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Notch2 Signaling Is Required for Potent Antitumor Immunity In Vivo

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CD8+ T cells play a central role in cancer immunosurveillance, and the efficient induction of CTLs against tumor Ags is required for successful immunotherapy for cancer patients. Notch signaling directly regulates the transcription of effector molecules in CTLs. However, it remains unclear whether Notch signaling in CD8+ T cells is required for antitumor CTL responses and whether modulation of Notch signaling can augment antitumor CTL responses. In this study, we demonstrate that signaling by Notch2 but not Notch1 in CD8+ T cells is required for antitumor CTL responses. Notch2flox/flox mice crossed with E8I-cre transgenic (N2F/F-E8I) mice, in which the Notch2 gene is absent only in CD8+ T cells, die earlier than control mice after inoculation with OVA-expressing EG7 thymoma cells. In contrast, Notch1flox/flox mice crossed with E8I-cre transgenic mice inoculated with EG7 cells die comparable to control mice, indicating that Notch2 is crucial for exerting antitumor CTL responses. Injection of anti-Notch2 agonistic Ab or delta-like-1 overexpressing dendritic cells augmented the antitumor response in C57BL/6 mice inoculated with EG7 cells. These findings indicate that Notch2 signaling in CD8+ T cells is required for generating potent antitumor CTLs, thus providing a crucial target for augmenting tumor immune responses. The Journal of Immunology, 2010, 184: 4673–4678.

The efficacy of antitumor CTLs critically depends on interaction of the TCR and MHC-presented tumor Ags as well as signals through costimulatory molecules or cytokine receptors (1). T cell costimulatory molecules include both activating and inhibitory receptors, and several attempts have been tried to modulate costimulatory molecule activity to augment antitumor immune responses (1, 2). For instance, blockade of feedback inhibition of costimulation through CD28 by blockade of CTLA-4 with a mAb induces remarkable therapeutic responses in tumor-bearing mice (3). Tumor-associated dendritic cells (DCs) overexpress PD-L1 and PD-1, inhibitory receptors for T cells, and PD-L1 blockade by anti–PD-L1-augmented DC-mediated T cell activation (4). Therefore, modulation of stimulatory or inhibitory receptors has a great impact on augmenting T cell immune responses against tumor cells (1).

Notch signaling controls both development and functional differentiation of T cells (5–7). Our group demonstrated that Notch2 signaling directly regulates granzyme B expression and promotes CTL differentiation (8). Notch-mediated CTL differentiation appears to be independent of Eomesodernin (8). Therefore, CTL differentiation is coregulated by several transcriptional pathways. Furthermore, we found that Notch signaling contributes to DC-mediated NK cell activation, which enhanced the killing activity of NK cells (9). These studies suggest that Notch signaling is crucial for exerting cytotoxic responses in immune cells.

In this report, we investigated whether Notch signaling is required for an efficient antitumor response and, if that is the case, whether modulation of Notch signaling augments antitumor responses. We found that Notch2- but not Notch1-deficient CD8+ T cells fail to expand in response to tumor inoculation. The injection of anti-Notch2 agonistic Ab in mice augmented antitumor immunity. These results indicate that stimulation of Notch2 could provide a new strategy to strengthen CD8+ T cell-mediated antitumor immune responses.

Materials and Methods

Mice

Six- to 8-wk-old C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Notch2flox/flox crossed with E8I-cre transgenic mice (N2F/F-E8I) were previously reported (8, 10). Notch1flox/flox mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with E8I-cre transgenic mice. OT-I TCR transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with E8I-cre transgenic mice. EG7 cells are EL4 cells transfected with OVA, which were obtained from American Type Culture Collection (Manassas, VA). Animals were injected s.c. on the flank with 3 × 106 EG7 cells. Cells were washed three times in 0.25% BSA-PBS and resuspended in RPMI 1640 medium plus 10% FBS.

Flow cytometry

PE-conjugated anti-CD8 mAb (eBioscience, San Diego, CA) was used for cell staining. In some experiments, cells were fixed with 4% paraformaldehyde and stained with allophycocyanin-conjugated anti-granzyme B mAb (Caltag Laboratories, Burlingame, CA) in saponin-containing buffer. Fluorescence intensity of ~106 cells was examined using a FACSCalibur or FACSFlow II flow cytometer (BD Biosciences, Mountain View, CA).

Inoculation of tumor cells

EG7 cells are EL4 cells transfected with OVA, which were obtained from American Type Culture Collection (Manassas, VA). Animals were injected s.c. on the flank with 3 × 106 EG7 cells. Cells were washed three times in 0.25% BSA-PBS and resuspended in RPMI 1640 medium plus 10% FBS.
endotoxin-free RPMI 1640 medium without FCS and once in endotoxin-free PBS before injection. In some experiments, anti-Notch2 mAb (HNM2-29) (8) or hamster IgG (100 μg/mouse) was injected on days 1, 3, 7, and 10 after tumor inoculation. The anti-Notch2 mAb specifically recognizes Notch2 but not Notch1 or Notch3 (Supplemental Fig. 1). Bone marrow-derived DCs (BMDCs) (2 × 10^6) were injected proximal to the tumor inoculation region 10, 12, and 14 d after tumor inoculation. The BMDCs were infected with delta-like I (DL1)- or Jagged2-encoding retrovirus three times as previously reported (8). Then BMDCs were further cultured with irradiated EG7 cells for 2 d before injection, and CD11c^+ cells were purified by CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) before injection into mice. The larger and smaller diameters of the s.c. tumors were measured using calipers at 2-d intervals; these two diameters were multiplied to obtain an estimate of the tumor area. The data are displayed as the mean ± SD of the tumor areas for each group of animals at a given time point.

Vectors and constructs
cDNA for mouse delta-like 1 or Jagged2 was cloned into the retroviral vector pEEO04 (11). Retroviruses were generated by transfecting Plat-E (Clontech Laboratories, Palo Alto, CA) with the cDNA for mouse delta-like 1 or Jagged2 encoding retrovirus three times as previously reported (8). Then BMDCs were further cultured with irradiated EG7 cells for 2 d before injection, and CD11c^+ cells were purified by CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) before injection into mice. The larger and smaller diameters of the s.c. tumors were measured using calipers at 2-d intervals; these two diameters were multiplied to obtain an estimate of the tumor area. The data are displayed as the mean ± SD of the tumor areas for each group of animals at a given time point.

Luciferase assay
CHO cells transfected with Notch1- or Notch2-encoding plasmids were further transfected with a reporter plasmid carrying HES-1 promoter regions (13) and 1.25 ng of the control Renilla luciferase reporter plasmid pRL-TK by a Gene Pulser II system (Bio-Rad, Hercules, CA). Anti-Notch2 mAb (10 μg/ml) was added soon after transfection. Twenty-four hours after transfection, luciferase activity was measured by Dual-Luciferase Reporter Assay (Promega, Madison, WI) according to the manufacturer’s protocol. Firefly luciferase activity was normalized to Renilla luciferase activity using pRL-CMV (Promega). All of the experiments were done in triplicate and repeated at least three times.

Cell purification and cell culture
CD8^+ T cells were purified from total spleen or lymph node cells by incubating cells with anti-CD8 mAb (53-6.72), followed by positive selection of CD8^+ cells with anti-rat IgG MicroBeads (Miltenyi Biotec). Purified CD8^+ T cells (5 × 10^6) were transferred into C57BL/6 mice that had received EG7 cells 12 d previously. Total spleen cells from OT-I TCR transgenic mice were stimulated with OVA peptide (SIINFEKL) (100 μM) in the presence of anti-Notch2 mAb (HNM2-29) or control hamster IgG (10 μg/ml) for 5 d. The resultant CD8^+ cells (1 × 10^6) were transferred into C57BL/6 mice that had received EG7 cells 12 d previously.

In vitro killing assay
Single-cell suspensions of lymph node cells (2 × 10^6) were cultured with 1 × 10^6 irradiated spleen cells for 5 d in the presence of OVA peptide (SIINFEKL) (1 μM) and 5 U/ml human recombinant IL-2. Five days after stimulation, live cells were pulsed with Lympohyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) and incubated with target EL4 pulsed with or without OVA peptide and labeled with ^51Cr (PerkinElmer, Waltham, MA) at the indicated E:T ratios. After 5 h of incubation, supernatants were harvested, and ^51Cr release was measured with a γ scintillation counter (Aloka, Tokyo, Japan). The corrected percentage of lysis was calculated as: corrected % lysis = 100 × (test ^51Cr released – spontaneous ^51Cr released)/(maximum ^51Cr released – spontaneous ^51Cr released).

Statistical analysis
All of the data expressed as mean ± SD are representative of at least three different experiments. Comparisons between individual data points were made using Student t tests. Differences in survival between experimental groups were analyzed using the Kaplan-Meier approach. The statistical significance of group differences was assessed using the log-rank test. p < 0.05 was considered significant.

FIGURE 1. Notch2 regulates tumor immune response against EG7. EG7 tumor cells were s.c. inoculated in (A, B) E8I-Cre (open) and N2F/F-E8I (closed) or (C, D) E8I-Cre (open) and N1F/F-E8I (closed) mice, and tumor size (A, C) and mice survival (B, D) were monitored. Results are shown as mean ± SD. *p < 0.01. The experiments are representative of four independent experiments.

FIGURE 2. Notch2-deficient T cells do not eradicate tumor cells. EG7 tumor cells were s.c. inoculated into E8I or N2F/F-E8I mice. The CD8^+ cells from E8I (open square) or N2F/F-E8I mice (open circle) were purified 12 d after inoculation and transferred into C57BL/6 mice that had received EG7 tumor cells 12 d previously. As a control, C57BL/6 mice that did not receive any primed T cells were used (closed square). Results are shown as mean ± SD. The experiments are representative of four independent experiments.
Results

Notch2-deficient mice have lower antitumor activity

We have previously reported that Notch2 has a crucial role in exerting CTL responses (8). To investigate the contribution of Notch2 signaling to CD8⁺ T cell antitumor immunity, we inoculated EG7 cells (EL4 thymoma cells transfected with the OVA gene) into Notch2flox/flox crossed with E8I-cre transgenic mice (Fig. 1A). Accordingly, E8I mice die later than N2F/F-E8I mice after inoculation of EG7 cells (Fig. 1B). These data suggest that Notch2 deficiency in CD8⁺ T cells attenuates EG7 antitumor immune responses.

Because Notch1 is also highly expressed on activated CD8⁺ T cells (8), we next used Notch1flox/flox crossed with E8I-cre transgenic mice (N1F/F-E8I) inoculated with EG7 cells to determine whether Notch1 deficiency on CD8⁺ T cells reduces T cell-mediated antitumor immune responses. In contrast to N2F/F-E8I mice, EG7 cells inoculated into N1F/F-E8I mice grow comparable to those in E8I mice (Fig. 1C), and N1F/F-E8I mice die with kinetics similar to those seen in E8I mice (Fig. 1D). These data indicate that Notch2 on CD8⁺ T cells contributes to antitumor immunity.

Notch2-deficient mice do not generate antitumor CTLs

We next examined whether tumor Ag-specific killing activity is defective in N2F/F-E8I mice. N2F/F-E8I or E8I CD8⁺ T cells were harvested from spleen and lymph nodes and enriched from mice 12 d after inoculation of EG7 cells. These cells were then transferred into naive C57BL/6 mice in which EG7 cells had been inoculated 12 d previously. EG7 cells grew poorly in C57BL/6 mice that received T cells from E8I mice, indicating the presence of strong tumor-specific CTLs (Fig. 2). In contrast, EG7 cells grew rapidly in C57BL/6 mice that received T cells from N2F/F-E8I mice, similar to mice in which naive T cells were transferred (Fig. 2). These data indicate that N2F/F-E8I mice are not able to generate enough or high-quality tumor Ag-specific CTLs.

Stimulation of Notch2 by a specific Ab augments antitumor immune responses

We next determined whether overstimulation of Notch2 augments antitumor immune responses. CHO cells were transfected with the genes for mouse notch1 or notch2. These cells were next transfected with Notch reporter plasmids and stimulated by anti-Notch2 mAb. The anti-Notch2 mAb stimulated Notch-mediated anti-Notch2 agonistic Ab augments antitumor CD8⁺ T cell responses. A. Anti-Notch2 mAb (clone HMN2-29) (black) or control hamster IgG (gray) were incubated with CHO cells transfected with control vector or the cDNA for Notch1 or Notch2. A Notch reporter plasmid was transfected, and luciferase activity was measured and compared with that without an Ab (white). Results are shown as mean ± SD. B and C, The spleen cells from OT-I TCR transgenic mice were stimulated with OVA peptide (100 pM) in the presence of anti-Notch2 mAb (open square) or control IgG (closed circle) for 5 d in vitro, and purified CD8⁺ T cells were transferred into C57BL/6 mice that were inoculated s.c. with EG7 tumor cells 12 d before. Then tumor size (B) and survival (C) of each mouse was monitored. Results are shown as mean ± SD. *p < 0.01. The experiments are representative of four independent experiments.

FIGURE 3. Anti-Notch2 agonistic Ab augments antitumor CD8⁺ T cell responses. A. Anti-Notch2 mAb (clone HMN2-29) (black) or control hamster IgG (gray) were incubated with CHO cells transfected with control vector or the cDNA for Notch1 or Notch2. A Notch reporter plasmid was transfected, and luciferase activity was measured and compared with that without an Ab (white). Results are shown as mean ± SD. B and C, The spleen cells from OT-I TCR transgenic mice were stimulated with OVA peptide (100 pM) in the presence of anti-Notch2 mAb (open square) or control IgG (closed circle) for 5 d in vitro, and purified CD8⁺ T cells were transferred into C57BL/6 mice that were inoculated s.c. with EG7 tumor cells 12 d before. Then tumor size (B) and survival (C) of each mouse was monitored. Results are shown as mean ± SD. *p < 0.01. The experiments are representative of four independent experiments.

FIGURE 4. Treatment of mice with anti-Notch2 agonistic Ab promotes antitumor immunity in vivo. EG7 tumor cells were s.c. inoculated into C57BL/6 mice that 12 d later received control IgG (open) or anti-Notch2 mAb (closed) 3, 6, 7, and 10 d after tumor inoculation. Tumor size (A) and mouse survival (B) were monitored every day. Results are shown as mean ± SD. *p < 0.01. We used at least five mice in each group, and the experiments are representative of four independent experiments.
signaling only in CHO cells transfected with Notch2 and not Notch1, as evaluated by a HES-1 reporter gene system (Fig. 3A). The anti-Notch2 mAb stimulates Notch2 in a concentration-dependent manner, and this stimulatory effect was blocked in the presence of γ secretase inhibitor (Supplemental Fig. 2).

The CD8+ T cells from OT-I TCR transgenic mice were activated with OVA peptide in the presence or absence of anti-Notch2 mAb for 5 d in vitro. Then activated CD8+ T cells were transferred into C57BL/6 mice that had received EG7 cells 12 d previously, and tumor size (Fig. 3B) and mouse survival (Fig. 3C) were monitored. CD8+ T cells stimulated with anti-Notch2 mAb exhibited greater suppression of tumor growth and enhanced mouse survival compared with those of mice receiving activated control cells (Fig. 3B, 3C). We did not observe any death at least until day 70 in anti-Notch2 mAb-treated mice that survived at day 50 (data not shown).

**Anti-Notch2 mAb promotes the eradication of tumor cells in vivo**

We next injected anti-Notch2 mAb or control hamster IgG in C57BL/6 mice that had received EG7 cells 12 d previously. Treatment of tumor-bearing C57BL/6 mice with anti-Notch2 mAb inhibited the growth of EG7 cells compared with that of control IgG (Fig. 4A). Similarly, treatment of C57BL/6 mice with anti-Notch2 mAb enhanced mouse survival, and significantly, all mice eventually eradicated the tumor cells (Fig. 4B). In addition, we have observed similar antitumor effects of anti-Notch2 mAb when 50 μg Ab in each injection was used (data not shown). These data indicate that stimulation of Notch2 promotes the eradication of tumor cells in vivo.

**Delta-like 1-transfected dendritic cells suppress tumor growth**

DC-mediated tumor immunotherapy is widely used in clinical settings currently. We have previously reported that DL1 upregulates CTL activity (8). To determine whether DCs overexpressing DL1 (DL1-DCs) are able to suppress tumor growth in vivo, we three times peritumorally injected DL1-DCs or control DCs (cont-DCs) that had been cultured with irradiated EG7 cells in vitro. Tumor cell growth was slower in DL1-DC–injected mice than in cont-DC–injected mice (Fig. 5A). The T cells from draining lymph nodes more highly expressed hes1, a Notch target gene, in DL1-DC–inoculated mice than those in cont-DC–inoculated mice (Supplemental Fig. 3). The draining lymph node cells from mice inoculated with EG7 and DL1-DCs or cont-DCs were cultured with irradiated EG7 cells in the presence of IL-2 for 5 d. The cytotoxic activity of T cells against EL4 cells pulsed with OVA peptide was evaluated by 51Cr release assay. No apparent lysis of EL4 cells not pulsed with OVA peptide was exerted by T cells from mice that received cont-DCs or DL1-DCs (Fig. 5B). Effector cells from mice that received DL1-DCs exhibited much stronger specific lysis of target EL4 cells pulsed with OVA than effector cells from mice that received cont-DCs (Fig. 5B). In addition, we could not detect any cytotoxic activity of T cells from DL1-DC–treated mice if T cells are not cultured in vitro in the presence of...
OVA peptide (data not shown), probably due to a very low number of Ag-specific T cells. We also tested whether another Notch ligand, Jagged2, has a similar ability to DL1. However, inoculation of DCs transduced with Jagged2 (Jagged2-DCs) did not suppress EG7 growth (Fig. 5C), and T cells from Jagged2-DCs did not exhibit cytolytic activity against OVA-pulsed EL4 cells (Fig. 5D). The hest expression in T cells from Jagged2-DC–inoculated mice was higher than that from cont-DC–inoculated mice but lower than that from DL1-DC–inoculated mice (Supplemental Fig. 3). Taken together, these data indicate that manipulation of Notch signaling induced by DL1 could be beneficial as a new strategy to augment antitumor CTL activity.

Discussion

Notch signaling controls mature T cell differentiation and activation by directly regulating transcription of effector molecules (7, 8, 14, 15). In particular, our group has recently demonstrated that Notch2 signaling directly controls CTL effector molecules, including granzyme B, by integrating RBP-J and CREB1 (8). In this study, we revealed that Notch2, but not Notch1, signaling in CD8+ T cells is required for efficient induction of antitumor CTLs. Furthermore, treating tumor-bearing mice with anti-Notch2 mAb or DL1-DCs strengthened antitumor CTL responses. These data indicate that Notch2 signaling is required for augmenting antitumor CTL activity and suggest that manipulation of Notch2 signaling might provide a new clinical approach for cancer immunotherapy.

We have recently demonstrated that Notch signaling controls cytotoxic responses in both CTL and NK cells (8, 9). These data suggest that Notch signaling is a crucial signaling pathway required for cytotoxic responses in immune cells. In the studies described in this report, we found that deficiency of Notch2 but not Notch1 decreased the antitumor responses in vivo, although Notch1 is highly expressed on activated CD8+ T cells (8). These data suggest a lower affinity of Notch1 and Notch ligands present in our tumor model relative to those that activate the Notch2 pathway in CD8+ T cells. Notch–Notch ligand interaction is tightly regulated by Notch glycosylation (16), which might be crucial for the distinct Notch receptor utilization controlling CTL responses that we observe. We also found that treatment with anti-Notch2 agonistic Ab in tumor-bearing mice increased antitumor responses. These data strongly suggest that the major target cells for anti-Notch2 mAb in terms of antitumor effects would be CD8+ T cells, although we cannot completely deny the possibility that anti-Notch2 mAb interacts with Notch2 on non-CD8+ T cells, which may indirectly affect Notch2 signaling in CD8+ T cells. Notch2 is widely expressed on many tissues, and thus treatment with anti-Notch2 mAb might have some adverse effects for the host, although we have never seen any macroscopic changes in mice after Ab treatment. Nevertheless, to reduce the possibility of adverse effects of anti-Notch2 mAb, the appropriate route or dose must be considered.

Our previous study showed that DL1 is able to augment CTL responses in vivo (8). We demonstrated in this study that injection of DL1-DCs peritumorally suppresses tumor growth compared with cont-DCs. These data suggest a potential therapeutic strategy to augment antitumor CTLs by injecting DL1-DCs pulsed with tumor-specific Ags. However, we should be cautious with this approach in terms of clinical use because Notch signaling regulates angiogenesis, which nurtures tumor cells (17–19). Those studies revealed that delta-like 4 contributes to angiogenesis. Although the contribution of DL1 to angiogenesis around tumor cells has not been reported, DL1 might also be able to activate Notch receptors that control angiogenesis. Thus, injection of DL1-DCs around the tumor burden may promote angiogenesis for tumor growth, although DL1-DCs would also help to strengthen CTL-mediated killing of tumor cells. The i.v. or s.c. route as a method to transfer DCs has been used in human clinical trials for treating cancer patients. Therefore, it would be important to carefully evaluate whether i.v. or s.c. injection of DL1-DCs affects angiogenesis at the tumor site before applying those methods for clinical use.

In the present work, we have focused on investigating the role of Notch2 signaling in tumor immunity and the effect of anti-Notch2 mAb or DL1-DC treatment on tumor eradication. We show that these treatments enhance survival and decrease the size of the tumor by augmenting CTL activity. The data suggest that stimulation of Notch2 would be a new way to stimulate antitumor immune responses. Combining anti-Notch2 mAb with other therapeutic approaches, such as DC-mediated tumor vaccines, is likely to yield further clinical benefits.

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Disclosures

The authors have no financial conflicts of interest.

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