

# Dendritic cell-mediated NK cell activation is controlled by Jagged2–Notch interaction

Mika Kijima\*, Takeshi Yamaguchi\*†, Chieko Ishifune\*, Yoichi Maekawa\*, Akemi Koyanagi‡, Hideo Yagita\*§, Shigeru Chiba¶, Kenji Kishihara\*, Mitsuo Shimada†, and Koji Yasutomo\*||

Departments of \*Immunology and Parasitology and †Digestive and Pediatric Surgery, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima 770-8503, Japan; ‡Division of Cell Biology, Bio-medical Research Center, and §Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan; and ¶Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, 7-3-1 Hongo, Tokyo 113-8421, Japan

Edited by Tak Wah Mak, University of Toronto, Toronto, ON, Canada, and approved February 29, 2008 (received for review October 18, 2007)

**Natural killer (NK) cells regulate various immune responses by exerting cytotoxic activity or secreting cytokines. The interaction of NK cells with dendritic cells (DC) contributes to NK cell-mediated antitumor or antimicrobial responses. However, the cellular and molecular mechanisms for controlling this interaction are largely unknown. Here, we show an involvement of Jagged2–Notch interaction in augmenting NK cell cytotoxicity mediated by DC. Enforced expression of Jagged2 on A20 cells (Jag2-A20 cells) suppressed their growth *in vivo*, which was abrogated by depleting NK cells. Moreover, Jag2-A20 cells exerted a suppression on the growth of nonmanipulated A20 cells in SCID mice in an NK-dependent manner. Consistently, coinoculation of A20 cells with DC overexpressing Jagged2 (Jag2-DC) suppressed the growth of A20 cells in mice. Stimulation of NK cells with Jagged2 directly enhanced their cytotoxicity, IFN- $\gamma$  production, and proliferation. Ligation of Notch2 on NK cells enhanced their cytotoxic activity, and Jag2-DC or CpG-treated DC-mediated NK cell cytotoxicity was suppressed by a  $\gamma$ -secretase inhibitor. These results indicate that the Jagged2–Notch axis plays a crucial role in DC-mediated NK cell cytotoxicity. Furthermore, manipulation of this interaction may provide an approach to induce potent tumor immunity or to inhibit certain autoimmune diseases caused by NK cell activation.**

cytotoxicity | tumor immunity

Natural killer (NK) cells are effector cells of the innate immune system that exhibit direct cytotoxic function (1, 2). NK cell activation is determined by a delicate balance of signals delivered by inhibitory and activating receptors (1–3). Inhibitory receptors bind to MHC class I molecules and down-regulate NK cell cytotoxicity and cytokine production. Thus, missing or low self-MHC expression activates NK cells and initiates target cell lysis (2, 3). Activating receptors are Ig-like or lectin-like molecules that initiate signaling cascades similar to those in T cells. Signaling through activating receptors can overcome inhibitory signaling when the ligands for activating receptors are abundant (3).

Recent studies have revealed that NK cells interact with dendritic cells (DC) (4, 5) and that this interaction can mediate cytotoxic T cell differentiation and NK cell activation/priming (6). For instance, DC-mediated NK cell activation is crucial for antiviral immunity (5). However, the underlying molecular and cellular mechanisms to control DC-mediated NK cell activation are poorly understood.

Notch is a transmembrane receptor activated by the interaction with Notch ligands. Mammals possess four Notch receptors and five Notch ligands. Notch ligands are categorized by two families, Delta (1, 3, and 4) and Jagged (1 and 2) (7). The interaction between a ligand–receptor pair cleaves the extracellular and transmembrane domains of Notch, resulting in the translocation of the intracellular domain of Notch to the nucleus. The interaction of the intracellular domain of Notch with RBP-J allows the formation of transcriptional DNA–protein complexes, leading to specific gene expression. Notch signaling generally controls cell fate choice, cell survival, and activation in many cell types. Recent studies have demon-

strated that Notch signaling controls both the development and activation of T cells (7–10). Furthermore, two papers reported that stimulation of hematopoietic stem cells by Jagged1 or Jagged2 promotes NK cell differentiation (11–12). However, whether Jagged controls mature NK cell activation or functional differentiation *in vivo* requires further investigation.

In this report, we addressed whether or not one of the Notch ligands, Jagged2, controls NK cell responses *in vivo*. We found that stimulation of NK cells by Jagged2 enhanced the antitumor cytolytic activity of NK cells *in vivo* and *in vitro*. In addition, Jagged2 expressed on DC augmented NK cell function through interaction with Notch. These results suggest that robust Notch signaling by enhanced Jagged2 expression may provide a useful strategy to eradicate tumor cells. Conversely, the inhibition of Jagged2–Notch interaction might help cure certain autoimmune diseases caused by the overactivation of NK cells.

## Results

**Overexpression of Jagged2 on A20 Cells Accelerated A20 Rejection in BALB/c Mice.** We first investigated the role of Jagged2 in NK cell activation by overexpressing Jagged2 on A20 cells. A20 is a BALB/c-derived B cell lymphoma cell line that expresses high levels of MHC class II, low levels of MHC class I, Jagged1 and Delta1, and no Jagged2 or Delta4 (Fig. 1*a*). We transfected Jagged2 (Jag2-A20 cells) or a control vector (c-A20 cells) into A20 cells, and  $\approx 75\%$  of Jag2-A20 cells expressed Jagged2 after zeocin selection (Fig. 1*b*). The expression levels of NKG2D ligands and MHC class I on Jag2-A20 cells were comparable with those of c-A20 cells (Fig. 1*c*).

To examine the effect of Jagged2 on antitumor immunity, both cell lines were inoculated s.c. in naive BALB/c mice, and tumor sizes were monitored (Fig. 1*d*). The growth of c-A20 cells was detectable 19 days after inoculation, and c-A20 cells continued to grow in BALB/c mice (Fig. 1*d*). In contrast, Jag2-A20 cells were detectable 28 days after inoculation, and the growth of tumors was greatly suppressed compared with that of c-A20 cells (Fig. 1*d*). NK cells infiltrated more in Jag2-A20 than c-A20 tumors at 10 days after inoculation [supporting information (SI) Fig. S1]. These results suggest that overexpression of Jagged2 on A20 cells enhances the immune-mediated rejection of A20 cells.

Author contributions: M.K. and T.Y. contributed equally to this work; M.S. and K.Y. designed research; M.K., T.Y., Y.M., and A.K. performed research; C.I., Y.M., A.K., H.Y., and S.C. contributed new reagents/analytic tools; M.K., T.Y., Y.M., A.K., H.Y., and K.K. analyzed data; and K.Y. wrote the paper.

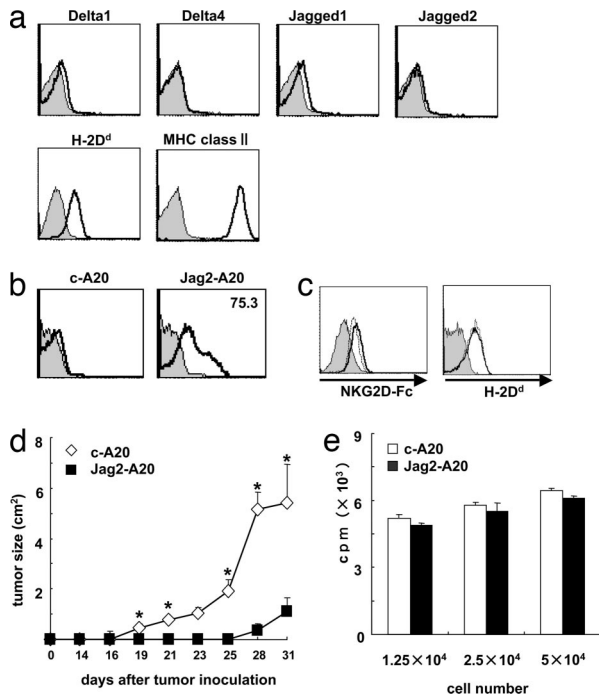
The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

||To whom correspondence should be addressed at: Department of Immunology and Parasitology, Institute of Health Biosciences, University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan. E-mail: yasutomo@basic.med.tokushima-u.ac.jp.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0709919105/DCSupplemental](http://www.pnas.org/cgi/content/full/0709919105/DCSupplemental).

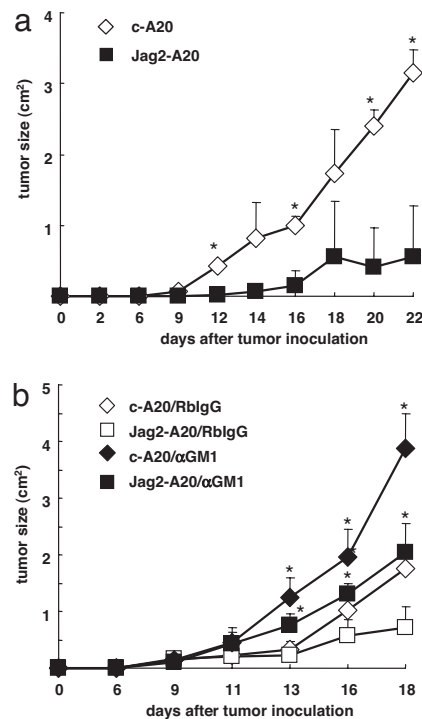
© 2008 by The National Academy of Sciences of the USA



**Fig. 1.** Overexpression of Jagged2 on A20 cells accelerates A20 rejection. (a) A20 cells were stained with biotin-conjugated anti-Jagged1, Jagged2, Delta1, or Delta4, followed by PE-streptavidin or FITC-conjugated anti-MHC class I (D<sup>d</sup>) or anti-MHC class II (solid line) and expression intensity was compared with PE-streptavidin- or control Ig-stained cells (shaded histogram). (b) A20 cells were transfected with a full-length murine Jagged2-encoding vector (Jag2-A20) or control vector (c-A20). The expression of Jagged2 on Jag2-A20 or c-A20 was tested by staining cells with biotin-conjugated anti-Jagged2 antibody, followed by streptavidin-PE (solid line). Cells stained with streptavidin-PE alone were used as a control (shaded histogram). (c) The expression of NKG2D ligands or MHC class I on Jag2-A20 (thick line) or c-A20 (thin line) was tested by staining cells with NKG2D-Fc followed by FITC-conjugated anti-human IgG or FITC-conjugated anti-D<sup>d</sup> antibody, respectively. Cells stained with streptavidin-PE or isotype control alone were used as a control (shaded histogram). (d) Jag2-A20 (filled squares) or c-A20 cells (open diamonds) were injected s.c. into BALB/c mice, and tumor size was monitored. The tumor size was calculated as (axial) × (horizontal) diameter. Data are shown as the mean ± SD. \*, statistical significance ( $P < 0.05$ ). These results are representatives of five independent experiments. (e) [<sup>3</sup>H]thymidine incorporation in c-A20 (open) or Jag2-A20 (filled) cells in cultured medium over an 8-hour period was measured. Data are shown as the mean ± SD.

To rule out the possibility that Jag2-A20 cells have acquired an intrinsic slow-proliferation phenotype, we compared the proliferation of Jag2-A20 cells and c-A20 cells *in vitro*. [<sup>3</sup>H]thymidine incorporation was quite similar in both cell lines under three different cell concentration conditions (Fig. 1e). Taken together, these results eliminated the possibility that the overexpressed Jagged2 directly slowed down the proliferation of A20 cells, suggesting that the retarded growth of Jag2-A20 in mice might be a consequence of augmented immune surveillance.

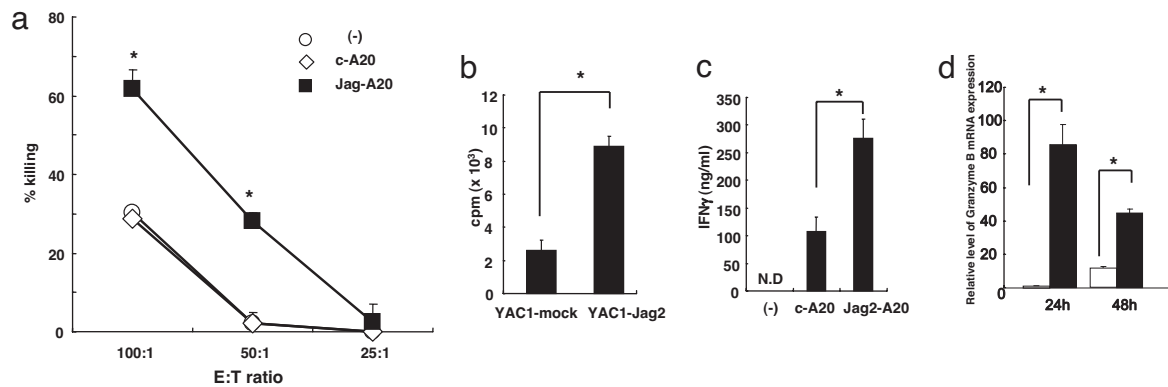
**Growth of Jag2-A20 Cells Is Suppressed Even in SCID Mice, and NK Cells Are Involved in Jagged2-Mediated Tumor Suppression.** We next examined whether mature T or B cells caused the reduced growth of Jag2-A20 cells by inoculating Jag2-A20 cells into T cell- and B cell-deficient SCID mice. The c-A20 cells grew in SCID mice similarly in BALB/c mice (Fig. 2a). In contrast, the Jag2-A20 tumor size was much smaller in SCID mice compared with that of c-A20 cells, which indicates that T and B cells are not major players in suppressing the growth of Jag2-A20 cells (Fig. 2a). Collectively, these results suggest a role for NK cells in suppressing Jag2-A20



**Fig. 2.** NK cells are responsible for the rejection of Jag2-A20 cells. (a) Jag2-A20 (filled squares) or c-A20 (open diamonds) cells were injected s.c. into SCID mice ( $n = 5$ ), and tumor size was monitored. The tumor size was calculated as (axial) × (horizontal) diameter. Data are shown as the mean ± SD. \*, statistical significance ( $P < 0.05$ ). These results are representatives of five independent experiments. (b) SCID mice injected with Jag2-A20 (squares) or c-A20 (diamonds) cells were treated with 200 μg of anti-asialo-GM1 (filled) or control rabbit (open) antibody (day -1, day 2, day 5, day 8;  $n = 5$ ). The tumor size was calculated as (axial) × (horizontal) diameter. Data are shown as the mean ± SD. \*, statistical significance ( $P < 0.05$ ) between anti-asialo-GM1-treated and control rabbit antibody-treated Jag2-A20-inoculated mice or between anti-asialo-GM1-treated and control rabbit antibody-treated c-A20-inoculated mice. These results are representatives of four independent experiments.

tumor growth. Thus, we next examined the tumor growth of Jag2-A20 cells in NK-depleted SCID mice that had been treated with anti-asialo-GM1 mAb (Fig. 2b). In fact, this treatment specifically restored the *in vivo* growth of Jag2-A20 cells (Fig. 2b). It is of note that the depletion of NK cells augmented the tumor growth of c-A20 cells in SCID mice, indicating that NK activation occurred to a moderate degree upon inoculation of c-A20 cells, although the activation was not strong enough to reject A20 cells. Additionally, the c-A20 tumor size was larger than that of Jag2-A20 after NK cell depletion (Fig. 2b), suggesting the presence of an NK cell-independent aspect of the Jagged2-mediated suppression in this context. Taken together, these results suggest that the overexpression of Jagged2 on A20 cells enhanced the antitumor response of NK cells against A20 cells.

**Enhanced Cytotoxic Activity of NK Cells from BALB/c Mice Inoculated with Jag2-A20 Cells.** To confirm that the enhanced tumor suppression of Jag2-A20 cells reflected augmented cytotoxicity of NK cells, we purified NK cells from the spleens of c-A20- or Jag2-A20-inoculated BALB/c mice and examined them in an *in vitro* killing assay. The cytotoxic activity against A20 cells was markedly elevated in NK cells harvested from Jag2-A20-inoculated BALB/c mice compared with those from c-A20-inoculated or untreated mice (Fig. 3a). Therefore, these results indicate that overexpression of Jagged2 in A20 cells enhanced the killing activity of NK cells *in vivo*.



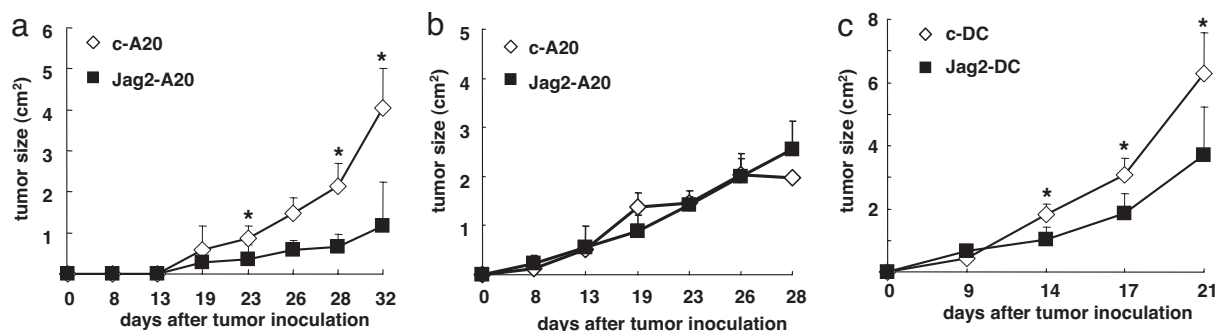
**Fig. 3.** Enhanced cytotoxic activity of NK cells from BALB/c mice inoculated with Jag2-A20 cells. (a) c-A20 or Jag2-A20 cells ( $2 \times 10^7$  per mouse) were injected i.p. in BALB/c mice. NK cells were purified from spleens 4 days after injection and were cultured with  $^{51}\text{Cr}$ -labeled A20 cells for 5 h. Cytotoxic activity of NK cells from unimmunized (open circles), c-A20 injected (open diamonds) or Jag2-A20 injected (filled squares) mice against A20 cells were evaluated by measuring  $^{51}\text{Cr}$  release from A20 cells. These results are representatives of three independent experiments. The data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). (b) Purified NK cells were stimulated with YAC-1 cells transfected with control or Jagged2-encoding vectors for 2 days. The [ $^3\text{H}$ ]thymidine incorporation for the final 8 h was counted. Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). (c) Purified NK cells were stimulated with A20 cells transfected with control or Jagged2-encoding vectors for 3 days. The concentration of IFN- $\gamma$  in the supernatant was measured by ELISA. Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). N.D., not detected. (d) Purified NK cells were stimulated with OP9 cells transduced with control (open symbols) or Jagged2-encoding vectors (filled symbols) for 24 or 48 h. The expression of granzyme B in NK cells was measured by real-time PCR. All data were normalized against the G3PDH mRNA levels and expressed as fold increases relative to control values  $\pm$  SD obtained from naïve NK cells. Data are shown as the mean  $\pm$  SD of four samples. \*, statistical significance ( $P < 0.05$ ).

We next tested whether Jagged2 augments the proliferation, IFN- $\gamma$  production and expression of effector molecules by NK cells. We isolated DX5 $^+$  NK cells from BALB/c mice and incubated them with YAC-1 cells transfected with a control vector or a Jagged2-encoding vector (Fig. 3*b*), with c-A20 or Jag2-A20 cells (Fig. 3*c*), or with OP9 cells transfected with a control vector or a Jagged2-encoding vector (Fig. 3*d*). The presence of Jagged2 on YAC-1, A20, or OP9 cells apparently enhanced the proliferation, IFN- $\gamma$  secretion, or granzyme B transcription of NK cells, respectively (Fig. 3*b*, *c*, and *d*). These results demonstrate that Jagged2 enhances NK cell functions, including cytotoxicity, proliferation and IFN- $\gamma$  production.

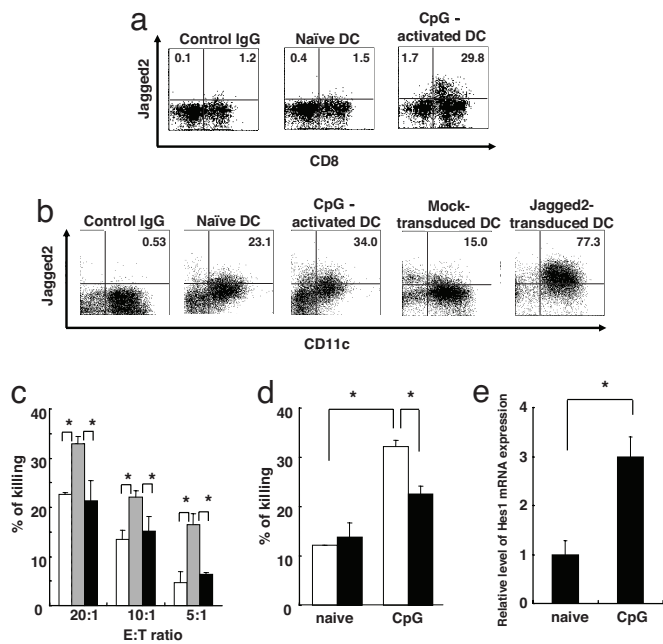
**Inoculation of Jag2-A20 Cells Enhanced the Antitumor Effect of NK Cells to Kill A20 Cells Transplanted at a Different Site.** We then postulated that the exposure of NK cells to Jagged2-expressing cells could condition them to become activated killers, and these

cells could subsequently eradicate bystander tumor cells that are defective for Jagged2 expression. To this end, we inoculated Jag2-A20 and A20 cells or c-A20 and A20 cells at different sites on the same SCID mouse and monitored the tumor size of the bystander A20 cells (Fig. 4*a*). The growth of A20 cells in SCID mice inoculated bilaterally with Jag2-A20 cells was suppressed compared with that of mice that received c-A20 cells (Fig. 4*a*). This effect of Jag2-A20 cells was diminished by treating SCID mice with anti-asialo-GM1 mAb, indicating that NK cells are responsible for this effect (Fig. 4*b*).

Transfection of Jagged2 in tumor cells is not a favorable clinical method to accomplish enhanced NK cell activity. Thus, we examined whether DC overexpressing Jagged2 (Jag2-DC) are able to augment NK cell activity. Jagged2 was transduced into bone marrow-derived DC by a retroviral vector. Then Jag2-DCs or control virus-transduced DC (c-DC) were coinjected with A20 cells, and the tumor size was monitored. The inoculation of



**Fig. 4.** Inoculation of Jag2-A20 cells enables NK cells to kill A20 cells transplanted at a different site. (a) Jag2-A20 and A20 cells (closed squares) or c-A20 and A20 cells (open diamonds) were inoculated in different sites of the same SCID mouse ( $n = 5$ ), and the tumor size of the A20 cells was monitored. The tumor size was calculated as (axial)  $\times$  (horizontal) diameter. Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). These results are representatives of three independent experiments. (b) SCID mice ( $n = 5$ ) injected with Jag2-A20 and A20 cells (filled squares) or c-A20 and A20 cells (open diamonds) were treated with 100  $\mu\text{g}$  of anti-asialo-GM1 antibody (day -1, day 2, day 5, day 8;  $n = 5$ ), and the tumor size of the A20 cells was monitored. Tumor size was calculated as (axial)  $\times$  (horizontal) diameter. Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). These results are representatives of three independent experiments. (c) A20 cells were inoculated with DC transduced with Jagged2 (filled squares) or control vector (open diamonds) in SCID mice, and the tumor size of A20 cells was monitored. The tumor size was calculated as (axial)  $\times$  (horizontal) diameter. Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). These results are representatives of three independent experiments.



**Fig. 5.** DC-mediated NK cell activation is controlled by Notch signaling. (a) The expression of Jagged2 on splenic DC from naïve or CpG-treated mice was evaluated by flow cytometry after staining cells with PE-conjugated anti-CD11c, FITC-conjugated anti-CD8 $\alpha$  mAb, and biotin-conjugated anti-Jagged2 mAb, followed by streptavidin-APC. Data shown are gated on CD11c<sup>+</sup> cells. (b) The expression of Jagged2 on bone marrow-derived untreated, CpG-treated, control vector-transduced, or Jagged2-transduced DC was evaluated by flow cytometry. Cells were stained with PE-conjugated anti-CD11c mAb and biotin-conjugated anti-Jagged2 mAb, followed by streptavidin-APC. Data shown are gated on CD11c<sup>+</sup> cells. (c) NK cells from BALB/c mice were cultured with Jag2-DC in the presence (black bars) or absence (gray bars) of 10  $\mu$ M GSI. The cytotoxic ability of NK cells against YAC-1 cells was measured by <sup>51</sup>Cr release from YAC-1 cells. The activity of NK cells without Jag2-DC stimulation was used as a control (white bars). Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). (d) NK cells from BALB/c mice were cultured with unstimulated or CpG (2  $\mu$ M)-stimulated bone marrow-derived DC for 16 h in the absence (white bars) or presence (black bars) of 10  $\mu$ M GSI. NK cells were enriched and cocultured with <sup>51</sup>Cr-labeled YAC-1 cells, and <sup>51</sup>Cr release from YAC-1 cells was measured. Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ). (e) NK cells from BALB/c mice were cultured with unstimulated or CpG (2  $\mu$ M)-stimulated bone marrow-derived DC for 16 h. The mRNA expression of HES1 in purified NK cells was quantified by real-time PCR. The value obtained from Notch1 expression in NK cells cocultured with unstimulated DC was set as 1. Data are shown as the mean  $\pm$  SD. \*, statistical significance ( $P < 0.05$ ).

Jag2-DC significantly suppressed the growth of A20 cells compared with c-DC (Fig. 4c). This result indicates the feasibility for the use of DC as a vehicle to enable Jagged2-mediated augmentation of NK cell activity *in vivo*, and opens up the possibility for therapeutic tumor intervention.

**Jagged2 on DC Directly Activates NK Cells.** To explore the physiological roles of Jagged2 in terms of NK cell activation, we examined whether Jagged2 is involved in DC-mediated NK cell activation. We first analyzed Jagged2 expression on splenic DC. Splenic DC from untreated BALB/c mice express little Jagged2, and CpG-administration to mice up-regulated Jagged2 expression on DC (Fig. 5a). Furthermore, the expression of Jagged2 on DC was limited to the CD8 $\alpha$ <sup>+</sup> subset. Bone marrow-derived DC cultured in the presence of GM-CSF express Jagged2 (Fig. 5b). CpG stimulation of bone marrow-derived DC up-regulated Jagged2 expression, although the level was lower than that of DC transduced with a Jagged2-expressing retrovirus (Fig. 5b). CpG stimulation of bone marrow-derived DC also up-regulated ex-

pression of Delta1 and Jagged1 but not Delta4, compared with controls (Fig. S2).

To test whether Jagged2 expressed on DC is able to directly activate NK cells, we examined NK cell cytotoxic activity after coculture with Jag2-DC. The coculture of Jag2-DC and NK cells increased the cytotoxic activity of NK cells over that of unstimulated NK cells (Fig. 5c). We examined whether blocking Notch signaling decreases DC-mediated NK cell cytotoxicity by treating NK cells with a  $\gamma$ -secretase inhibitor (GSI). GSI treatment inhibited NK cell activation mediated by Jag2-DC (Fig. 5c). We next tested whether Jagged2-untransfected DC also activate NK cells in a Notch signaling-dependent manner (Fig. 5d). NK cells cocultured with CpG-activated DC exhibited increased NK cell cytotoxicity, which was suppressed by GSI treatment (Fig. 5d). Furthermore, NK cells cocultured with CpG-activated DC up-regulated the Notch target gene, HES1 (Fig. 5e). Taken together, these studies indicate that Notch signaling, possibly through Jagged2, is crucial for DC-mediated enhancement of NK cell cytotoxicity.

**Notch2 Signaling Is Involved in DC-Mediated NK Cell Activation.** To identify the receptor that interacts with Jagged2 in our system, we tested the expression of Notch1, Notch2, Notch3, and Notch4 on unstimulated and IL-2-activated NK cells. We detected the expression of Notch1, Notch2, and Notch4 mRNA in unstimulated NK cells by RT-PCR, and we could detect the expression of Notch1–4 after IL-2 stimulation (Fig. 6a). We could also detect the expression of Notch1 and Notch2 in naïve NK cells by flow cytometry, and their expression was apparently up-regulated by IL-2 stimulation (Fig. 6b).

We next examined whether ligation of Notch2 increases NK cell activity. NK cells were incubated with anti-Notch2 agonist antibody for 16 h, and NK cell cytotoxic activity against YAC-1 cells was measured. The ligation of Notch2 enhanced NK cell activity, and the treatment of NK cells by GSI inhibited the effect of the anti-Notch2 antibody (Fig. 6c), indicating that Notch2 is one of the functional receptors for up-regulating NK cell function.

## Discussion

Notch signaling controls the development and activation of a variety of immune cells (7, 8, 10, 13, 14). Recent studies have demonstrated that Jagged and Delta are able to promote the development or activation of NK cells, at least in *in vitro* culture systems (11, 12, 15). However, it remains unclear whether Notch signaling controls mature NK cell activation and functional differentiation *in vivo*, and, if so, it should be important to know which cells affect NK cell activation through the expression of Notch ligands. In this report, we shed light on this issue by demonstrating that stimulation of NK cells with Jagged2 strengthens mature NK cell-killing activity *in vivo* and *in vitro* and that Jagged2 expressed on DC plays a crucial role in DC-mediated NK cell activation through interaction with Notch. These findings provide an insight that the modulation of Notch signaling could be a strategy to eradicate tumors or to suppress NK cell-mediated diseases.

Recent studies have provided evidence that NK cells are activated by DC and macrophages (5, 6, 16–19), although cellular and molecular mechanisms controlling DC-mediated NK cell cytotoxicity remain unclear. We have addressed this issue by examining whether Notch-signaling plays a crucial role. We observed that Jagged2 expressed on DC is able to enhance NK cell cytotoxic activity through interaction between these cells and that blockade of Notch signaling significantly attenuated the DC-mediated NK cell activation. Furthermore, stimulation of NK cells with an agonist antibody recognizing Notch2 increased NK cell-killing activity. Therefore, these results collectively indicate that Notch signaling is crucial for DC-mediated NK cell activation, possibly through Jagged2 interaction with Notch2. As for the receptor binding Jagged2 on NK cells, we cannot completely rule out the involvement of other Notch receptors, in particular Notch1, because



Bone marrow cells from BALB/c mice were collected and cultured in RPMI medium 1640 supplemented with 10% FCS and culture supernatant containing GM-CSF (provided by T. Iizuka, University of Minnesota, Minneapolis). The retroviral vector pKE004 encoding murine Jagged2 was transfected into plat-E cells. Bone marrow cells were infected with the retrovirus 0, 1, and 2 days after initial culture. The CD11c<sup>+</sup> and GFP<sup>+</sup> cells were sorted by the JSAN cell sorter (Bay Bioscience) 6 days after initial culture. In some experiments, bone marrow-derived CD11c<sup>+</sup> cells were stimulated with 2  $\mu$ M CpG (Hycult Biotechnology) for 24 h.

**Flow Cytometry.** NK cells, bone marrow-derived DC, or A20 cells were resuspended in staining buffer (2% FCS, 0.05% sodium azide in PBS) at a cell density of 2  $\times$  10<sup>6</sup> per milliliter. In some experiments, spleen cells from BALB/c mice injected with 50  $\mu$ g of CpG or NK cells stimulated with mouse IL-2 (100 units/ml) (R&D Systems) for 3 days were resuspended in staining buffer. PE-conjugated anti-DX5 (eBiosciences), PE-conjugated anti-CD11c (eBiosciences), FITC-conjugated anti-CD8 $\alpha$  (eBiosciences), FITC-conjugated anti-H-2D<sup>d</sup> (BD Biosciences), FITC-conjugated anti-MHC class II, mNKG2D-Fc chimera (R&D Systems), and FITC-conjugated anti-human IgG (Chemicon) were used for cell staining. The biotin-conjugated anti-Jagged1 (HMJ1-29), Jagged2 (HMJ2-1), Delta1 (HMD1-5), Delta4 (HMD4-3), Notch1 (HMN1-12), or Notch2 (HMN2-29) mAbs were used for cell staining together with streptavidin-PE or streptavidin-APC (eBiosciences) (*SI Text*). Anti-Jagged1, Jagged2, Delta1, Delta4, Notch1, or Notch2 antibodies were established by immunizing Armenian hamsters with Jagged1-, Jagged2-, Delta1-, or Delta4-expressing CHO cells or Notch1-Fc or Notch2-Fc recombinant protein. The specificities for anti-Notch ligand antibodies or Notch antibodies are shown in Figs. S4 and S5, respectively. The fluorescence intensity of  $\approx$ 10<sup>5</sup> cells was examined by using a FACSCalibur flow cytometer (BD Biosciences).

**NK Cell Purification and Functional Analysis.** Spleen cells from unimmunized mice or mice that received 2  $\times$  10<sup>6</sup> c-A20 or Jag2-A20 cells i.p. were collected, and IgG-positive cells were depleted by anti-mouse IgG beads (Qiagen). DX5<sup>+</sup> cells were purified from the IgG-negative fraction by anti-DX5-coated beads (Miltenyi Biotec). The purity of the DX5<sup>+</sup> cells was  $\approx$ 90%. NK cells were stimulated with 1  $\mu$ g/ml anti-Notch2 antibody for 16 h or cultured with bone marrow-derived control DC or Jagged2-transduced DC (1  $\times$  10<sup>5</sup>) for 16 h in the absence or presence of 5  $\mu$ g/ml (10  $\mu$ M)  $\gamma$ -secretase inhibitor (DAPT; Sigma). After 4 hours of coculture of NK cells with <sup>51</sup>Cr-labeled targets at various effector/target ratios, supernatants were harvested, and their radioactivity was determined. The percentage of specific cytotoxicity (% cytotoxicity) was calculated by using the following formula: % cytotoxicity = (experimental lysis - spontaneous lysis)/(maximum lysis - spontaneous lysis)  $\times$  100. All cytotoxicity assays were carried out in triplicate. Data are presented as the mean  $\pm$  SD. Proliferation of NK cells was assessed by using purified NK cells stimulated with YAC-1 cells transfected with pKE004 or pKE004 encoding Jagged2 for 48 h. One  $\mu$ Ci of [<sup>3</sup>H]thymidine (PerkinElmer Life Sciences) was added for the final 8 hours. The incorporation of [<sup>3</sup>H]thymidine was counted by a liquid scintillation counter. In some experiments, purified NK cells (2  $\times$  10<sup>5</sup>)

were stimulated with A20 cells transfected with murine Jagged2 (2  $\times$  10<sup>5</sup>) for 3 days, and culture supernatants were collected. The concentration of IFN- $\gamma$  in the supernatant was measured by ELISA according to the manufacturer's protocol (eBiosciences). To test the mRNA expression of NK cells stimulated by Jagged2, purified NK cells (2  $\times$  10<sup>5</sup>) were stimulated with OP9 cells transfected with murine Jagged2 (1  $\times$  10<sup>5</sup>) for 24 or 48 h, and then NK cells were purified by anti-DX5 antibody-coated beads.

**Real-Time PCR.** RNA was isolated from NK cells by Trizol (Invitrogen), and cDNA was generated by using Omniscript Reverse Transcription (Qiagen) according to the manufacturer's instructions. The mRNA expression levels were quantified by using the ABI 7500 Real-Time PCR System (Applied Biosystems). cDNAs were amplified with TaqMan probes using SYBR Premix Ex Taq (TaKaRa). The mRNA expression levels were normalized relative to G3PDH gene expression levels. Fold differences in mRNA levels were determined by using SDS System Software ver.1.4.0 (Applied Biosystems). Primer sequences are as follows:

Hes1 (forward: 5'-GGACAAACCAAGACGGCTCTGAGCACAG-3', reverse: 5'-TGCCGGAGCTATCTTTCTTAAGTGCATCC-3'), Granzyme B (forward: 5'-GGATATAAGGATGGTTACC-3', reverse: 5'-CACCTGTCTAGAGCAATCC-3'), G3PDH (forward: 5'-TCCACCACCTGTGTGTA-3', reverse: 5'-ACCACAGTCCATGCCATCAC-3'), Notch1 (forward: 5'-TGAGACTGCCAAAGTGTTC-3', reverse: 5'-GTGGGAGACAGAGTGGGTG-3'), Notch2 (forward: 5'-ACAAATACTGTGCAGACACTTCAA-3', reverse: 5'-AGCACCAGATGATCAGGT-3'), Notch3 (forward: 5'-CAGCGAAAGCGAGAACAC-3', reverse: 5'-GGCCATGTTCTTATCCCA-3'), Notch4 (forward: 5'-GAACGTGGATCCCTCAAGT-3', reverse: 5'-AGAGAGAGGGCAAGGACTCAT-3')

**Tumor Inoculation.** Mice were inoculated s.c. on the flank with 1  $\times$  10<sup>6</sup> A20 or A20-Jag2 cells in PBS. The long and short diameters of the tumors were measured by using calipers at 2-day intervals; these two diameters were multiplied to obtain an estimate of the tumor area. Depletion of NK cells was accomplished by i.p. injections of 200  $\mu$ g of polyclonal rabbit anti-asialo GM1 (Wako Chemicals) 2 days before the first tumor inoculation and then at 3-day intervals. In some experiments, 1  $\times$  10<sup>6</sup> A20 and A20-Jag2 or c-A20 cells were injected s.c. in different sites on the same mouse, or 1  $\times$  10<sup>5</sup> bone marrow-derived dendritic cells transduced with Jagged2 were mixed with 1  $\times$  10<sup>6</sup> A20 cells and s.c. injected. The data are displayed as the mean  $\pm$  SD of the tumor areas for each group of animals at a given time point.

**Statistical Analysis.** The Mann-Whitney *U* test was applied to compare data from noninterval scales, such as tumor size. Normally distributed data from interval scales were analyzed by Student's *t* test with *P* < 0.05 considered to be statistically significant.

**ACKNOWLEDGMENTS.** We thank Dr. Kitamura and Dr. Iizuka for providing reagents; Dr. Tagaya (National Cancer Institute, Bethesda, MD) for critically reading the manuscript; Mrs. Kinouchi for technical assistance; and Mrs. Yamakawa for secretarial assistance. This work was supported by grants-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Knowledge Cluster Initiative from the Ministry of Education, Science and Technology.

1. Lanier LL (2005) NK cell recognition. *Annu Rev Immunol* 23:225-274.
2. Di Santo JP (2006) Natural killer cell developmental pathways: A question of balance. *Annu Rev Immunol* 24:257-286.
3. Raulet DH, Vance RE (2006) Self-tolerance of natural killer cells. *Nat Rev Immunol* 6:520-531.
4. Adam C, et al. (2005) DC-NK cell cross talk as a novel CD4+ T-cell-independent pathway for antitumor CTL induction. *Blood* 106:338-344.
5. Andoniou CE, et al. (2005) Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. *Nat Immunol* 6:1011-1019.
6. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A (2007) Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* 26:503-517.
7. Radtke F, Wilson A, Mancini SJ, MacDonald HR (2004) Notch regulation of lymphocyte development and function. *Nat Immunol* 5:247-253.
8. Maekawa Y, et al. (2003) Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 19:549-559.
9. Amsen D, et al. (2004) Instructive of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117:515-526.
10. Tsukumo S, Yasutomo K (2004) Notch governing mature T cell differentiation. *J Immunol* 173:7109-7113.
11. DeHart SL, Heikens MJ, Tsai S (2005) Jagged2 promotes the development of natural killer cells and the establishment of functional natural killer cell lines. *Blood* 105:3521-3527.
12. Lehar SM, Dooley J, Farr AG, Bevan MJ (2005) Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. *Blood* 105:1440-1447.
13. Pear WS, Radtke F (2003) Notch signaling in lymphopoiesis. *Semin Immunol* 15:69-79.
14. Minato Y, Yasutomo K (2005) Regulation of acquired immune system by notch signaling. *Int J Hematol* 82:302-306.
15. Carotta S, Brady J, Wu L, Nutt SL (2006) Transient notch signaling induces NK cell potential in Pax5-deficient pro-B cells. *Eur J Immunol* 36:3294-3304.
16. Baratin M, et al. (2005) Natural killer cell and macrophage cooperation in MyD88-dependent innate responses to *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 102:14747-14752.
17. Ferlazzo G, Munz C (2004) NK cell compartments and their activation by dendritic cells. *J Immunol* 172:1333-1339.
18. Andrews DM, et al. (2003) Functional interactions between dendritic cells and NK cells during viral infection. *Nat Immunol* 4:175-181.
19. Krug A, et al. (2004) TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21:107-119.
20. Iwatsubo T (2004) The gamma-secretase complex: machinery for intramembrane proteolysis. *Curr Opin Neurobiol* 14:379-383.
21. Shimizu K, et al. (2000) Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. *Mol Cell Biol* 20:6913-6922.
22. Bruckner K, Perez L, Clausen H, Cohen S (2000) Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* 406:411-415.
23. Moloney DJ, et al. (2000) Fringe is a glycosyltransferase that modifies Notch. *Nature* 406:369-375.
24. Tsukumo S, Hirose K, Maekawa Y, Kishihara K, Yasutomo K (2006) Lunatic fringe controls T cell differentiation through modulating notch signaling. *J Immunol* 177:8365-8371.
25. Morita S, Kojima T, Kitamura T (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 7:1063-1066.