In this report we demonstrate that Notch signaling promotes the development of novel Thy1-expressing DCs (Thy1$^+$DCs) in the thymus. Thy1$^+$DCs express CD11c, Thy1, cII, CD40, CD86, CD80 and CD8α but not NK1.1 and B220. Thy1$^+$DCs constitute about 5% of thymic CD11c^{high}cII$^+$ DCs and possess the ability to delete CD4$^+$CD8$^+$ double-positive thymocytes. These data indicate that Notch controls a novel type of DC and suggest that Notch signaling may regulate negative selection in the thymus through Thy1$^+$ DC development.

Results

Notch signaling promotes the differentiation of Thy1$^+$ DCs

Culturing bone marrow cells (BMCs) in the presence of GM-CSF promotes the differentiation of DCs. In order to investigate the role of Notch in DC development, BMCs were cultured in the presence of GM-CSF on OP9-DL1 cells, which are OP9 stromal cells that overexpress Delta-like 1 (DL1) [13]. Culturing BMCs on OP9-DL1 cells induces a greater differentiation of CD11c$^+$cII$^+$ cells compared with culture on control OP9-GFP cells (Fig. 1A). The gating strategy for analyzing DCs is shown in the Supporting Information Fig. 1. By examining a variety of cell surface markers on CD11c$^+$cII$^+$ cells that were differentiated on OP9-GFP or OP9-DL1 cells, we found that about 70% of CD11c$^+$cII$^+$ cells cultured on OP9-DL1 express Thy1.2, while only about 20% of cells express Thy1.2 when differentiated on OP9-GFP cells (Fig. 1A). The level of Thy1.2 on DCs cultured with OP9-GFP was lower than that with OP9-DL1. The total number of Thy1-expressing DCs was also increased by culturing BMCs on OP9-DL1 cells compared with that on OP9-GFP (Fig. 1A). We hereafter designate Thy1-expressing DCs as Thy1$^+$DCs. In order to examine the characteristics between Thy1$^+$DCs and pDCs, we compared their expression profiles for a number of lymphocytic and monocytic markers in in vitro differentiated Thy1$^+$DCs and pDCs. We cultured BMCs in the presence of FMS-like tyrosine kinase 3-ligand (Flt3-L), which favors pDC development, in the absence of OP9 cells [14]. We found that few Thy1$^+$DCs developed from BMCs in the presence of Flt3-L, and only a small number of Thy1$^+$DCs differentiated in the presence of GM-CSF (Fig. 1A)(Supporting Information Fig. 2), suggesting that there are distinct differentiation requirements for Thy1$^+$DCs and pDCs. Thy1$^+$DCs express CD11b, but not CD3ε, CD4, CD8α, B220, NK1.1 or PDCA-1, indicating that Thy1$^+$DCs have a phenotype distinct from T cells, NK cells and pDCs (Supporting Information Fig. 3A). Thy1$^+$DCs highly express CD80 and CD86, comparable to Thy1-negative DCs (Thy1^{neg}DCs) differentiated from BMCs cultured on OP9 cells in the presence of GM-CSF, and to DCs differentiated from BMCs cultured in the presence of GM-CSF without OP9 cells (GM DCs) (Supporting Information Fig. 3B). CD80 and CD86 expression on Thy1$^+$DCs was higher than on DCs differentiated from BMCs cultured in the presence of Flt3-L (Flt3-L DCs) (Supporting Information Fig. 3B). CD40 expression was low on all DC types in our studies (Supporting Information Fig. 3B). Furthermore, Thy1$^+$DCs were smaller than Thy1^{neg}DCs, but larger than Flt3-L DCs (Supporting Information Fig. 3C). Thy1$^+$DCs have a dendritic morphology similar to Thy1^{neg}DCs (Fig. 1B).

Mastermind-like 1 (MamL1) is required for Notch signaling [15]. We tested if Thy1$^+$DC development depended on Notch signaling by transducing a dominant negative form of MamL1 (MamL1-DN) into BMCs cultured on OP9-DL1. Transduction of MamL1-DN into BMCs did not affect the development of DCs cultured on OP9 cells but almost completely inhibited the development of Thy1$^+$DCs from BMCs cultured on OP9-DL1 cells (Fig. 1C). These data show that Notch signaling in BMCs is required for Thy1$^+$DC development.

DCs are characterized by their ability to efficiently present antigens to T cells. We tested if Thy1$^+$DCs are able to present exogenous protein antigens by co-culturing TCR transgenic

Figure 1. Differentiation of Thy1-expressing DCs requires Notch signaling. (A) Cell surface expression of Thy1.2 on DCs (CD11c$^+$cII$^+$) was assessed by flow cytometry after gating cells as shown in Supporting Information Fig. 1. The percentage or absolute number of CD11c$^+$cII$^+$ cells or CD11c$^+$cII$^+$Thy1.2$^+$ cells (Thy1$^+$DCs) was counted after culturing BMCs for 6 days on control OP9-GFP or OP9-DL1 cells in the presence of GM-CSF, or in the presence of Flt3-L without OP9 stromal cells or GM-CSF without OP9 stromal cells. (B) Morphology of pDCs (PDCA-1$^+$) from BMCs cultured with Flt3-L, Thy1^{neg}DCs (CD11c$^+$cII$^+$Thy1.2$^-$) cultured with OP9-GFP cells, or Thy1$^+$DCs (CD11c$^+$cII$^+$Thy1.2$^+$) cultured with OP9-DL1 evaluated by Giemsa staining. Original magnifications, ×400. Scale bars = 10 μm. (C) BMCs were cultured on OP9-GFP or OP9-DL1 cells in the presence of GM-CSF for 6 days after triple infection with a control virus (EV) or a retrovirus carrying MamL1-DN. Cells were stained with anti-CD11c, and Thy1.2 mAbs and analyzed gated on GFP$^+$ cells after exclusion of debris and OP9 stromal cells by gating on forward and side scatter and dead/autofluorescent cells by gating out 7AAD$^+$ cells. Percentage of Thy1$^+$DCs in total cells is shown in the lower bar graph. (D) Thy1^{neg}DCs or Thy1$^+$DCs were obtained by co-culture of BMCs with OP9-GFP or OP9-DL1 cells, respectively. Thy1^{neg}DCs or Thy1$^+$DCs (2×10^4) were cultured with CD4$^+$ T cells (4×10^4) from 5C.C7 TCR transgenic mice (left and middle graph) or CD8$^+$ T cells (4×10^4) from OT-I TCR transgenic mice (right graph) for 3 days with PCC$_{81-104}$ (left graph), PCC protein (middle graph) or SIINFEKL (right graph), respectively. [^{3}H]-thymidine incorporation during the final 6 h was measured. NS, not significant. (E) Expression of *Thy1*, *Ptcra*, *Rag1*, *Rag2*, *Cd2*, *Gata3*, *Pax5*, *Sfpi1*, *E2-2*, *Spib*, *Dtx1* and *Hes1* in total thymocytes (Thy), total splenocytes (Spl), pDCs (PDCA-1$^+$ from Flt3-L DC), GM DCs (CD11c$^+$cII$^+$Thy1.2$^-$), Thy1^{neg}DCs from OP9 DCs (CD11c$^+$cII$^+$Thy1.2$^-$), and Thy1$^+$DCs from OP9-DL1 DCs (CD11c$^+$cII$^+$Thy1.2$^+$) by real-time PCR. The expression of each gene was normalized to *Actb* mRNA and presented as 'fold increase' relative to the expression of each gene in total thymocytes RNA. The *p* value indicated in (E) is the data compared with expression between DC samples. ND, not detected. (F) Western blotting of Hes1 protein in B3Z-EV (negative control), B3Z-N2IC where B3Z T-cell hybridoma cells were transduced with intracellular domain of mouse Notch2 (positive control), total thymocytes (Thy), total splenocytes (Spl), pDCs (PDCA-1$^+$ from Flt3-L DCs), GM DCs (CD11c$^+$cII$^+$Thy1.2$^-$), Thy1^{neg}DCs from OP9 DCs (CD11c$^+$cII$^+$Thy1.2$^-$) and Thy1$^+$DCs from OP9-DL1 DCs (CD11c$^+$cII$^+$Thy1.2$^+$). The filled and opened arrowheads indicate specific bands of Hes1 or Actin and non-specific bands (NS), respectively. The lower of the two bands indicate expression of Hes1 protein. Actin protein was used as an internal control. The data are representatives of at least three independent experiments. $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. NS, not significant.

5C.C7 T cells (specific to a pigeon cytochrome c (PCC) peptide (81–104) presented by IE^k) with a PCC peptide (81–104) or whole PCC protein. $Thy1^+$DCs were able to stimulate $CD4^+$ 5C.C7 T cells when either PCC peptide or PCC protein was used as an antigen although the ability to present protein antigens is slightly lower than that of $Thy1^{neg}$DCs (Fig. 1D; left or middle panel, respectively). Additionally, $Thy1^+$DCs were able to stimulate $CD8^+$ OT-I T cells that express TCRs specific to OVA peptide (SIINFEKL) presented by D^b in the presence of SIINFEKL peptide (Fig. 1D: right panel). These data demonstrate that $Thy1^+$DCs are able to stimulate $CD8^+$ and $CD4^+$ T cells when mixed with MHC class I or class II peptides, as well as processing and presenting exogenous antigens on cII.

We next investigated the expression of lineage-specific molecules in $Thy1^+$DCs by real-time PCR in order to analyze the lineage relationship of $Thy1^+$DCs with lymphocytes and other DC subtypes (Fig. 1E). Thymocytes highly express *Thy1*, *Ptcra*, *Rag1*, *Rag2*, *Cd2*, *Gata3* and *Pax5*, whereas spleen cells express *Thy1*, *Cd2*, *Gata3* and *Pax5*, but not *Ptcra*, *Rag1* and *Rag2*. pDCs express *Ptcra*, *Sfpi1*, *E2-2* (also known as *Tcf4*) and *Spib*, as previously reported [16]. $Thy1^+$DCs do not express *Ptcra*, *Rag1*, *Rag2*, *Cd2*, *Pax5*, *E2-2* or *Spib*, but do express *Thy1*, *Gata3* and *Sfpi1* (Fig. 1E). $Thy1^+$DCs highly expressed the Notch target genes *Dtx1* and *Hes1* when compared with other DC types (Fig. 1E). We found that $Thy1^+$DCs expressed Hes1 protein, but that the other DC types did not (Fig. 1F). The high expression of *Thy1* and *Gata3* in $Thy1^+$DCs, but not other DCs, suggests that $Thy1^+$DCs express, at least partially, T-cell lineage-specific transcriptional programs together with DC-specific genes, and that $Thy1^+$DCs have molecular signatures distinct from other DCs.

$Thy1^+$DCs are present in the thymus and not in the spleen or lymph nodes

We next determined if DCs possessing phenotypes similar to those of in vitro-generated $Thy1^+$DCs are present in spleen, lymph nodes and thymus. We gated on cells negative for B220, NK1.1 and TCR-β and positive for CD11c and cII, and measured Thy1-positive cells in the thymus, spleen, and inguinal/brachial (pLNs) and mesenteric lymph nodes (mLNs) (Fig. 2A). The gating strategy is described in detail in the Supporting Information Fig. 4A. We found $Thy1^+$DCs in the thymus, constituting about 5% of total thymic DCs; however, we observed few $Thy1^+$DCs in spleen and lymph nodes (Fig. 2A).

A previous paper indicated that DCs in the thymus capture CD4 or CD8 from thymocytes and express those molecules even though the DCs themselves do not transcribe those particular mRNAs [17]. In order to determine if Thy1 expressed on $Thy1^+$DCs comes from adjacent T cells, we cultured BMCs from B6 Thy1.1 and B6 Thy1.2 mice at a 1:1 ratio on OP9-GFP or OP9-DL1. Only about 1% of the cultured cells were Thy1.1 and Thy1.2 double-positive, and most of the Thy1-expressing DCs on OP9-DL1 were $Thy1.1^+Thy1.2^-$ or $Thy1.1^-Thy1.2^+$ cells (Fig. 2B). For a more stringent in vivo test of the ability of DCs to capture Thy1, we co-transplanted BMCs from B6 Thy1.1 and RAG2-deficient (Thy1.2) mice into irradiated B6 Thy1.2 mice. If the $Thy1^+$DCs from RAG2-deficient mice capture Thy1.1 protein expressed on thymocytes, we would expect to see Thy1.1 and Thy1.2 double-positive DCs in the recipient thymus. We analyzed Thy1.1 and Thy1.2 expression on $CD11c^{hi}cII^+$ cells in the thymus 4 wk after transplantation and found low numbers (5–12%) of Thy1.1 and Thy1.2 double-positive DCs, with most DCs (88–95%) expressing only Thy1.2 (Fig. 2C), which is not consistent to Thy1 pick-up theory. The gating strategy is described in detail in the Supporting Information Fig. 4B. We performed short-term culture experiments in order to determine whether the Thy1 molecules present on $Thy1^+$DCs are due to intrinsic expression (Fig. 2D). If Thy1 on DCs comes from adjacent T cells or other Thy1 expressers, the expression of Thy1 on $Thy1^+$DCs themselves should decrease over time during short-term in vitro culture. However, the population of $Thy1^+$DCs and the intensity of Thy1 expression were constant throughout the culture period (Fig. 2D). Furthermore, we found higher *Thy1* mRNA expression in thymic $Thy1^+$DCs compared to $Thy1^{neg}$DCs (Fig. 2E). Taken together, these data indicate that the Thy1 expressed on $Thy1^+$DCs is mainly attributed to Thy1 transcription in $Thy1^+$DCs.

Thymic $Thy1^+$DCs express CD8α and some of $Thy1^+$DCs express CD11b (Fig. 3A), whereas about half of the thymic $Thy1^{neg}$DCs were $CD8\alpha^-$. Both $Thy1^+$DCs and $Thy1^{neg}$DCs express Notch2 and Notch3 at low levels, but not Notch1 (Fig. 3B). Thymic pDCs do not express Notch1, Notch2 or Notch3 (Fig. 3B). $Thy1^+$ DCs and thymic $Thy1^{neg}$DCs express comparable levels of CD40, CD80 and CD86 (Fig. 3C). These data demonstrate that DCs with a phenotype similar to $Thy1^+$DCs differentiated in vitro on OP9-DL1 cells are present in vivo in the thymus.

$Thy1^+$DCs are able to present exogenous antigens

In order to know if $Thy1^+$DCs in the thymus are able to process and present exogenous antigens, Alexa Flour 488-conjugated OVA protein was injected into B6 mice and the uptake of OVA protein by DCs was evaluated 18 h after injection. $Thy1^+$DCs in the thymus and $Thy1^{neg}$DCs took up OVA protein equally well, and the OVA uptake ability of $Thy1^+$DCs was higher than that of pDCs (Fig. 4A).

We next tested if $Thy1^+$DCs in the thymus are able to delete immature $CD4^+CD8^+$ T cells by co-culturing $Thy1^+$DCs and thymocytes from OT-II TCR (specific to OVA peptide presented by IA^b) transgenic mice in the presence of OVA protein or peptide. Thymic $Thy1^+$DCs were able to delete $CD4^+CD8^+$ T cells comparable to thymic $Thy1^{neg}$DCs, demonstrating that $Thy1^+$DCs are able to capture exogenous antigens in the thymus and delete $CD4^+CD8^+$ thymocytes (Fig. 4B).

RBP-J is required for $Thy1^+$DC development in vivo

RBP-J is required for canonical Notch signaling [18]. In order to determine if deletion of RBP-J impacts $Thy1^+$DC development,

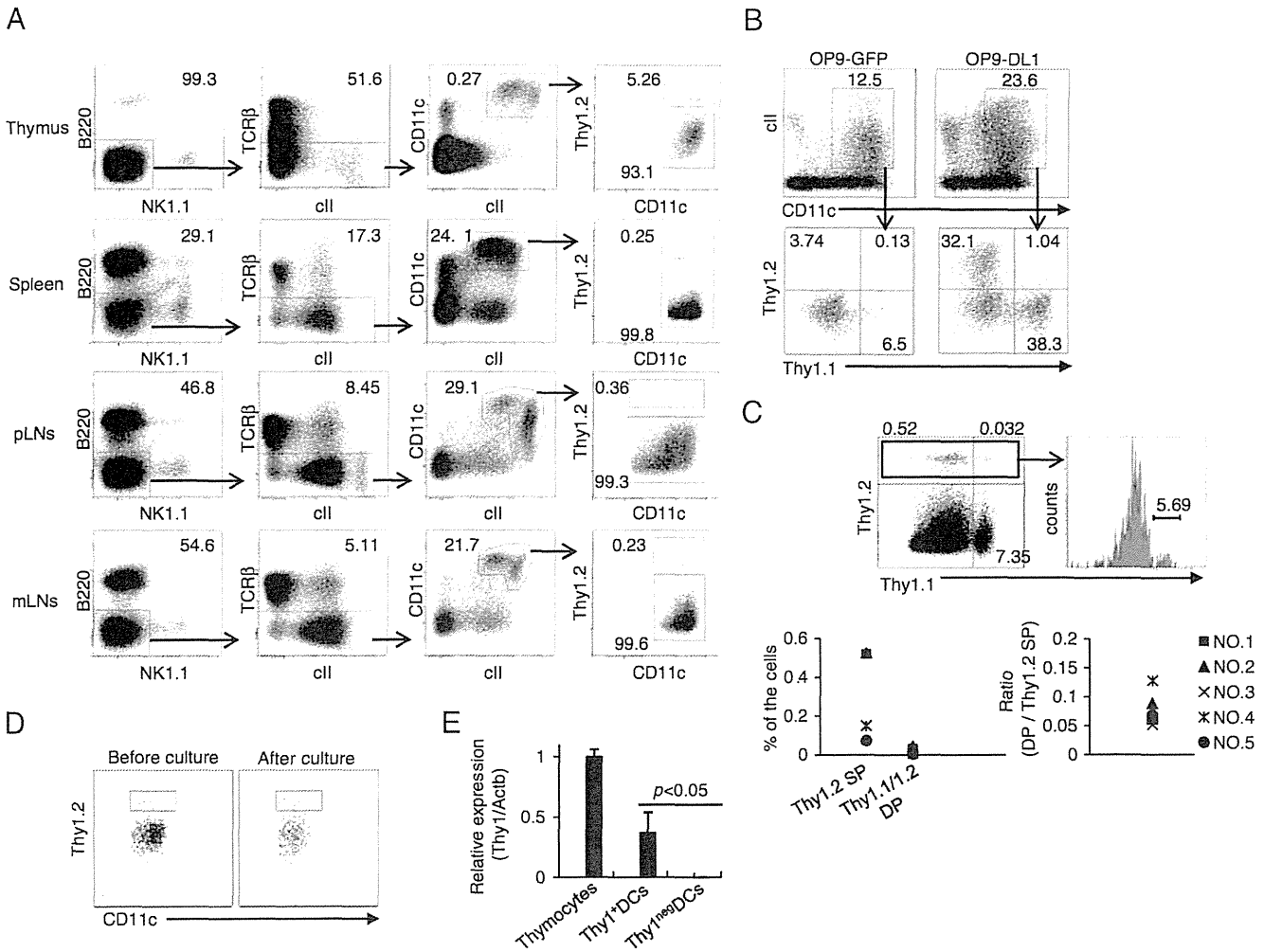

Figure 2. Thy1$^+$DCs are present in vivo. (A) CD11c^{hi}cII$^+$Thy1.2$^+$ cells in the thymus, spleen, inguinal and brachial lymph nodes (pLNs), or mesenteric lymph nodes (mLNs) were analyzed by gating cells negative for B220, NK1.1 and TCR-β as shown in Supporting Information Fig. 4A. (B) BMCs from B6 mice (Thy1.2) and B6 Thy1.1 mice at a 1:1 ratio were cultured for 6 days in the presence of GM-CSF and OP9-GFP or OP9-DL1. The expression of Thy1.1 and Thy1.2 on CD11c$^+$cII$^+$ cells was analyzed after gating cells as shown in Supporting Information Fig. 1. (C) Lin$^-$ BMCs from B6 Thy1.1 and Rag2-deficient mice (Thy1.2) were injected intravenously into 9-Gy-irradiated B6 mice. B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$ cells were analyzed for Thy1.1 and Thy1.2 expression 4 wk after transplantation using the gating strategy shown in Supporting Information Fig. 4B. Five recipient mice were used in each group. The left graph shows the percentage of Thy1.1$^-$Thy1.2$^+$ cells (Thy1.2 SP) and Thy1.1$^+$Thy1.2$^+$ cells (Thy1.1/Thy1.2 DP) in CD11c^{hi}cII$^+$ DCs in each of five mice (NO. 1–5). The right graph indicates the ratio of Thy1.1/Thy1.2 DP per Thy1.2 SP in CD11c^{hi}cII$^+$ DCs in each of five mice. The FACS data shown in this figure are from mouse No. 1. (D) MACS-purified Thymic cII$^+$ cells were analyzed immediately (left panel) or after culture (right panel) with GM-CSF (10 ng/mL) and Flt3-L (10 ng/mL) for 18 h. Thy1$^+$DCs are indicated after gating on forward and side scatter to exclude debris and 7AAD$^-$NK1.1$^-$B220$^-$TCR-β$^-$CD11c^{hi}cII$^+$ cells. (E) Real-time PCR analysis of Thy1 mRNA from thymocytes sorted thymic Thy1$^+$DCs (B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^+$) and Thy1^{neg}DCs (B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^-$). The expression of each gene was normalized to *Actb* mRNA and presented as 'fold increase' relative to the expression of each gene in total thymocytes RNA. The data are representative of three independent experiments.

we deleted RBP-J in CD11c$^+$ cells by crossing RBP-J$^{flox/flox}$ [19] mice with CD11c-Cre transgenic mice to generate RFF11c mice. As previously reported [12], the number of CD8$^-$DCs in the spleen was decreased in RFF11c mice relative to control mice (data not shown). In contrast, the number of Thy1$^+$DCs in the thymus of RFF11c mice was comparable to that of control mice (Fig. 5A), suggesting that Notch signaling is required before the Thy1$^+$DC progenitors express CD11c. In order to delete RBP-J at an earlier stage of Thy1$^+$DC development, we retrovirally transduced the Cre recombinase (Cre) gene into Lin$^-$ BMCs from RBP-J$^{flox/flox}$ mice and transplanted those cells into irradiated B6 mice (Fig. 5B). We found that the development of thymic Thy1$^+$ DCs from RBP-J-deficient BMCs was impaired, although not completely abolished, in the absence of RBP-J (Fig. 5B). As previously reported, the number of splenic CD11c$^+$cII$^+$ cells and CD8$^-$DCs was decreased by RBP-J deficiency in the hematopoietic cells of the reconstituted mice (Supporting Information Fig. 5A), although the number of thymic CD8$^-$DCs did not change in the context of RBP-J deficiency (Supporting Information Fig. 5B) [12].

A previous paper demonstrated that a CD44$^+$CD25$^+$ population in thymus contains DC progenitors [20]. In order to assess

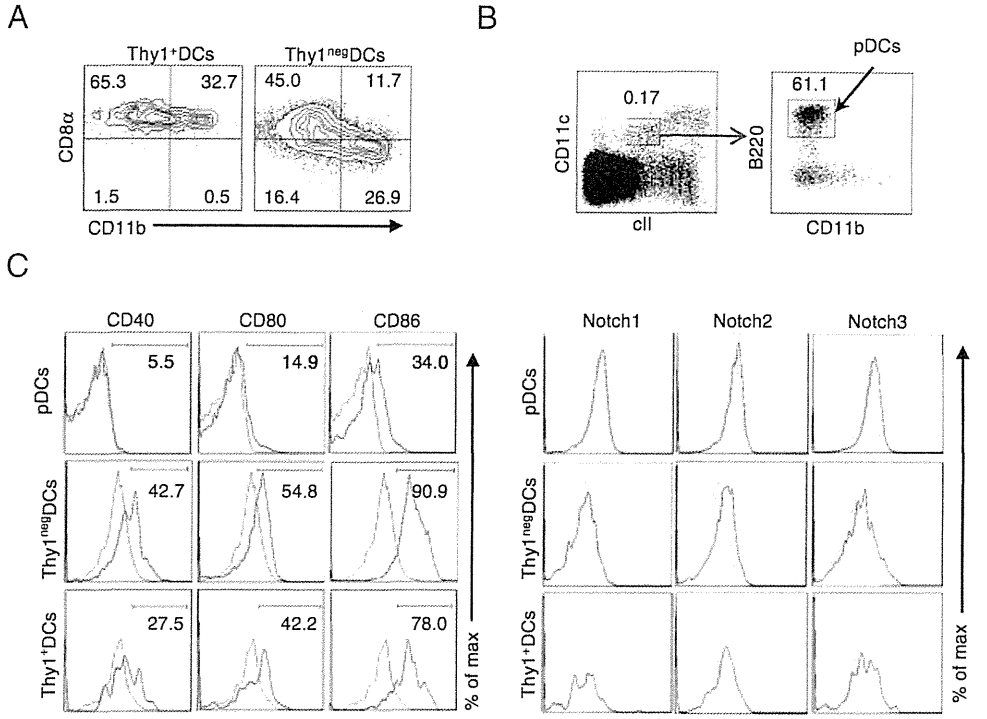

Figure 3. In vivo Thy1$^+$DC phenotypes. (A) The expression of CD11b and CD8α on thymic Thy1^{neg}DCs (B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^-$) or Thy1$^+$DCs (B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^+$) was analyzed by flow cytometry. Expression of Notch1, Notch2 or Notch3 (B) or CD40, CD80 or CD86 (C) on thymic Thy1$^+$DCs, Thy1^{neg}DCs or pDCs (CD11c^{lo}cII^{lo}CD11b^{neg}B220$^+$) was analyzed by flow cytometry. The shaded histogram shows each cell type stained with streptavidin-PE-Cy7 alone. The gating strategy is the same as in Fig. 2A. The data shown in this figure are representative of three independent experiments.

which cells are the progenitors for thymic Thy1$^+$DCs, we isolated CD44$^+$CD25$^-$ (DN1) and CD44$^+$CD25$^+$ (DN2) cells from B6 mice and cultured each population on OP9-GFP or OP9-DL1 cells in the presence of IL-7, TNF-α, SCF, IL-3 GM-CSF and Flt3-L. We found that Thy1$^+$DCs are able to differentiate from both DN1 and DN2 cells (Fig. 5C). The gating strategy for this experiment is described in detail in the Supporting Information Fig. 6. We then transferred DN1 or DN2 cells from B6 Thy1.1/Thy1.2 mice into the thymus of B6 Thy1.2 mice and analyzed the development of Thy1$^+$DCs 2 wk after transfer. Similar to our in vitro experiments, we found that thymic Thy1$^+$DCs are able to differentiate from DN1 and DN2 cells, although the efficiency of differentiation was quite low with DN2 cells (Fig. 5D). These data demonstrate that DN1 and DN2 cells both contain progenitors that are capable of differentiating into thymic Thy1$^+$DCs in vivo, although DN2 cells are less capable of supporting Thy1$^+$DC differentiation in vivo.

Discussion

DCs are classified into several subtypes depending on phenotypic and functional differences [1]. However, it remains unclear if differential transcription factor expression controls the development of each DC subtype. In this report, we demonstrated that overstimulation of BMCs with DL1 promotes Thy1$^+$DC development, and that deletion of RBP-J in Lin$^-$ BMCs blocks Thy1$^+$DC development in the thymus. The expression pattern of lineage-specific transcription factors and a number of cell surface markers is different among Thy1$^+$DCs, pDCs and Thy1^{neg}DCs. For instance, Thy1$^+$DCs, but not other DC subtypes, express *Gata3*, which is required for T-cell development [21]. Thy1$^+$DCs do not express pDCs specific transcription factor E2-2, PDCA-1 and B220, suggesting that Thy1$^+$DCs population is not a part of pDCs. Thy1$^+$DCs are able to capture exogenous antigens in the thymus and delete CD4$^+$CD8$^+$ thymocytes. These data provide evidence that the thymus possesses a novel type of DC, Thy1$^+$DCs, that their development is controlled by Notch signaling, and that this novel cell type might have a role in promoting negative selection in the thymus.

A previous paper indicated that DCs in the thymus are able to differentiate from DN2 cells [20]. We found that both DN1 and DN2 cells could support the differentiation of Thy1$^+$DCs in vivo. We also found that RBP-J deletion at a relatively late stage of development when CD11c is expressed did not disturb Thy1$^+$DC development, whereas deletion of RBP-J at an earlier developmental stage in Lin$^-$ BMCs did suppress Thy1$^+$DC development. These data indicate that Notch signaling is required during an early phase of Thy1$^+$DC development, but is dispensable for the maintenance of Thy1$^+$DCs. However, we cannot rule out the possibility that RBP-J-independent Notch signaling could control the maintenance of Thy1$^+$DCs at later developmental stages, such as after CD11c is expressed. It is noteworthy that a small number of Thy1$^+$DCs still developed even when RBP-J was deleted in Lin$^-$ BMCs. There are at least two possibilities to

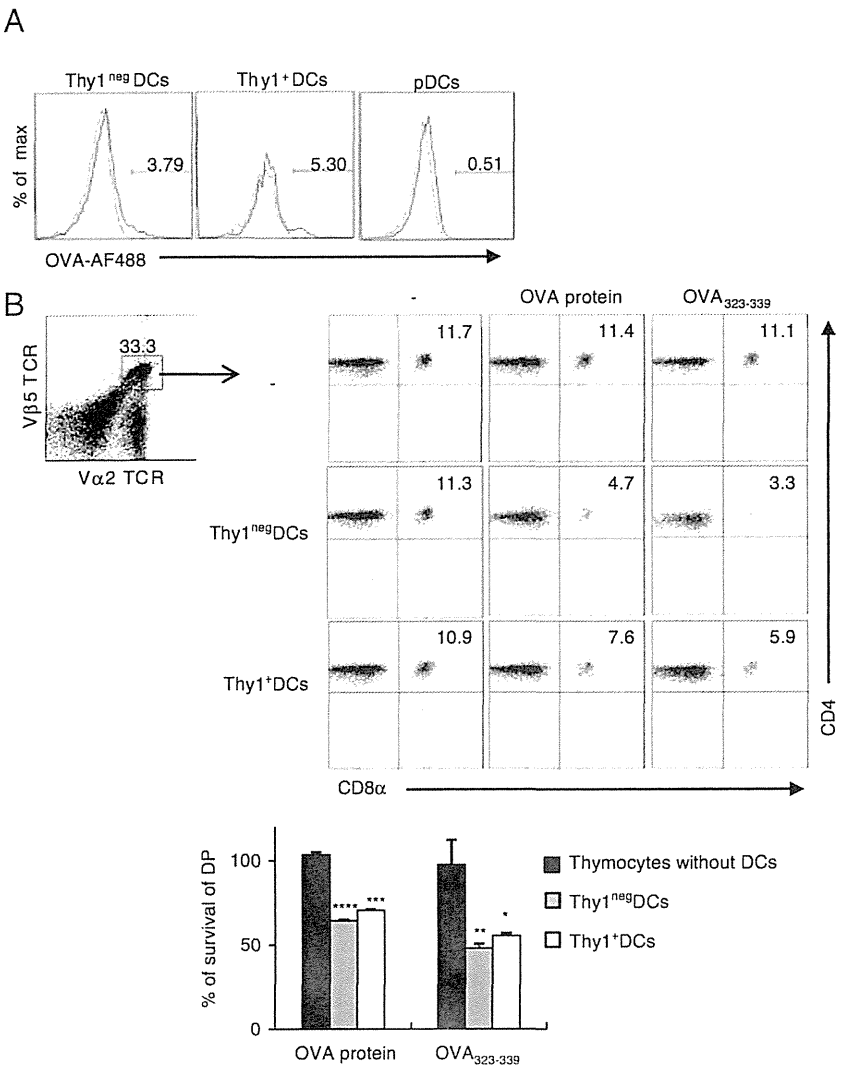

Figure 4. Thy1$^+$DC function. (A) Alexa Flour 488-conjugated OVA (OVA-AF488) was injected into B6 mice and the uptake of the OVA-AF488 by thymic Thy1^{neg}DCs, Thy1$^+$DCs and pDCs was analyzed by flow cytometry 18 h after injection. The shaded histogram shows the value of thymocytes in mice injected with unconjugated OVA. Five mice were used for each group. The gating strategy is the same as described in Figs. 2A and 3B. (B) CD4$^+$CD8$^+$ thymocytes from OT-II TCR transgenic mice were cultured with sorted thymic Thy1$^+$DCs or Thy1^{neg}DCs in the presence of OVA peptide or OVA protein for 38 h after which live cells were counted. The panels show the expression of CD4 and CD8α gated on live (7AAD$^-$) Vα2$^+$Vβ5$^+$ cells. The lower bar graph indicates percent survival of Vα2$^+$Vβ5$^+$ TCR CD4$^+$CD8$^+$ cells (DP) based on the ratio of cell counts compared to cultures without antigen. $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. The data shown are representative of four independent experiments.

explain this observation. The first is that Thy1$^+$DCs are further subdivided into multiple populations and the development of only some subtypes of Thy1$^+$DCs is dependent on Notch signaling. The second is that the Lin$^-$ BMCs are mixtures of cells at different stages of differentiation and deletion of RBP-J in Thy1$^+$ DC progenitors that have already passed some critical step requiring Notch signaling allows such cells to support Thy1$^+$DC differentiation. In any case, it would be interesting to know at which differentiation stage Notch signaling is required for Thy1$^+$ DC development.

Notch signaling is an absolute requirement for T-cell development, as evidenced by the lack of T cells in mice with genetic deficiencies of Notch1 or RBP-J in T-cell precursors [19, 22]. These data suggest that Notch signaling is crucial for expressing a set of T-cell-specific genes, although it remains unclear which Notch target genes are required for T-cell development. In this report we demonstrated that Thy1$^+$DCs highly express *Gata3*, which is necessary for T-cell development [21] and a known target gene of Notch, suggesting that Thy1$^+$DCs share some T-cell lineage-specific genetic programs together with DC-specific genes. The clarification of the roles of T-cell lineage genes expressed in Thy1$^+$DCs will contribute to understanding the functional characteristics of Thy1$^+$DCs. As for the roles of Thy1$^+$ DCs, we found that Thy1$^+$DCs were able to delete immature CD4$^+$CD8$^+$ thymocytes in the presence of specific antigens, suggesting that Thy1$^+$DCs contribute to negative selection in the thymus. Considering the essential role of Notch in T-cell development, these results suggest that Notch signaling plays a role

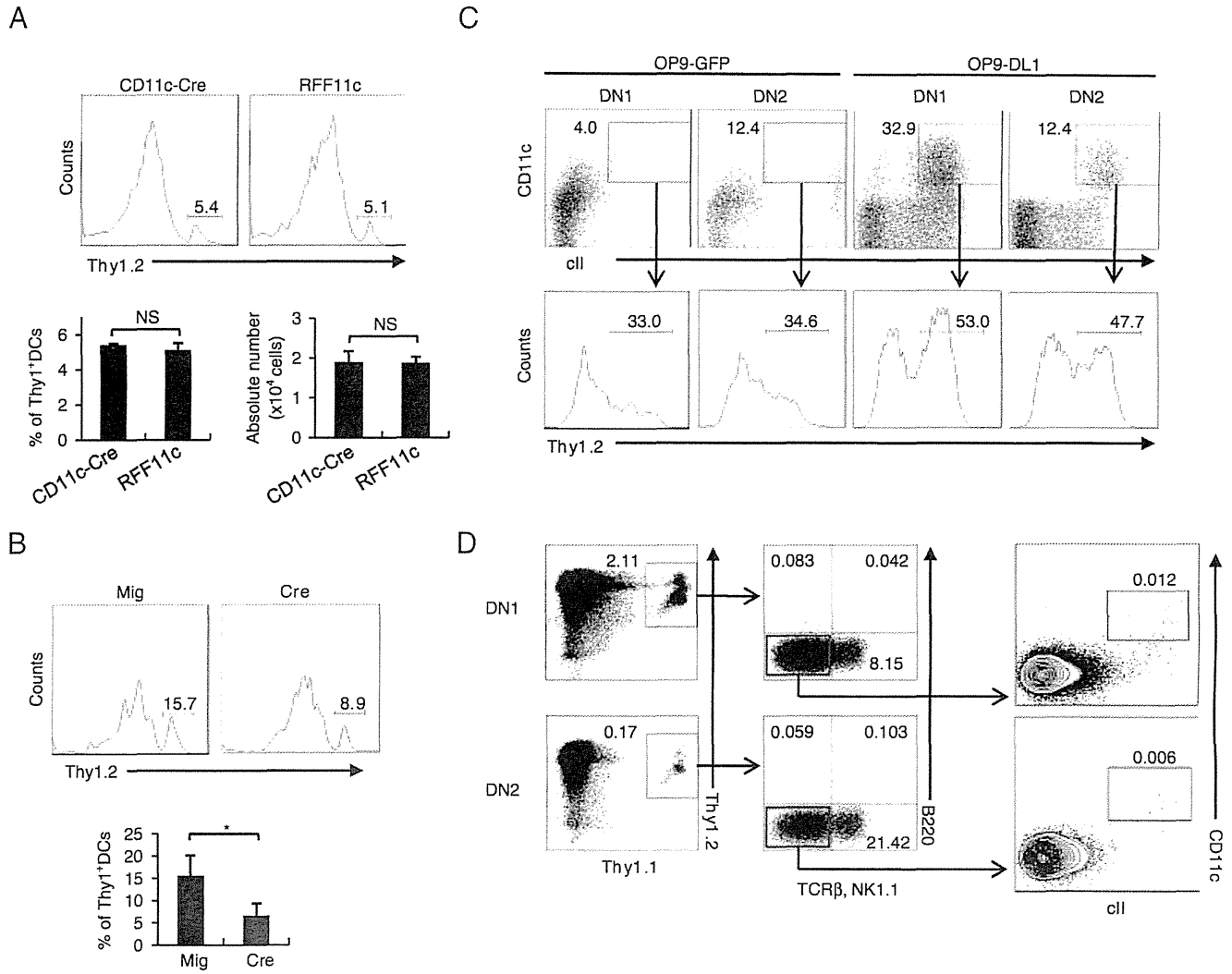

Figure 5. Thy1$^+$DC development. (A) Thymic Thy1$^+$DCs from CD11c-Cre transgenic mice or CD11c-Cre transgenic mice crossed with RBP-J$^{flox/flox}$ (RFF11) mice were analyzed after gating on B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$ cells, as described in Fig. 2A. The lower bar graphs show the percentage of Thy1$^+$DCs in B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$ cells. NS, not significant. (B) Thymic Thy1$^+$DCs from 9-Gy-irradiated B6 mice reconstituted with Lin$^-$ BMCs from RBP-J$^{flox/flox}$ mice infected with a retrovirus carrying Cre or a control virus were analyzed 4 wk after transplantation. The cells were analyzed by gating on B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$ cells in the GFP$^+$ fraction, as described in Fig. 2A. The lower bar graphs show the percentage of Thy1$^+$DCs in GFP$^+$NK1.1$^-$B220$^-$TCR-β$^-$CD11c^{hi}cII$^+$ cells. *p<0.05. Five recipient mice were used in each group. (C) Sorted DN1 or DN2 thymocytes from B6 mice were cultured on OP9 or OP9-DL1 cells for 6 days. The histogram shows Thy1.2 expression on CD11c^{hi}cII$^+$ cells after gating on 7AAD$^-$B220$^-$NK1.1$^-$TCR-β$^-$GFP$^-$, as shown in Supporting Information Fig. 6. (D) Sorted DN1 or DN2 thymocytes from B6 mice heterozygous for Thy1.1 and Thy1.2 were injected into the thymii of 5-Gy-irradiated B6 Thy1.2 mice. After 14 days, Thy1$^+$DCs expressing both Thy1.1 and Thy1.2 were analyzed by measuring Thy1.1$^+$Thy1.2$^+$B220$^-$NK1.1$^-$TCR-β$^-$ cells, gated on forward and side scatter to exclude debris and 7AAD$^-$ to remove dead/autofluorescent cells. Five recipient mice were used in each group. The data are representative results from four experiments.

not only in the cell-autonomous regulation of early T cells but also in ensuring negative selection of T cells by promoting the generation of Thy1$^+$DCs. Unfortunately, we have not found Thy1$^+$DC-specific markers to delete Thy1$^+$DCs in vivo, which precludes a rigorous analysis of whether Thy1$^+$DCs have roles distinct from Thy1^{neg}DCs in the thymus. We are currently attempting to find molecules specifically expressed on Thy1$^+$DCs.

Previous papers have shown that thymic DCs capture CD4 or Thy1 from T cells and express those molecules on their surface [17] by analyzing DCs gated on cII$^+$ cells. In order to examine if Thy1 expressed on Thy1$^+$DCs comes from adjacent T cells, we co-transplanted BMCs from RAG2-deficient (Thy1.2) mice or Thy1.1 B6 mice into irradiated mice and analyzed Thy1.2 expression, which clearly distinguished T cells from DCs. We identified Thy1.2 single-positive Thy1$^+$DCs by gating on B220$^-$NK1.1$^-$TCR-β$^-$CD11c^{hi}cII$^+$ cells, indicating that Thy1$^+$ DCs intrinsically express Thy1. However, we also observed Thy1.2 and Thy1.1 double-positive DCs, which would be attributed to the capture of Thy1 from adjacent Thy1-expressing cells. The discrepancy between our results and previous results could be due to using different flow cytometry gating strategies for analyzing cells. We used more parameters to distinguish Thy1$^+$

DCs than previous studies, which might account for our success in detecting about 5% of total thymic CD11c^{high}cII^{+} DCs as being Thy1^{+}DCs. Furthermore, we did not see any reduction in Thy1 expression during short-term cultures of Thy1^{+}DCs. Taken together, these data indicate that Thy1^{+}DCs transcribe and express Thy1 intrinsically.

In this report we showed that Thy1^{+}DCs highly express cII and several costimulatory molecules and are able to delete CD4^{+} CD8^{+} thymocytes in vitro. Thus far, we have not found any functional characteristics of Thy1^{+} DCs distinct from conventional DCs. We are currently attempting to identify molecules that are specifically expressed on Thy1^{+}DCs to delete Thy1^{+}DCs in vivo in order to carefully analyze this novel cell type's roles in immune responses.

In conclusion, we have demonstrated that Notch signaling controls the development of Thy1^{+}DCs, a novel type of DC located in the thymus. Thy1^{+}DCs are able to capture exogenous antigens and delete CD4^{+}CD8^{+} thymocytes. These data indicate that Notch signaling controls not only the differentiation of T cells but also DC development in the thymus.

Materials and methods

Mice

C57BL/6 (B6) and B10.A mice were purchased from Japan SLC (Hamamatsu, Japan). 5C.C7, OT-II and OT-I TCR transgenic and RAG2-deficient mice were purchased from Taconic (Germantown, NY, USA). CD11c-Cre transgenic and B6 Thy1.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). RBP-J$^{flox/flox}$ mice were described previously [19]. All mice were maintained under specific pathogen-free conditions in the animal research center of the University of Tokushima. All animal studies were approved by the animal research committee of the University of Tokushima.

Antibodies and flow cytometry

Fluorochrome-labeled anti-CD11c (N418), anti-CD4 (GK1.5), anti-CD8α (53–6.7), anti-Thy1.2 (53–2.1), anti-CD80 (16–10A1), anti-CD86 (GL1), anti-CD40 (1C10), anti-NK1.1 (PK136), anti-TCR-β (H57-597), anti-Thy1.1(HIS51), anti-CD25 (PC61.5) and anti-CD44 (1M7) mAbs were from eBioscience (San Diego, CA, USA). Anti-B220 (RA3-6B2), anti-cII (M5/114.15.2) and anti-CD11b (M1/70) mAbs, streptavidin-PE-Cy7 and streptavidin-APC were from BioLegend (San Diego, CA, USA). Anti-Vα2 TCR (B20.1) and anti-Vβ5.1/5.2 TCR (MR9-4) mAbs were from BD Biosciences (Mountain View, CA, USA). Fc receptors were blocked with anti-Fc receptor mAb (2.4G2). 7-amino actinomycin D (7AAD; Sigma) was used to eliminate dead/autofluorescent cells. Fluorescence intensity was evaluated using a FACSCalibur or FACSCanto II (BD Biosciences). Flow cytometry data were analyzed by FlowJo (Tree Star) or FACSDiva software (BD Biosciences). Anti-Notch1, 2 and 3 antibodies were reported previously [9, 23].

Vectors and constructs

The cDNA for Cre recombinase was cloned into the pKE004 retroviral vector [7]. MamL1-DN cloned into MSCViresGFP was provided by Dr. Pear [15]. Retroviruses were generated by transfecting each vector into Plat-E cells [24] using GeneJuice (Novagen, Darmstadt, Germany).

Induction of BMC-derived Thy1^{+} DCs

BMCs were recovered after flushing the femurs and tibias of mice with RPMI 1640 medium. After lysis of RBC with NH_4Cl, cells were plated at 1.5×10^7 cells in HEPES modified RPMI complete medium containing 10% fetal bovine serum, 2 mM L-glutamine, 40 U/mL of *penicillin*, 40 μg/mL of *streptomycin* and 55 μM of 2-mercaptoethanol with 4×10^5 OP9-GFP or OP9-DL1 cells [13] and 20% culture supernatant from GM-CSF-producing cells (provided by T. Iizuka, University of Minnesota, Minneapolis, MN, USA) for 6 days. As a control for induction of cDCs or pDCs, BMCs were cultured without OP9 cells in the presence of 20% GM-CSF or Flt3-L (10 ng/mL) (Roche) for 6 days, respectively. In some experiments, a retrovirus carrying MamL1-DN was used to infect BMCs cultures three times (days 0, 1 and 2).

T-cell proliferation assay

Splenic CD4^{+} or CD8^{+} T cells were positively purified with streptavidin microbeads (Miltenyi Biotec) after staining cells with biotin-conjugated anti-CD4 or anti-CD8 mAb, respectively. BMC-derived DCs were stained with anti-CD11c and anti-Thy1.2 mAb, and Thy1^{neg}DCs (CD11c^{+}Thy1^{-}) or Thy1^{+}DCs (CD11c^{+}Thy1^{+}) were sorted by a cell sorter (JSAN; Bay Bioscience, Kobe, Japan). The purity of sorted cells was >95%. T cells were cocultured with Thy1^{neg}DCs or Thy1^{+}DCs in the presence of PCC protein (Sigma), PCC_{81-104} or SIINFEKL (both from Biologica) for 3 days. To assess the proliferation of T cells, 1 μCi of [^{3}H]-thymidine was added for the last 6 h of culture. The incorporation of [^{3}H] was analyzed by a β liquid scintillation counter (Aloka, Tokyo, Japan)

Real-time PCR

BMC-derived pDCs were purified by using biotin-conjugated anti-PDCA-1 mAb (eBioscience) followed with streptavidin microbeads. The BMC-derived GM DCs, Thy1^{neg}DCs or Thy1^{+}DCs were purified using a cell sorter (FACSAriaII, BD Biosciences) gated on Thy1^{+}or Thy^{-}, CD11c^{+} and cII^{+} cells. The purity of sorted cells

was >95%. Thymii were digested with 400 U/mL Collagenase D (Roche) and 0.5 mg/mL DNase I in complete RPMI 1640 medium injected into several parts of the thymus, cut into small pieces, and incubated for 1 h at 37°C. The cII$^+$ cells were purified by staining cells with biotin-conjugated anti-cII mAb followed by streptavidin microbeads. Thy1$^+$DCs (NK1.1$^-$B220$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^+$) and Thy1^{neg}DCs (NK1.1$^-$B220$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^-$) were purified with a cell sorter. Total RNA was extracted with TRIzol (Invitrogen) or RNeasy Plus Micro Kits (Qiagen) and cDNA was synthesized using an Omniscript RT Kit (Qiagen) after digestion of genomic DNA by DNase (Promega). Gene expression was analyzed using SYBR *Premix Ex Taq* II (TAKARA) and primer sets (Supporting Information Table 1).

Giemsa staining

For Giemsa staining, BMC-derived DCs were purified with CD11c microbeads (Miltenyi Biotec). The BMC-derived Thy1^{neg}DCs (Thy1.2$^-$cII$^+$), Thy1$^+$DCs (Thy1.2$^+$cII$^+$) or GM DCs (Thy1.2$^-$cII$^+$) were sorted using a cell sorter. The BMC-derived Flt3-L DCs were purified using the PDCA-1$^+$ cell purification protocol described above. Purified cells were cultured on glass slides for 1 day. Adherent cells were dried and fixed for 5 min with ethanol. After drying the slides, cells were stained for 30 min with Giemsa staining solution (Sigma) diluted twenty times with phosphate buffer (pH6.4).

Western blotting

Cells were washed three times with cold PBS and then lysed with lysis buffer (50 mM Tris HCl (pH 7), 1% NP-40, 150 mM NaCl, 1 mM EDTA and a protease inhibitor cocktail (Roche)) for 20 min on ice. Cell lysates were collected after centrifugation at 10 000 rpm for 3 min. Cell lysates (30 μg) were fractionated on 10% polyacrylamide gels by SDS-PAGE and transferred to polyvinylidene fluoride membranes (ATTO, Tokyo, Japan). The marker protein was from Precision Plus Protein Western C Standards from Bio Rad Laboratories (CA, USA). Membranes were incubated with rabbit anti-mouse Hes1 Ab (H-140; Santa Cruz Biotechnology, CA, USA) at 4°C for overnight after blocking non-specific binding with TBST containing 3% gelatin (Sigma) at room temperature (RT) for 1 h. Next, membranes were incubated with HRP-conjugated anti-rabbit IgG Ab (Bio Rad) and HRP-conjugated StrepTactin (Bio Rad) at room temperature for 1 h. An enhanced chemiluminescence HRP Substrate (Millipore, Bedford, MA, USA) was used for detection. For analysis of control protein by re-probing, Abs bound to membranes were released with 100 mM 2-mercaptoethanol, 2% SDS and 6.25 mM Tris HCl (pH6.7), washed with TBST, and incubated with anti-actin Ab (Sigma) after blocking with TBST containing 5% BSA. B3Z-EV and B3Z-N2IC, previously reported [9], were used as negative or positive controls for detecting Hes1, respectively.

In vivo antigen uptake

B6 mice were injected intravenously with 500 μg of Alexa Fluor 488-conjugated OVA (Invitrogen) or unconjugated OVA protein (Sigma) dissolved in PBS. Thymii were collected 18 h after transfer and digested with Collagenase D and DNase I. Antigen uptake by each DC subset was assessed by flow cytometry.

Clonal deletion assay

Thymic Thy1$^+$DCs (NK1.1$^-$B220$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^+$) and Thy1^{neg}DCs (NK1.1$^-$B220$^-$TCR-β$^-$CD11c^{hi}cII$^+$Thy1.2$^-$) were purified with a cell sorter. Thy1$^+$DCs or Thy1^{neg}DCs (10^4 cells) were cultured with thymocytes from OT-II TCR transgenic mice depleted of cII$^+$ cells with anti-cII mAb followed by rat IgG beads (BioMag; Qiagen) (10^5 cells) and 50 μg/mL of OVA protein or 1 μM of OVA$_{323–339}$ (NH_2-ISQAVHAAHAEI-NEAGR-COOH; ABGENT) for 38 h in 96-well round-bottom plates after centrifugation.

BMCs chimera mice

Lin$^+$ cells were depleted from BMCs from RBP-J$^{flox/flox}$ mice, RAG2-deficient mice or Thy1.1 B6 mice using a Lineage cell depletion kit (Miltenyi Biotec). Lin$^-$ cells from RAG2-deficient and Thy1.1 B6 mice (1:1) were injected intravenously into irradiated (9 Gy) B6 Thy1.2 recipient mice. In some experiments, Lin$^-$ cells were cultured in HEPES modified complete RPMI medium containing SCF (25 ng/mL), IL-6 (10 ng/mL), and IL-3 (6 ng/mL) (Miltenyi Biotec) and infected three times with retroviruses encoding Cre (days 0, 1 and 2). Four days after the initial culture, cells were injected intravenously into 9-Gy-irradiated B6 mice.

Short-term culture of thymic DCs

Thymii were digested with enzymes and cII$^+$ cells were purified by staining cells with biotin-conjugated anti-cII mAb followed by streptavidin microbeads. The cII$^+$ cells were cultured in HEPES modified complete RPMI medium containing GM-CSF (10 ng/mL) and Flt-3L (10 ng/mL) (Roche) for 18 h at a concentration of 5×10^5/mL.

Induction of Thy1$^+$DCs from T-cell precursors

CD4$^+$ and CD8$^+$ thymocytes from B6 mice were stained with purified anti-CD4 and anti-CD8 mAbs and positive cells were depleted using rat IgG beads. After staining cells with anti-CD25, CD44 and CD3 mAbs, DN1 (CD3$^-$CD4$^-$CD8$^-$CD25$^-$CD44$^+$) and DN2 (CD3$^-$CD4$^-$CD8$^-$CD25$^+$CD44$^+$) cells were sorted using a cell sorter. The purity of sorted cells was >95%. DN1 or DN2

cells (3×10^4 cells) were cultured on OP9-GFP or OP9-DL1 cells (1×10^4 cells) in the presence of IL-7 (10 ng/mL) (Roche), TNF-α (1 ng/mL) (eBioscience), SCF (400 ng/mL) (Roche), IL-3 (400 ng/mL) (Miltenyi Biotec), Flt-3L (100 ng/mL) and GM-CSF (200 ng/mL) for 6 days.

Intrathymic injection

Sorted DN1 or DN2 (2.5×10^4) cells from B6 Thy1.1/Thy1.2 mice were injected into the left thymic lobes of B6 Thy1.2 mice in a volume of 50 μL of PBS with a 26-G needle between the 1st and 2nd rib. Fourteen days after transfer, thymii were digested and $Thy1.1^+Thy1.2^+$ cells were analyzed by flow cytometry.

Statistical analysis

Distributed data from interval scales were analyzed with Student's *t*-test; a *p* value of less than 0.05 was considered statistically significant.

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Abbreviations: BMCs: bone marrow cells · **cII:** MHC class II · **DL1:** Delta-like 1 · **Flt3:** FMS-like tyrosine kinase 3 · **Lin:** lineage · **MamL1:** mastermind-like 1 · **PCC:** pigeon cytochrome c · **pDCs:** plasmacytoid DCs · **RBP-J:** recombination signal binding protein for immunoglobulin kappa J region

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Lecture

解　説

禁煙に伴う抑うつとその対策*

橋本和典**　岸本年史**

Key Words : smoking cessation, depression, withdrawal symptom, nicotine

はじめに

たばこは，肺がんをはじめとして，口腔・咽頭がん，喉頭がん，食道がん，胃がん，膀胱がん，膵がんなどの多くの悪性腫瘍や，虚血性心疾患，脳血管障害，慢性閉塞性肺疾患などの疾患や，低出生体重児や流産・早産など妊婦に関連した以上の危険因子である．本人の喫煙のみならず，周囲の喫煙者のたばこの副流煙による受動喫煙も肺がんや虚血性心疾患，呼吸器疾患などの危険因子である．喫煙の本質はニコチン依存症であるという認識から，禁煙の動きは世界的に普及し，2003年に「WHOタバコ規制枠組条約」が採択され，2008年には日本を含む国々により「タバコ規制枠組条約(FCTC)」が採択された．わが国では喫煙防止，禁煙支援，非喫煙者の保護などを目標に「健康日本21」が2000年に公表され，2003年には健康増進法の制定により，受動喫煙の防止が義務づけられた．さらに2010年には厚生労働省から全国自治体に学校や飲食店など公共の場では原則全面禁煙とする方針が通告された．このように，健康施策の整備のもと，わが国でも急速に喫煙対策が進められている．たばこ産業の「平成20年全国たばこ喫煙者率調査」によると，成人女性の喫煙率は横ばいといった状況であるが，成人男性の喫煙率は昭和41年のピークの83.7%から39.5%に減少しており，日本では国民の禁煙に対する意識が高まるとともに，実際に喫煙率は減少傾向を示している．喫煙率を減少させる一つの手段として，禁煙治療がある．わが国では2006年から，一定の基準を満たす患者における禁煙治療に対して保険適用が認められた．厚生労働省がまとめた禁煙成功率の実態調査結果(平成21年度調査)では，指導終了9か月後の禁煙率は49.1%であったが，精神疾患を合併している患者では28.6%と低くなっていた．うつ病患者では喫煙率が高く，禁煙成功率が低いことが知られており[1][2]，禁煙を困難にする要因の一つに，抑うつ状態との関連が報告されている[3]．本稿では喫煙と抑うつの関係，禁煙に伴う抑うつとその対応について述べたい．

喫煙と抑うつ

喫煙と抑うつについての報告は数多くみられ，その関連についてさまざまな視点から検討されている．

喫煙の本質であるニコチン依存と抑うつが併発するモデルとしていくつか考えられている[4]．

1つは抑うつ状態が先行し，ニコチン依存の形成に関与するというモデルである．ニコチンは，セロトニン系の活性化[5]をとおして，抗うつ作用を有する[6]と考えられている．つまり，喫煙を自らの抑うつ症状に対する自己治療の手段として用いていることにより，ニコチン依存が形成されるということである．このことを支持する研究として，Escobedoら[7]が行った10歳代を対象と

* The depressive symptoms in smoking cessation and the treatment for it.

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したコホート研究では，抑うつ症状のない対象者では12.9％が喫煙を開始したのに対して，抑うつ症状のある対象者のうち19％が喫煙を開始し(odds ratio[OR] 1.3[95％CI 1.1～1.6])，抑うつ症状の重症度が高いほど，喫煙を開始しやすい傾向を認めたという結果であった．

2つ目として喫煙が先行し，抑うつ症状が出現するというモデルである．いくつかの研究で報告されているが，喫煙者の方が，非喫煙者よりモノアミンオキシダーゼ(MAO)のレベルが下がっていることが報告されており，この結果さまざまな精神疾患をひき起こすと考えられている[8)9)]．また，ニコチンの離脱症状もこれらの経路に影響を与え，うつ病に対しての脆弱性を増加させている可能性も指摘されている[10)]．喫煙がうつ病を発症させる一つの要因であることを支持する疫学的なデータも多くみられる．8～14歳の抑うつ症状のない2,000人の住民を対象とした前向き調査では[11)]，喫煙により抑うつ気分の出現のリスクが高まる(adjusted hazard ratio 1.66[95％CI 1.28～2.16])ことが示された．

3つ目として，喫煙と抑うつが継続的に相互に作用を及ぼし合うことによって，この二つが高い合併率をもって出現する原因になっているということである．たとえば，生物学的に脆弱性を持っている人において，喫煙がうつ病の発症の引き金となり，継続的に喫煙を行うことによって，その脆弱性を維持させるというようなことである[12)]．

4つ目として，うつ病と喫煙について共通の遺伝的背景があるという，双方に関連した共通因子があるという考え方である．つまり遺伝子的な脆弱性によりうつ病の発症のリスクが高くなり，また，ニコチンのポシティブな効果を感じる傾向が強くなるかもしれないということである[13)]．遺伝的な背景としての喫煙のしやすさが，同様にうつ病のなりやすさによって説明されうることをKendlerら[14)]は見出した．ほかの共通因子として，社会的な問題やストレスとなる出来事などがうつ病と喫煙のリスクを増加させることが示されている[15)]．

禁煙と抑うつ

うつ病の喫煙者は精神疾患のない喫煙者に比べて禁煙率は低いことがいわれており，禁煙の失敗率は健常者に比べうつ病患者で約2倍と報告されている[16)]．

禁煙に伴う抑うつ症状の代表としてニコチンの離脱症状がある．抑うつ症状に加えDiagnostic and Statistical Manual-IV(DSM-IV)のニコチンによる離脱症状の診断基準には①不眠，②いらだたしさ，欲求不満，または怒り，③不安，④集中困難，⑤落ち着きのなさ，⑥心拍数の減少，⑦食欲増加または体重増加がある．これらの診断基準のうち少なくとも5つがDSM-IVの大うつ病性障害の診断基準と重なっている．

離脱症状の出現については，過去にうつ病の既往があるかどうかが関連している．Coveyら[17)]の報告ではニコチンの離脱症状が出現する期間において，うつ病の既往のある喫煙者の方がその既往のない喫煙者より抑うつ症状が強く出現することが示された．

一方で離脱症状とは無関係な禁煙と抑うつ症状との関連性についての報告もある．Glassmanら[18)]の報告によるとうつ病の既往があり，さらに禁煙を行った人は，喫煙を続けた人に比べ，禁煙の開始後6か月以内に新たなうつ病エピソードを7倍経験しやすいという結果であった．新たなうつ病エピソードの出現が離脱症状を反映しているのであれば，そのリスクは離脱症状が出現する期間を過ぎれば，もとのレベルに戻るはずである．しかし，Glassmanら[18)]の報告では，そのリスクは禁煙後数週間でピークに達し，その後もとのレベルに戻るということではなかった．つまり，離脱症状とは別にうつ病エピソードが出現することや，また，ニコチンによりうつ病に対する脆弱性に変化をもたらすことを示している．

禁煙に伴う抑うつへの対応

1．ニコチン代替療法

ニコチン依存に対してのニコチン代替療法の有効性については多くの無作為対照化試験によって証明されている[19)]．禁煙に伴う抑うつ症状への

効果は，禁煙後の脳内でのニコチンの減少に対して，ニコチンを経皮あるいは経口投与することで離脱症状を和らげることとして現れる．このようなニコチン依存や，禁煙の離脱症状への効果に加えて，ニコチン代替療法はそれ自体に抗うつ効果の性質も持っている．608名の喫煙者(3分の1は抑うつ症状あり)を対象に，行動療法に加えて，ニコチンガムかプラセボのガムのどちらかの投与を行った調査[19]では，12か月の禁煙成功率は，抑うつ症状がありニコチンガムを投与されていた群で15.1%(抑うつ症状がありプラセボガムを投与されていた群で5.7%)であり，また，抑うつ症状についての自己記入式のスケールでは有意に症状は改善していた．ニコチン代替療法の抗うつ効果については，大うつ病性障害の非喫煙者を対象にした調査も報告されている．Salin-Pascualら[20]はニコチンパッチによってこれらの対象者の抑うつ症状が軽減されることを示したが，ニコチンパッチを中止した3,4日後には再発し，また副作用も認められるなど実際の臨床での適応は推奨していない．

2．抗うつ薬

抗うつ薬にはニコチンの離脱症状に伴う抑うつ症状を軽減し，また喫煙行動を継続させるような不快感を軽減する重要な役割を持っている[21]．大うつ病性障害の既往のあるなしにかかわらず，bupropion(OR 1.69[95%CI 1.53～1.85])とノルトリプチリン(OR 2.03[95%CI 1.48～2.78])は再喫煙率を下げるには同等に有効である[22]．しかし，選択的セロトニン再取り込み阻害薬には禁煙を促進する効果は認めなかった[22][23]．これまでの研究でbupropionとノルトリプチリンの生理学的な作用はセロトニン系の経路というよりは，ノルアドレナリン系またはドパミン系の経路を通してのものである可能性が示唆されている．

多くの研究で神経伝達物質であるセロトニンの障害が，うつ病や，ニコチンの離脱期にみられることが示されている[24]．選択的セロトニン再取り込み阻害薬(SSRI)については禁煙治療への効果がないようであり，fluoxetineを禁煙開始の2～3週間前に使用することで禁煙の成功率に対しての効果は認められなかったものの，抑うつ気分，怒り，緊張といった症状に対してはプラセボに比べ効果を認めたという報告がある[25]．また，SSRIはノルトリプチリンと比べ，ニコチンの離脱症状や，喫煙欲求をより軽減させるかもしれないが[26]，禁煙率は改善されなかったという報告がある．これらのことから，ノルトリプチリンは離脱症状を軽減させたり，喫煙欲求を抑えたりする以外の方法で禁煙率を高める可能性があると考えられる．ノルトリプチリンはノルアドレナリンの再取り込みを阻害することでシナプスでのノルアドレナリンのレベルを上げている．喫煙はノルアドレナリン系の伝達に影響を与え，禁煙することで反跳性の変化をもたらす．このため，ニコチン代替療法とノルアドレナリンとの併用療法は合理的であるかもしれないし，また禁煙を促進するための異なった角度からの補完的な方法かもしれない．このことを検証した報告では[27]，併用療法の方がニコチン代替療法単独よりオッズ比2.62(95%CI：1.06～8.44)で禁煙に対する効果があったとしている．

3．バレニクリン

バレニクリンはα4β2ニコチン受容体の部分アゴニストであり，かつα7ニコチン受容体の完全アゴニストで，最近になって日本でも禁煙治療に使用できるようになった．ニコチンと拮抗し喫煙の満足感を抑制するとともに，ニコチンの作用で放出されるよりも少量のドパミンを放出させ，禁煙に伴う離脱症状やたばこに対する切望感を軽減する作用の両面を持つ．

海外の第III相試験[28]では抑うつ気分，いらだたしさ，不安，睡眠障害といったニコチンの離脱症状に伴う症状を和らげることが示された．このことからバレニクリンは単独で抗うつ効果を持っているという可能性が考えられる．

バレニクリンは抗うつ効果をもたらすような薬理学的な特性を持っている．側坐核のα4β4ニコチンレセプターへの作用により，バレニクリンは神経伝達物質であるドパミンを調整する．この調整の異常はうつ病の症状であるアンヘドニア(無快楽)と関連があるとされている．ほかにもニコチン性アセチルコリンシステムが抑うつ気分の調整と関連があるというエビデンスはあるものの，はっきりとした機序についてはわかっていない[29]．

以上のことからバレニクリンはうつ病を伴った喫煙者の抑うつ症状を改善するような治療効果をもたらすことがわかるが，バレニクリンによる精神症状の悪化や，自殺念慮，自殺などの報告もなされており，投与に際して注意を要する[30]．

おわりに

これまでの研究で喫煙，禁煙と抑うつ症状にはさまざまな関連性があることが示されており，禁煙に伴う抑うつ症状は，禁煙が成功するかどうかにも大きくかかわっていると考える．日本で用いられている禁煙治療薬のニコチン製剤(パッチ，ガム)やバレニクリンにはそれ自体に離脱症状を軽減し，それに伴う抑うつ症状を軽減する効果を持っている．SSRIには離脱症状を軽減する作用はあるものの，禁煙率を改善させる作用はなく，単独での使用では効果が乏しいと考えられる．日本ではノルトリプチリンのみが抗うつ薬の中で禁煙に対しての効果が認められており，離脱症状を軽減する作用もあり，抑うつを伴う場合，選択肢として考えてよいと思われる．

喫煙や禁煙に関連した抑うつ症状のある喫煙者に対しての禁煙治療を行うにあたっての決定的なデータはないものの，抑うつ症状を伴う喫煙者には抗うつ薬の併用療法が必要であり，また，抑うつ症状のない喫煙者に行うよりもより長期間に及ぶ治療が必要であると考える．

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■第30回日本社会精神医学会（奈良）：シンポジウム1「精神科病院における禁煙対策」

統合失調症と喫煙

橋本和典[1)]，岸本年史[1)]

はじめに

精神科病棟の喫煙室での統合失調症患者の喫煙風景は独特のものがある。彼らは煙の立ちこめる閉鎖されたスペースで黙々とタバコを吸い，中指と人差し指をやけどするほどにタバコが短くなっても吸い続けている。精神科医であれば，一度はそのような光景を目にしたことがあるだろう。精神科において喫煙は比較的望ましい嗜好品として職員にも患者にも愛されてきた歴史があり，「喫煙をよし」とする精神科病院の独特の文化があり，精神科における禁煙対策については，日本においてだけではなく世界的にも「neglected problem」[2)]であった。

統合失調症患者の喫煙率は健常者に比べるとその喫煙率は高い（68～88%）[5, 26)]ことが知られている。統合失調症患者の喫煙については他に，健常者と比べ，禁煙成功率が低い[14)]，1日の消費本数が多い[23)]，より強いタバコを好む，タバコからより多くのニコチンを吸い出す[16)]といった特徴がある。また，統合失調症患者の非喫煙者と喫煙者を比べると，喫煙者のほうが統合失調症発症の年齢が若い，入院回数が多い，重症度が高い（PANSSが高い）[21)]，抗精神病薬の服用量が多い[19)]といった報告がなされている。

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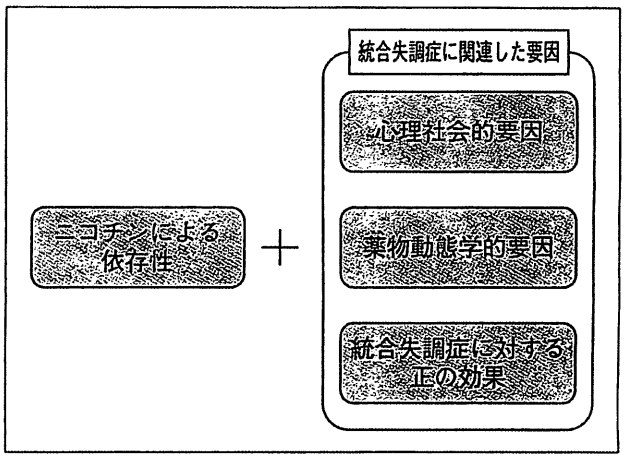

図1　統合失調症患者の喫煙の理由

以上のように統合失調症と喫煙との間には深い関係があるようだが，ではどうして統合失調症患者は喫煙するのであろうか。

統合失調症患者はなぜ喫煙するのか？

喫煙の本態は，ニコチンという依存性薬物による依存症である。統合失調症患者においてもその喫煙にはニコチン依存が大きく影響をしている。しかし，先に述べたような，健常者と比べた統合失調症患者の喫煙状況を考えると，単なる依存症以外にも喫煙をする理由がありそうである。そこで，統合失調症患者の喫煙する理由として，図1のように，ニコチンの依存性に加え，心理社会的要因，薬物動態学的な要因，統合失調症に対するニコチンの正の効果が挙げられる。これらの要因について考えてみる。

1．ニコチン依存

喫煙によりニコチンは肺から直接血流にのり，数秒で血液脳関門を越え，脳内に達する。そし

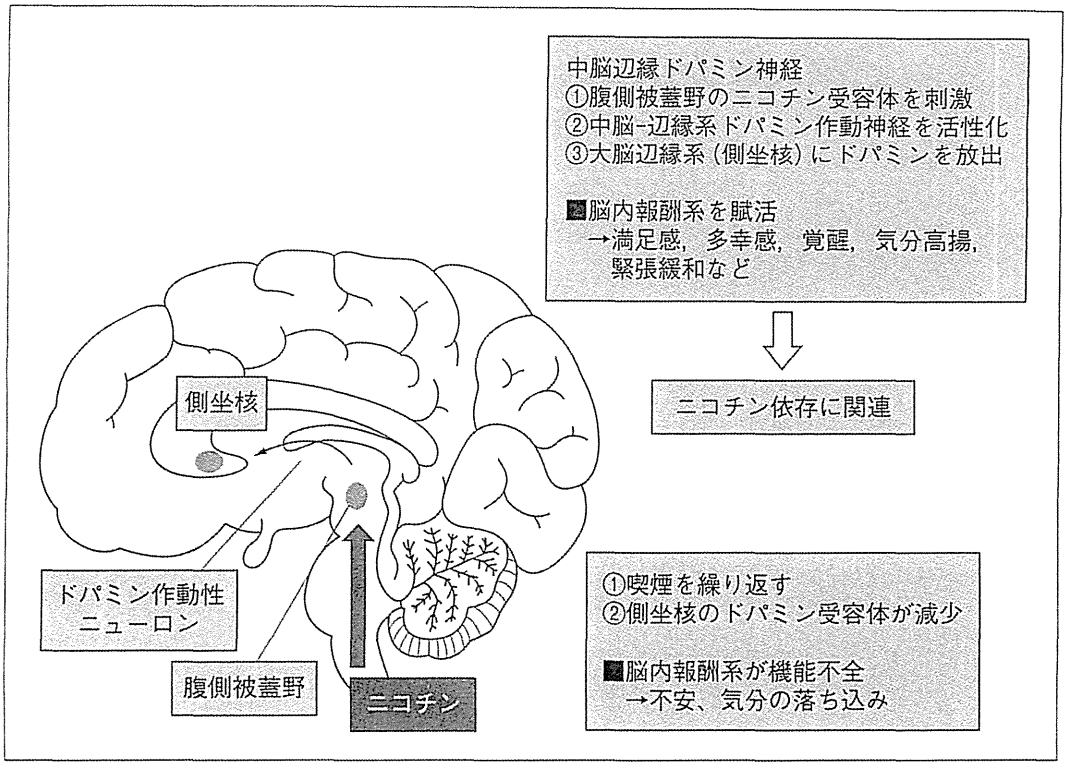

図2　ニコチン依存の形成

喫煙により肺から吸収されたニコチンは，数秒でBBBを超えて脳内に達する。
腹側被蓋野のα4β2ニコチン受容体にニコチンが結合するとドパミン動性ニューロンにより側坐核にドパミンが放出される。
喫煙をしてから，この快刺激が得られるまでの時間が短ければ短いほど，依存を生じやすい。
オピオイド（モルヒネ，ヘロイン）などは側被蓋野のGABA作動神経を抑制し，間接的に報酬系を賦活する
覚醒剤，コカインはドパミンの再吸収を阻害

て，中脳にある腹側被蓋野のニコチン性アセチルコリンレセプターに結合すると側坐核に伸びた神経末端からドパミンが放出され，側坐核細胞群のドパミンレセプターを介して脳内報酬系が賦活される（図2）。これにより満足感，多幸感，気分高揚，緊張緩和，覚醒といった効果を得る。ニコチンによる効果を期待してから数秒でその効果を感じることができ，この間隔が短いほど精神依存をきたしやすくなる。さらに喫煙を繰り返すと側坐核細胞群でのドパミンレセプターが減少し機能不全に陥り，その結果非喫煙時にニコチン離脱症状としての不安，焦燥感，うつなどを生じる。すなわち報酬系の賦活による快刺激の自覚の後にニコチン離脱に伴う神経，身体症状の出現が次の喫煙を引き起こし，結果，禁煙が困難になっていくというのがニコチン依存の形成過程である。

2．心理社会的要因

健常双生児を対象にした研究では，喫煙の開始には遺伝的要因が39％，環境要因が54％関与していると報告[12]されており，喫煙には環境要因も大きく影響していることがわかる。統合失調症患者では喫煙者のほうが非喫煙者に比べて，入院期間が長かったということから，入院といった特殊な環境下で，ひまな時にタバコを吸うしかないということや，集団生活の中での人間関係から生じるストレスに対しての安定剤としてのタバコの役割などが，心理社会的，あるいは環境要因として，喫煙行動に影響を与えると考えられる。また，入院以外の生活でも，統合失調患者では一般の人々より何もしないで過ごす時間が多い[18]との報告もあり，何もしない時間を埋める手段として，統合失調症患者と喫煙を結びつける要因になっていると考えられる。

表1　抗精神病薬と薬物代謝酵素

薬剤名	1A1/1A2	2C19	2D6	3A4
クロルプロマジン			●	●
ハロペリドール	●		●	●
ペルフェナジン			●	
チオリダジン			●	
オランザピン	●	●	●	
リスペリドン			●	●
アリピプラゾール			●	●
クエチピン				●

（戊亥敬一郎ら：日精協誌27(10)：22，2008．より一部引用）

3．薬物動態学的要因

タバコの不完全燃焼によって生じる多環状芳香族炭化水素は肝代謝酵素であるCYP1A2/1A1，2E1を誘導することが知られている。これらの酵素は抗精神病の代謝に影響を与えており（表1），例えばハロペリドール，オランザピンについては喫煙により血中濃度が有意に低下する。逆にリスペリドン，クエチアピン，アリピプラゾールについてはこれらの酵素の影響はほとんど受けない。喫煙により誘導された肝代謝酵素は，ハロペリドールやオランザピンの代謝を促進させることで，血中濃度を低下させる。その結果，これらの抗精神病薬による副作用を軽減させることとなる。この効果が統合失調症患者の喫煙を促す要因になっていることが考えられる。

4．統合失調に対する正の効果

統合失調症に対しての正の効果について精神症状，Sensory gating，認知機能に分けて述べる。

① 精神症状への効果

Smith[22]らは統合失調症患者を対象にニコチン含有量の高いタバコと，ニコチンの入っていないタバコを喫煙した場合の精神症状について評価した。その結果，ニコチンを含有したタバコを吸った患者のほうで陰性症状が低くなっていた。陰性症状が前頭皮質でのドパミンの低下によって引き起こされて[25]いると言われており，またニコチンは側坐核や前頭前野でのドパミン濃度を上昇させる[4]ため，ニコチンによる陰性症状に対する効果は，この領域におけるドパミン濃度の上昇によって引き起こされると考えられる（図3）。

② Sensory gatingに対する効果

Sensory gatingは感覚情報の入力に対する反応を制御する機能で，統合失調症の中間表現型であり，健常者と比べ障害されている。Sensory gatingの指標としては大きな音を聞かせ得たときの驚愕反応が，直前に小さな音を先行させると抑制されるというプレパルスインヒビション（PPI）や，事象関連電位の1つで刺激後50ms後の脳波のピークを表すP50が用いられる。

ニコチンによってSensory gatingが改善すること[1,13]が報告されており，この効果が自己治療として統合失調症患者の喫煙行動を強化していると考えられる。

③ 認知機能に対する効果

脳内のニコチンレセプターが多くみられる領域はワーキングメモリーや注意，自発性に関係しており[20]，それらがニコチンにより影響を受けている[24]と報告されている。

統合失調症患者の認知機能に対するニコチンの影響についてはいくつかの報告がある。二重盲検でニコチンパッチを用いた研究[6]では，注意機能の改善がみられ，また，禁煙中にニコチンパッチを用いた研究[11]では，ワーキングメモリー，注

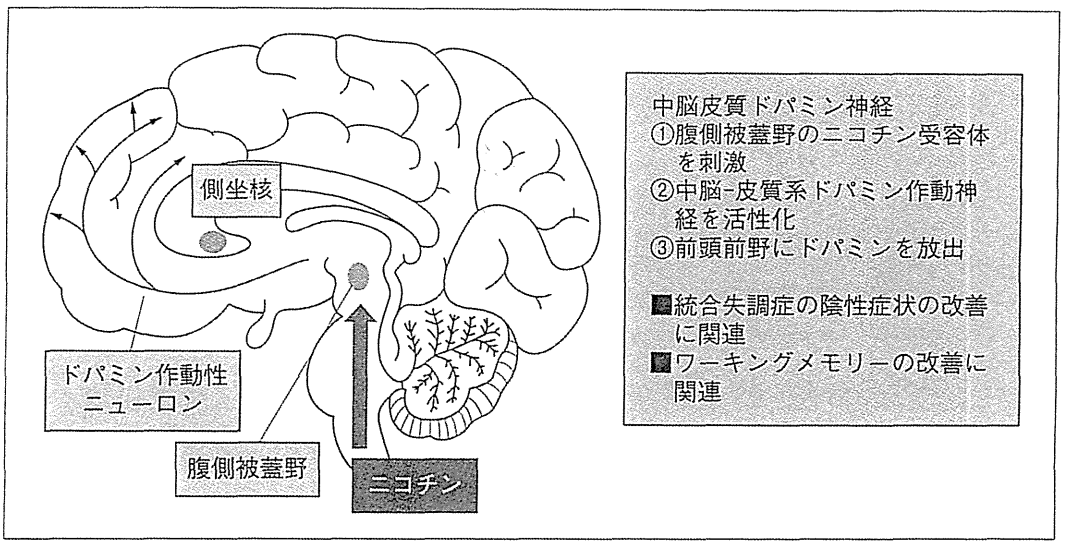

図3　ニコチンの前頭葉への影響

意力で改善がみられたことが報告された。しかし，即時再生，遅延記憶，空間視覚に関してはニコチンによる改善効果はみられなかった[9]と報告されている。

これらの改善効果は離脱症状がニコチンにより改善したことによる作用とも考えられるが，健常者を対象にした研究で，非喫煙者[7,15]にニコチンを投与した場合，注意機能やワーキングメモリーが改善したという報告がある。つまり，離脱症状の出ない非喫煙者でも同様の改善がみられたことから，ニコチンによる認知機能の改善作用が認められるということである。

以上のように統合失調症における精神症状，Sensory gating，認知機能に対するニコチンの正の効果は，統合失調症患者において喫煙行動に対して，正の強化因子となり，喫煙を続ける要因となっている。

このような効果と，喫煙，統合失調症を結びつけるものとして，ニコチン性アセチルコリンレセプターのサブタイプの一つであるα7ニコチン受容体の関与が報告されており，ニコチンによるこの受容体の刺激が統合失調症に対して正の効果を引き起こしていると考えられている。実際にこの効果を利用した薬剤が開発されている。α7ニコチン受容体作動薬である3-[(2, 4-dimethoxy) benzylidene] anabaseine (DMXB-A) が統合失調症を対象として，臨床試験が行われており，統合失調症に対する無作為化比較試験では認知機能やSensory gatingの改善[17]や陰性症状の改善[8]を認めたことが報告された。

統合失調症患者の禁煙

上述したように統合失調症に対しての有用な効果がニコチンに認められる。しかし，喫煙は心血管障害のリスクを2倍にし，統合失調症患者の寿命を20％縮める[3,10]，といった健康面への影響や，さらに喫煙行動に潜在する火災の危険から，住居確保困難となるなど，生活権にまで影響が及んでおり，統合失調症患者においても禁煙は必要であると思われる。統合失調症と喫煙の独特の関連を念頭に置き，ニコチンの効果を補った上での禁煙治療など禁煙には特別な配慮が必要であると思われる。

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