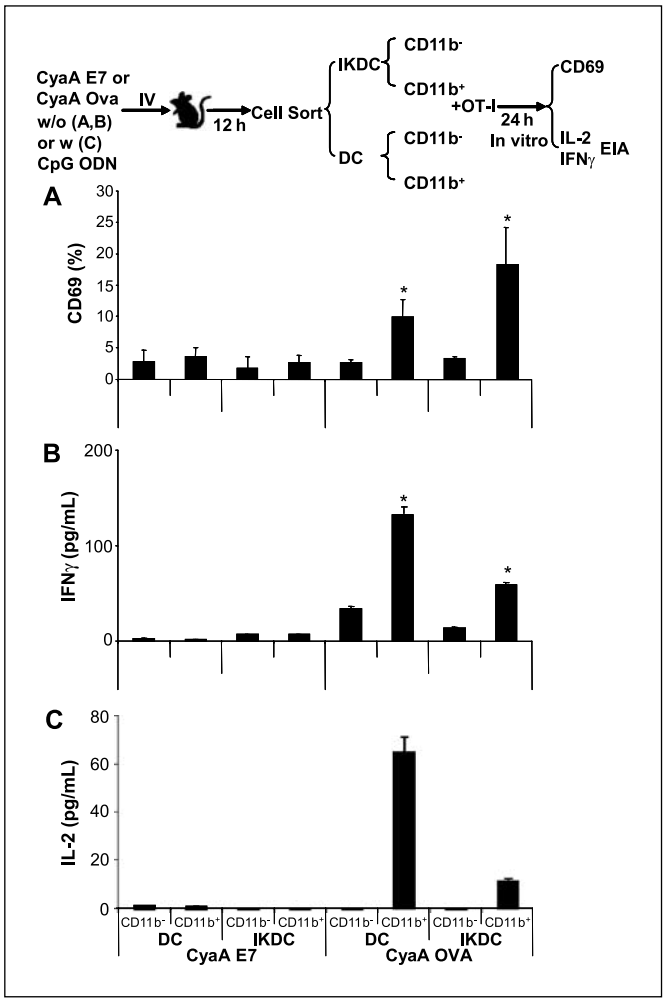

Figure 6. Targeted antigen delivery via CD11b/CD18 revealed CD11b$^+$IKDC cross-priming capacities. *In vivo* targeting of CD11b/CD18 expressing cells using systemic inoculation of CyaA-Ova or CyaA-E7 as negative controls (50 μg/mouse) in the absence (*A* and *B*) or presence (*C*) of CpG 1668 oligodeoxynucleotide (0.5 μg i.v.). Twelve hours later, cDC and IKDC were sorted according to CD11b expression and incubated with resting OT-I cells for 24 h. CD69 expression on OT-I was examined in flow cytometry (*A*) and mouse IFNγ (*B*) and/or IL-2 (*C*) secretion was monitored in the supernatants of APC/OT-I cocultures at 24 h. Each experiment contained two to three mice per group and was performed twice to thrice, yielding identical results. *, $P < 0.05$.

Discussion

When first identifying the CD11c^{int}CD49b$^+$MHCII$^+$ cell detectable at low frequency in RagxIL-2Rγ$^{-/-}$ mice, we and others decided to name this new cell type "dendritic" (8, 10, 30). Subsequently, several groups challenged this view and proposed to classify the population that we had apostrophed as IKDC among the pool of "activated NK cells" that undergo cell divisions yet lack APC functions (11, 13, 14). The aim of the present study was to revisit this notion and to address the regulation of APC functions in IKDC harvested from naïve spleens. Here, we produce evidence in favor of a DC-like function of IKDC. Indeed, our data indicate that (*a*) IKDC can prime naïve CD4$^+$ and CD8$^+$T cells *in vivo*, in the soluble Ova protein model system, in a CD206 and B7-dependent manner (Figs. 2, 3, 4, and 5); (*b*) the DC-like functions of IKDC reside in the CD11b$^+$ subset (Figs. 2, 3, 4, and 5); (*c*) the DC-like functions of IKDC are dictated by their contact with tumor cells (Figs. 1, 2, 3, and 4); (*d*) CD11b$^+$IKDC targeted by *Bordetella pertussis* CyaA *in vivo* were able of cross-presenting exogenous antigens to CD8$^+$T cells (Fig. 6). In all experimental conditions explored in this work, B220$^-$NK cells failed to function as APC, in conditions in which CD11b$^+$IKDC did present MHC class I or class II–restricted antigens. Antigen transfer to endogeneous cDC is unlikely because CD11b$^+$IKDC remained capable of stimulating CD4$^+$T cells in CD11c^{hi}DC-depleted mice (CD11c-DTR; Fig. 3) or in mice lacking surface expression of MHCII (CD83$^{-/-}$; Fig. 3), and CD8$^+$T cells in H2D$^{-/-}$K$^{-/-}$β2m$^{-/-}$ mice (Fig. 4). Thus, CD11b$^+$IKDC possess a unique APC activity that is not shared by other NK cell populations.

Like cDC, IKDC subserve different functions at various stages of their differentiation. When isolated from mice treated with rIL-15 or CpG or imatinib mesylate plus IL-2, IKDC proliferated and accumulated in lymphoid organs in an IL-15Rα/IL-15–dependent manner (9, 12). In these circumstances, IKDC down-regulated MHCII molecules, both at the mRNA and protein levels and failed to activate naïve OT-II *in vitro* (9) or tumor-specific T cells *in vivo*.[10] Following *trans*-presentation of IL-15, IKDC acquired an enhanced killing potential (9). Thus, upon signaling through the IL-2Rβ/γ chain, IKDC lost their DC-like functions and became NK-like effectors. IKDC remained distinguishable from the other B220$^-$NK cells by a set of characteristics that were all inducible by IL-15Rα/IL-15, namely a high proliferative potential, CCR2 expression, and TRAIL-dependent killing (9, 12).

After encountering a variety of distinct tumor cell types (B16F10, B16-Ova, B16-Rae1, MCA205, CT26), spleen-derived IKDC acquired

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the cell surface expression of MHCII and CD86 molecules (Fig. 1). Thus, tumor-driven licensing of IKDC stimulated their phenotypic conversion into DC-like cells capable of migrating to DLN and of triggering the activation and differentiation of naïve TCR transgenic OT-I and OT-II into polarized T cells (Figs. 2–4). Pletneva and colleagues (20) could corroborate that licensing of IKDC by a bystander cell was required to switch on the DC-like properties of IKDC. Licensing of IKDC by B16-Rae1 for T-cell priming involved an autocrine loop of IFNγ production because anti-IFNγ neutralizing Ab abolished this process and because IFN type IIR$^{-/-}$ IKDC failed to acquire MHCII and CD86 molecules in contact with tumor cells (data not shown; Fig. 1*C*).

Why do IKDC diverge from B220^{-}NK cells with regard to their APC function? Several arguments pertaining to antigen uptake and "maturation" or "licensing" can be enumerated.

First, genome-wide transcriptome analysis of resting IKDC and B220^{-}NK cells sorted from the spleens of C57Bl/6 mice revealed few but important differences. For instance, IKDC overexpressed CCR7 and CD83, compared with B220^{-}NK cells,[11] results that were corroborated by Pletneva and colleagues (20).

Second, the difference between IKDC and other NK subsets might also rely on their distinct abilities to capture antigens. Significant internalization of soluble proteins or fluid phase markers was observed for IKDC (Fig. 5*A*). Accordingly, CD11b^{+}IKDC expressed higher levels of the C-type lectin mannose receptor (CD206, MMR; Fig. 5*B*) than B220^{-}NK cells. MMR has been involved in the recognition and clearance of microorganisms and serum glycoproteins, mostly by macrophages (31, 32). A role in antigen presentation has been reported based on its expression on cDC and its role in the uptake of mannosylated structures into MHCII-enriched cellular compartments (22–24). Here, we reveal a functional role for MMR in the DC-like functions of IKDC (Fig. 5*C*).

The results presented in this work underscore the potential of CD11b^{+}IKDC to exhibit DC-like functions. However, as for NK myeloid DC or DC-like NK cells, future studies will be required to identify their pathophysiologic relevance *in vivo*.

[10] G. Mignot, unpublished data.
[11] E. Ullrich and J.L. Schultze, unpublished data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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