Notch3 activation is sufficient but not required for inducing human T-lineage specification.

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Abstract

While the role for the individual Notch receptors in early hematopoiesis have been thoroughly investigated in mouse, studies in human have been mostly limited to the use of pan-Notch inhibitors. However, such studies in human are important to predict potential side-effects of specific Notch receptor blocking reagents since these are currently being considered as therapeutic tools to treat various Notch-dependent diseases. Here, we studied the individual roles of Notch1 and Notch3 in early human hematopoietic lineage decisions, particularly during T-lineage specification. While this process in mice is solely dependent on Notch1 activation, we recently reported Notch3 expression in human uncommitted thymocytes, raising the possibility that Notch3 mediates human T-lineage specification. Although expression of a constitutive activated form of Notch3 (ICN3) results in the induction of T-lineage specification in human $CD34^+$ hematopoietic progenitor cells, similar to ICN1 overexpression, loss-of-function studies using blocking antibodies reveal that only Notch1, but not Notch3, is critical in this process. Blocking of Notch1 activation in OP9-DLL4 co-cultures resulted in a complete block in T-lineage specification and induced monocytic and plasmacytoid dendritic cell differentiation instead. In fetal thymus organ cultures, impeded Notch1 activation resulted in B and dendritic cell development. In contrast, Notch3 blocking antibodies only marginally affected T-lineage specification and hematopoietic differentiation with a slight increase in monocyte development. No induction of B or dendritic cell development was observed. Thus, our results unambiguously reveal a non-redundant role for Notch1 in human T-lineage specification, despite the expression of other Notch receptors.

Introduction

In mammals, the Notch pathway is composed of a highly conserved family of four different Notch receptors (Notch1-4) that can be activated through five different ligands (Delta like 1, 3 and 4 and Jagged 1 and 2). Activation results in cleavage of the transmembrane Notch receptor, thereby releasing the intracellular part of the protein (intracellular Notch, ICN) that subsequently migrates to the nucleus to activate downstream Notch target gene expression (1). Notch signaling is involved in various developmental programs and cell fate decisions (2). As a result, Notch mutations have been implicated in various malignancies, including neurological disorders (3), cancers (4) and immune-related diseases. A well-known example includes aberrant Notch1 activation that is involved in over 60% of T-acute lymphoblastic leukemia cases (5), while activating Notch3 mutations have been implicated in various tumors (6,7). Due to the broad activity of Notch signaling, the therapeutic potential of pan-Notch blocking reagents, such as gamma-secretase inhibitors, has been hampered as a result of significant side-effects which may be overcome with Notch receptor specific reagents such as monoclonal antibodies (8,9).

Unfortunately, limited information is available on the effects that Notch receptor specific blocking reagents might have on human hematopoiesis. However, such knowledge is crucial because of the involvement of Notch signaling in the development of various normal and malignant blood cell types (10-14). While specific gene deletion studies in mice have revealed critical roles for Notch1 and Notch2, Notch3 seems less critical during hematopoietic differentiation in the mouse. Since species differences exist, studies in human are of critical translational importance. Indeed, previous work from our lab and others, although mostly limited to pan-Notch activation and inhibition experiments, confirmed certain roles for Notch activation in early hematopoietic lineage decisions (15-18), but also revealed some subtle differences during intrathymic stages of T cell development (16,19-22). In more recent work,

we revealed a critical role for Jagged2-mediated Notch3 activation in human TCR-γδ T cell development (23), a mechanism that seems absent in mouse (24,25). In that study, we also observed that $CD34^{+}CD1a^{-}$ human thymocytes, immature uncommitted T-lineage progenitors in the human postnatal thymus, express significant *NOTCH3* mRNA levels, in addition to *NOTCH1* mRNA. While it is clear in the mouse that Notch1 (26) is the only receptor that is involved in the specification of multipotent hematopoietic progenitor cells into the T cell pathway as a result of activation through Delta-like-4 (27,28), it is still unclear which Notch receptors mediate this process in human. The question is particularly relevant since Jagged2, a strong Notch3 ligand, is abundantly expressed by cortical thymic epithelial cells (18), the region inside the thymus where the first Notch signals are provided to early thymic progenitors (29). While DLL4 is also expressed within that region, it is inefficient at binding and activating Notch3 (23,25).

Given our recent findings that Notch3 is expressed early during human T cell development and that this receptor modulates human T-cell lineage decisions (23), we investigated in this study the requirement of both Notch1 and Notch3 in the early stage of human T cell specification by specific overexpression or inhibition of one of these Notch receptors. Our results show that Notch3 is able to induce T cell lineage specification in the absence of Notch1 activation, but that Notch3 is not essential in this process. Thus, in accordance with observations in the mouse model, Notch1 is the only Notch receptor that is essential to induce early T-lineage specification.

Materials and methods

Cell samples

Cord blood units that did not meet the criteria for banking were obtained from the Navelstrengbloedbank UZ Gent and thymus tissue was obtained from children undergoing cardiac surgery (UZ Gent). Both were obtained and used according to the guidelines of the Medical Ethical Commission of Ghent University Hospital (Ghent, Belgium).

Mononuclear cells were collected after centrifugation over Lymphoprep and were, if necessary, cryopreserved in 10% dimethylsulfoxide, 90% fetal calf serum until required.

Cord blood cells or thymocytes were enriched for $CD34^+$ cells using magnetic microbeads (Miltenyi Biotec), according to the manufacturer's instructions. Cord blood cells were then stained with CD34-APC (Miltenyi), CD3-FITC, CD14-FITC, CD19-FITC, CD56-FITC (BD Biosciences) and sorted for $CD34^+lin^-$ using a FACSAriaII cell sorter (BD Biosciences). Purity of the sorted cells was always >95%. Purity of thymocytes following $CD34^+$ magnetic purification was always >98%.

Generation of plasmids and viruses.

cDNA encoding constitutively active Notch3 was subcloned from previously described constructs (30) into the multicloning site of the retroviral vector MSCV-EGFP. Generation of the plasmid containing ICN1 has been described previously (16). Retroviral transduction of cord blood has been described (31). ICN1 and ICN3 protein expression levels following transduction of human progenitor cells was validated previously (23).

OP9 cocultures and Fetal thymus organ cultures.

Retrovirally transduced progenitors were first sorted for $EGFP^+$ cells and subsequently seeded onto plates (24-well or 96-well) containing a confluent layer of OP9-control or OP9-DLL4 cells. OP9 cocultures were all performed in α-MEM media (Invitrogen) supplemented with

20% heat-inactivated FCS (Hyclone) plus 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamin (all from Invitrogen) (18,32). $CD34^{+}Lin^{-}$ cells were cultured in the presence of 5 ng/ml IL-7, 5 ng/ml Flt-3L and 5 ng/ml SCF. In blocking experiments, 5 µg/ml isotype control, anti-Notch1 (9) or anti-Notch3 antibody (8) was added to the medium and half of the medium was refreshed every 3-4 days to keep the antibody concentration stable.

Fetal thymus organ cultures (FTOCs) were performed as described previously (33) NOD-LtSz-scid/scid (NOD-SCID) mice, originally purchased from The Jackson Laboratory (Bar Harbor, ME), were obtained from our own specific pathogen-free breeding facility. NOD-SCID mice were treated according the guidelines of the Laboratory Animal Ethical commission of the University Hospital of Ghent. Fetal thymic lobes from these mice were isolated at fetal day 15-15.5 of gestation. 2000-10000 human cord blood cells were added to each lobe in medium containing 20 µg/ml G3 isotype control, anti-Notch1 (9) or anti-Notch3 antibody (8). After 2 days in hanging drop, lobes were transferred to FTOC in medium containing 15 µg/ml G3 isotype control, anti-Notch1 or anti-Notch3 antibody. Half of the medium was refreshed every 3-4 days.

Lymphocytes from cultures were counted using a hematocytometer and human cellularity was quantified following determination of the frequency of human $CD45^{+}$ cells (and EGFP in case of transduced cells) by flow cytometry.

Monoclonal antibodies and flowcytometry

Cell suspensions obtained from cocultures were first blocked with anti-mouse FcRγII/III (clone 2.4.G2) and human IgG (Fcblock, Miltenyi) to avoid non-specific binding. Cell suspensions obtained after FTOCs were also blocked with anti-mouse FcRγII/III mAb and stained with rat anti-mouse monoclonal antibody CD45-cychrome to gate out mouse cells during flowcytometry. Subsequently, cells were stained with combinations of anti-human monoclonal antibodies as indicated and previously described (18). Cells were examined for

the expression of cell surface markers on a LSRII (BDIS) and human viable cells were gated by excluding propidium iodide positive cells from analysis.

Quantitative RT-PCR

Two days after transduction with ICN1, ICN3 or control, cells were sorted for eGFP expression, were resuspended in RLT-buffer and stored at -70°C prior to RNA isolation. RNA was extracted using RNeasy RNA isolation kit (Qiagen) and converted into cDNA using Superscript RT II (Invitrogen).

Real-time PCR reactions were performed using qPCR Core kit for SYBR® Green I (Eurogentec) on a 7300 Real-time PCR system (Applied Biosystems). Relative expression levels were calculated for each gene using the ΔCt method using β-actin for normalization.

Statistical analysis

Statistical significance was calculated using the non-parametric paired Wilcoxon test from SPSS version 22.0 software.

Results

Notch3 is expressed in the majority of human early thymocyte progenitors (ETPs).

While the precise identity of the human equivalent of mouse ETPs is still a matter of debate (34-37), it is clear that the most immature human thymocytes reside within the $CD34^+CD1a^-$ population. We have previously shown that bulk $CD34^+CD1a^-$ thymocytes express both *NOTCH1* and *NOTCH3* mRNA (23), but now extend these results by investigating protein expression for Notch3 at the single cell level using flow cytometry. As illustrated in Figure 1, over 90% of these cells display Notch3 protein expression at the cell surface. While this frequency further increases in T cell committed $CD34^+CD1a^+$ thymocytes (Figure 1), these findings indicate that Notch3 activation can occur very early during human T cell development and thus may influence the T-lineage specification process.

Notch3 overexpression can support early T cell development in vitro.

The high frequency of Notch3 expressing cells within the most immature human thymocytes urged us to investigate whether Notch3 has a role in the induction of human T-lineage specification. We first determined if Notch3 has the potential to induce human T cell development in the absence of other Notch receptor stimuli. Therefore, human $CD34^+lin^-$ hematopoietic progenitor cells from cord blood were transduced (Figure 2A) with an EGFP control virus, or with viruses encoding EGFP in addition to the intracellular activated forms of Notch1 or Notch3 (ICN1 or ICN3, respectively), and, following sorting to start with a nearly 100% transduced and homogeneous population (Figure 2B), cultured on OP9-control or OP9-DLL4 stromal cells in cytokine conditions that promote T cell differentiation.

As expected, EGFP control transduced progenitor cells did not differentiate into $CD34^+CD7^+$ (Figure 2C, 2D) and $CD7^+CD5^+$ T-lineage specified cells (Figure 2G, 2H) on OP9-control stromal cells, in contrast to on OP9-DLL4 cells. Consistent with previous results, ICN1 was

sufficient to induce T cell development in human precursors, and this was also the case when ICN3 was continuously expressed, indicating that Notch3 activation could be sufficient in the absence of Notch1 activation to induce human T lineage specification (Figure 2C, 2D, 2G, 2H). Levels of transduction remained consistent throughout the coculture (Figure 2E, 2F). Interestingly, ICN3 was more efficient at inducing T-lineage specification compared to ICN1 when cocultured on OP9-DLL4 (although not statistically significant, Figure 2D, 2H), suggesting a synergistic effect of Notch1 (activated through DLL4) and Notch3 activation on early T cell development, similar as documented previously in T-lineage committed human thymocytes (23) but in contrast to earlier reports that suggested a negative feedback of Notch3 signaling on Notch1 activity (38).

Consistently, gene expression analysis revealed that the direct Notch target genes *HES1*, *DTX1*, *NRARP* and *IL7R* were upregulated 48 hours after transduction with both ICN1 and ICN3 (Figure 3) and in agreement with earlier work (23), ICN3 was a stronger inducer of the most sensitive Notch target genes (DTX1 and NRARP) (22) compared to ICN1. Interestingly, while ICN1 has the potential the induce *NOTCH3* expression, the reverse seems not possible as ICN3 transduced cells display virtually no upregulation of *NOTCH1* expression (Figure 3). In agreement with the capacity of ICN1 and ICN3 transduced cells to differentiate along the T-cell lineage, myeloid genes such as *CSFR1* (encoding MCSFR) and *SPI1* (encoding PU.1) were downregulated by both Notch receptors.

In conclusion, these results show that Notch3 activation is sufficient to induce T-lineage specification in human multipotent hematopoietic progenitor cells.

Notch3, but not Notch1, is dispensable for induction of human T-lineage specification.

Experiments in human have thus far revealed that Notch signaling is essential to induce T-lineage specification, but it is still unclear which Notch receptor is driving this process. While

the above experiments reveal that both Notch1 and Notch3 are expressed in the most immature thymocyte subsets and that both can induce T-lineage specification, they did not reveal their requirements in this process. Therefore, we used Notch1 and Notch3 specific blocking antibodies at concentrations known to fully block receptor activation (8,9) to reveal which Notch receptors are critical in this process. Their specificity was confirmed using quantitative RT-PCR for Notch target genes in $CD34^+$ thymocytes exposed to either DLL4 or JAG2 in OP9 cocultures. Consistent with the fact that DLL4 is a good Notch1 but a poor Notch3 ligand, blocking of Notch1 completely abolished Notch target gene expression in OP9-DLL4 cocultured cells, while blocking Notch3 antibodies had very little effect (Figure 4A). In contrast, Jagged2 can activate both Notch1 and Notch3, and consistently, blocking Notch3 antibodies now also efficiently blocked Notch target gene expression, although residual Notch activity was observed as a result of remaining Notch1 activation. Notch1 blocking antibodies also fully blocked Notch3 activity in OP9-JAG2 cocultures since Notch3 is a downstream Notch1 target during early T cell development in mouse and human (22,39).

In the presence of a Notch1 blocking antibody, $CD34^+lin^-$ hematopoietic progenitors from CB fail to differentiate into $CD34^+CD7^+$ and $CD7^+CD5^+$ T-lineage precursors on OP9-DLL4 stromal cells, in contrast to when a control antibody is added (Figure 4B, 4C). In the presence of a Notch3 blocking antibody, $CD34^+CD7^+$ and $CD7^+CD5^+$ thymocytes can develop (Figure 4B) with only a small, but significant, reduction in the number of $CD7^+CD5^+$ cells (Figure 4C).

Notch-induced T-lineage specification is accompanied by inhibition of myeloid differentiation and consistently, blocking of Notch1 activation resulted in a significant increase in the development of conventional $CD4^+$HLA-DR^+ dendritic cells, $CD11b^+CD14^+$ monocytes and $CD123^+CD303^+$ plasmacytoid dendritic cells (Figure 5A, 5B). Inhibition of Notch3 activation

resulted in a small increase in the development of conventional dendritic cells and monocytes, but no difference in plasmacytoid dendritic cell differentiation was observed (Figure 5A, 5B).

To test the requirement for Notch1 and Notch3 in a more physiological setting, we added these inhibiting monoclonal antibodies in an FTOC since DLL4 is not the only Notch ligand that is expressed within the thymus (18,21,40,41). Consistent with our findings in OP9-DLL4 cocultures, however, Notch1 inhibition resulted in a block in $CD7^{+}CD5^{+}$ T-lineage specification and an overall reduction in cell numbers, while Notch3 inhibition did not significantly influence this process (Figure 6A, 6B). In agreement, myeloid differentiation as well as $CD19^{+}$HLA-DR^{+} B-lineage development was only increased compared to the control when Notch1 signaling was inhibited, not upon Notch3 inhibition (Figure 6A, 6B). Overall, these results show that T-lineage specification in human is dependent on Notch1 activation, not Notch3.

Discussion

We have previously illustrated that human uncommitted $CD34^{+}CD1a^{-}$ thymocytes not only express Notch1, but also Notch3 (23). Here, we further analyzed Notch3 protein surface expression within the most immature population of human postnatal thymocytes and reveal that the majority of these cells already express Notch3 protein. Given that we have previously revealed important differences in Notch signaling activity between mouse and human (20-23,42-44), this prompted us to investigate whether Notch3 activation was critical at the earliest stages of human T cell development, during T-lineage specification. While activation of Notch1 or Notch3 by itself was sufficient to induce T cell development in human multipotent hematopoietic progenitors, specific blocking monoclonal antibodies revealed that only Notch1, not Notch3, is critical to drive this process and to inhibit alternative lineage differentiation. Thus, our results show that Notch1 activation is the first driver of T cell development in both mouse and human.

The ability of ICN3 to induce T-lineage commitment in the absence of Notch1 activation is in line with recent findings in mice that show that ICN1, ICN2, ICN3 and ICN4 all can induce T cell development when overexpressed in murine hematopoietic progenitor cells (45). While the intracellular regions of Notch1 and Notch2 possess a transactivation domain between the ANK and PEST sequences, Notch3 lacks a conventional version of this domain (46). Nevertheless, the precise role of this transactivation domain is still unclear and earlier work has suggested that this domain is weaker at activating downstream target gene expression in case of Notch3 compared to the conventional TAD in Notch1, and that it even can inhibit Notch1-mediated transactivation (38). Based on their capacity to induce T-lineage specification on OP9-GFP stromal cells in the absence of Notch ligands, our findings suggest that ICN3 on its own is indeed a weaker Notch activator compared to ICN1 since this process is strongly dependent on Notch signal strength (22). While this may seem in contrast with the

gene expression profiles in which ICN3 induces stronger activation of the most sensitive Notch target genes DTX1 and NRARP, both these targets are considered to be negative regulators of Notch activity (47,48), leaving it unclear whether Notch3 truly is weaker at activating downstream target genes, a phenomenon that may also be cell type specific (46). However, in conjunction with Notch1 activation on OP9-DLL4, ICN3 synergizes with ICN1 to induce a stronger Notch activation signal as observed through a more efficient induction of T-lineage specification compared to ICN1 by itself. Given that ICN dimerization can critically influence downstream target gene expression (49), further studies that can specifically study ICN1 homodimers and ICN1-ICN3 heterodimers could be very informative.

Previous work from our lab has shown that a large subset of human cortical thymic epithelial cells express the Notch ligand Jagged2 (18) and that this ligand preferentially binds and activates Notch3 (23). Although cortical epithelial cells are responsible for inducing T cell development in immigrating precursors and despite the fact that the majority of immature $CD34^{+}CD1a^{-}$ thymocytes express Notch3, the results from this manuscript suggest that the Jagged2/Notch3 interaction is not critical during T-lineage specification. Although these findings are in line with data from other species, such as unambiguous data obtained from genetic mouse models (24-26), the fact that Notch3 is expressed at such a high level at these earliest stages of human T cell development, combined with the abundant expression of Jagged2 on cTECs, suggests however a prominent role for this receptor/ligand interaction during early T cell development in human. While we were unable to reveal a role in the T-lineage specification process, one caveat may involve the technical approaches that we used since foetal thymus colonization occurs differently compared to postnatal (50) and thus some caution is required in the interpretation of our FTOC results. Nevertheless, the gene expression analysis that was performed to validate the specificity of the blocking monoclonal antibodies indicates that Notch3 function is highly dependent on Notch1 activity during the

earliest stages of human T cell development, but not vice versa. While this can be explained by the observation that Notch3 is a downstream target of Notch1 in both mouse (39) and human(22), it further confirms that Notch1 triggering is the first critical Notch signaling event that is required to induce T-lineage specification. Since extrathymic progenitor cells express Notch1 and Notch2, but not Notch3, the induction of Notch3 upon Notch1 activation seems to reflect ETPs that have received initial Notch1 signaling events. We now show that, besides Notch1 activation, triggering of the additional expressed Notch3 receptor that is induced through Notch1 activation is not required to complete the T-lineage specification process. Given that DLL4 is a stronger Notch1 activator compared to JAG2 (18), that this ligand is also expressed by human cTECs (18) and that T-lineage specification is dependent on strong Notch activation (22), it seems likely that the DLL4/Notch1 interaction, similar as in the mouse (26-28), is the major Notch signaling event that induces T cell development in immigrating thymic progenitors. As also illustrated in this manuscript, this specification event coincides with the repression of B and myeloid cell development, lineage potentials that are lost during the T-cell specification process that occurs immediately upon thymic entry of multipotent progenitor cells and of which we now reveal that they are Notch3 independent. Further studies will be required to investigate whether Notch3 activation is involved in inducing T-lineage commitment, a process that follows T cell specification and that is characterized by the loss of NK cell potential. In each case, Notch1-induced upregulation of Notch3 following thymus colonization of thymic progenitors does play a critical role later during human T cell development as we have recently illustrated that the Jagged2/Notch3 interaction mediates human TCR-γδ T cell development (23).

Together, while our findings confirm previous work from mice, the results from this manuscript are the first experiments in human to unambiguously reveal that Notch1 is the driving force to initiate T cell development from multipotent hematopoietic precursors since

virtually all previous human studies on hematopoiesis used pan Notch inhibitors, including gamma-secretase inhibitors or the dominant-negative mastermind-like 1 protein. To reveal the specific requirement for Notch1 in the initial stages of human T cell development was important, not only from a fundamental perspective because of the abundant Notch3 expression early during T cell development, but also from a clinical perspective since Notch3 blocking antibodies may have significant therapeutic potential in patients that display Notch3 driven tumors (6,7). Our findings indicate that administration of Notch3 blocking antibodies should have limited impact on early hematopoietic lineage decisions since Notch3 function seems limited to synergizing with Notch1, thereby limiting potential side effects.

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Authorship Contributions

Els Waegemans: performed and designed research, analyzed and interpreted data, wrote the manuscript

Inge Van de Walle: performed and designed research, analyzed and interpreted data

Jelle De Medts: performed research

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Tessa Kerre: provided critical reagents

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Tom Taghon: performed and designed research, analyzed and interpreted data, wrote the manuscript

Disclosure of Conflicts of Interest

The authors have no competing financial interests.

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525

Figure legends

Figure 1

Notch3 is expressed by the majority of human early thymocyte progenitors.

(A) Gating strategy for $CD34^+$ human postnatal thymocytes following CD34 MACS enrichment. (B) Flow cytometric analysis of cell surface Notch3 expression on $CD34^+CD1a^-$ uncommitted and $CD34^+CD1a^+$ committed thymocyte populations as indicated. Data shown is representative for at least 4 independent stainings on 4 different thymus donors.

Figure 2

Notch3 activation induces T cell development.

(A) Control, ICN1 or ICN3 transduced $CD34^+Lin^-$ cord blood progenitors were (B) sorted for EGFP expression and (C-H) subsequently cultured on OP9 stromal cells that express the Notch ligand Delta-like-4 or control OP9 cells that express no Notch ligand, as indicated. Dot plots in (C) and (G) are gated on human $CD45^+EGFP^+$ cells. Numbers in the quadrants indicate the percentage of cells for the corresponding populations after 6 (C) or 10 (G) days of coculture. (E) and (F) show, within human $CD45^+$ gated cells, the frequency of EGFP positive cells following 10 days of coculture on OP9-control or OP9-DLL4, respectively. Dot plots shown are representative for 5 independent experiments. (D) and (H) show the absolute cell numbers for the corresponding populations in panels C (day 6) and G (day 10), respectively. Graphs show the average of 5 independent experiments, error bars indicate SEM (* $p<0.05$).

Figure 3

Notch1 and Notch3 activation induces Notch target gene expression.

549 Quantitative RT-PCR analysis of Notch target genes and genes that are critical for driving non
550 T-cell lineages in human $CD34^+$ cord blood cells, sorted for EGFP expression 2 days after
551 transduction with ICN1, ICN3, or control virus. The expression levels are normalized to β-
552 actin levels. Data shown are the mean of 5 sets of independent samples and error bars show
553 SEM (* $p<0.05$).
554

555 ## Figure 4

556 **Notch3, but not Notch1, is dispensable for induction of human T-lineage specification.**
557 (A) Notch target gene expression analysis in $CD34^+$ thymocytes following 48 hours of
558 coculture on OP9-DLL4 or OP9-JAG2 stromal cells in the presence of control, Notch1 or
559 Notch3 blocking antibodies. mRNA levels are normalized to β-actin levels and shown relative
560 to the control antibody for each culture condition. Data are the mean of two sets of
561 independent samples and error bars show SEM. (B) Flow cytometric analysis of $CD34^+Lin^-$
562 cord blood progenitors after 12 days of coculture on OP9 stromal cells that express the Notch
563 ligand Delta-like-4 and in the presence of control or blocking anti-Notch1 or anti-Notch3
564 antibody. Dot plots are gated on human $CD45^+$ cells and numbers in quadrants indicate the
565 percentage of cells for the corresponding populations. Dot plots shown are representative for
566 6 independent experiments. (C) Absolute cell numbers for the corresponding populations in
567 (B), as indicated. Graphs show the average of 6 independent experiments, error bars indicate
568 SEM (* $p<0.05$).
569

570 ## Figure 5

571 **Notch1 inhibition induces myeloid lineage differentiation.**
572 (A) Flow cytometric analysis of myeloid differentiation from $CD34^+Lin^-$ cord blood
573 progenitors after 2 weeks of coculture on OP9-DLL4 stromal cells in the presence of control

or blocking anti-Notch1 or anti-Notch3 antibody. Dot plots are gated on human $CD45^+$ cells and numbers in the dot plots indicate the percentage of cells for the corresponding populations. Dot plots shown are representative for 6 independent experiments. (B) Absolute cell numbers for the corresponding populations from (A), as indicated. Graphs display the average of 6 independent experiments, error bars indicate SEM (* $p<0.05$).

Figure 6

Intrathymic Notch1 inhibition induces alternative lineage differentiation.

$CD34^+Lin^-$ cord blood progenitors were submitted to FTOC, in the presence of control, anti-Notch1 or anti-Notch3 blocking antibody. (A) Dot plots are gated on human lymphocytes and numbers in dot plots indicate the percentage of cells for the corresponding populations after 2 weeks of FTOC. Dot plots shown are representative for 7 independent experiments. (B) Absolute cell numbers for the corresponding populations from (A), as indicated. Graphs show the average of 7 independent experiments, error bars indicate SEM (* $p<0.05$).

Figure 1

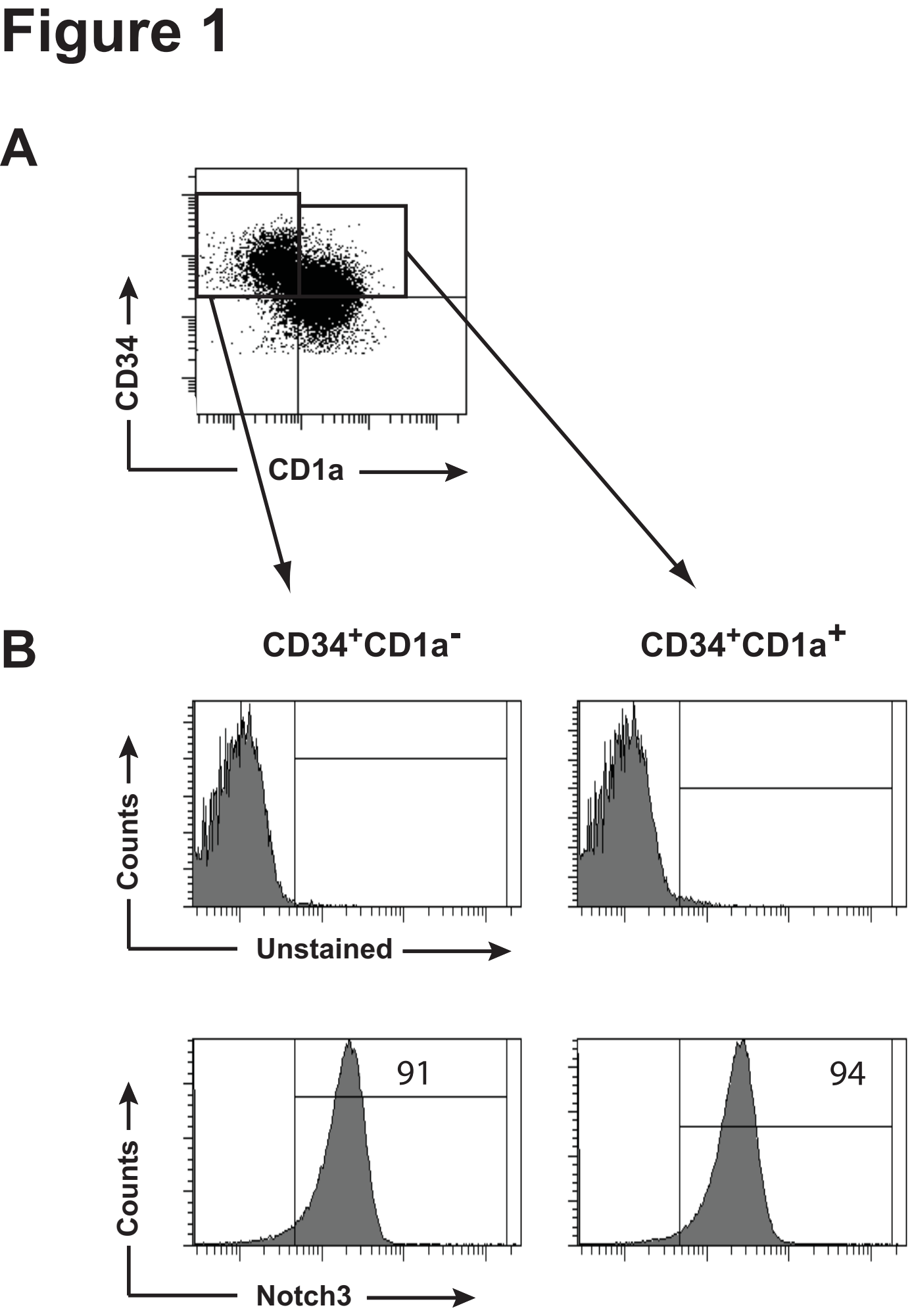

A
CD34
CD1a
B
CD34+CD1a-
CD34+CD1a+
Counts
Unstained
Counts
91
94
Notch3

Figure 2

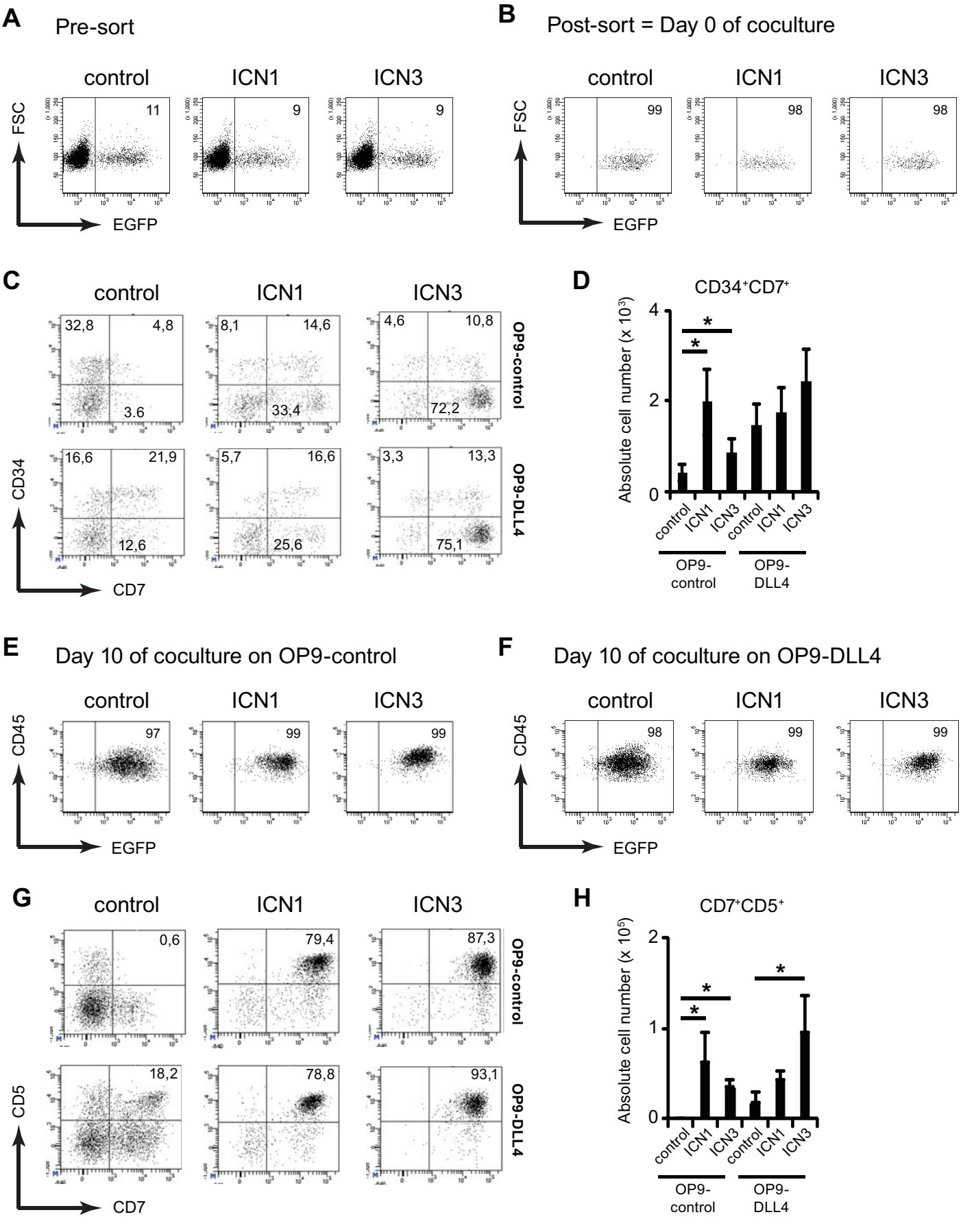

A
Pre-sort
control
ICN1
ICN3
FSC
EGFP
11
9
9
B
Post-sort = Day 0 of coculture
control
ICN1
ICN3
FSC
EGFP
99
98
98
C
control
ICN1
ICN3
CD34
CD7
32,8
4,8
3.6
8,1
14,6
33,4
4,6
10,8
72,2
OP9-control
16,6
21,9
12,6
5,7
16,6
25,6
3,3
13,3
75,1
OP9-DLL4
D
CD34+CD7+
Absolute cell number (x 10³)
4
2
0
*
*
control
ICN1
ICN3
control
ICN1
ICN3
OP9-control
OP9-DLL4
E
Day 10 of coculture on OP9-control
control
ICN1
ICN3
CD45
EGFP
97
99
99
F
Day 10 of coculture on OP9-DLL4
control
ICN1
ICN3
CD45
EGFP
98
99
99
G
control
ICN1
ICN3
CD5
CD7
0,6
79,4
87,3
OP9-control
18,2
78,8
93,1
OP9-DLL4
H
CD7+CD5+
Absolute cell number (x 10⁵)
2
1
0
*
*
*
control
ICN1
ICN3
control
ICN1
ICN3
OP9-control
OP9-DLL4

Figure 3

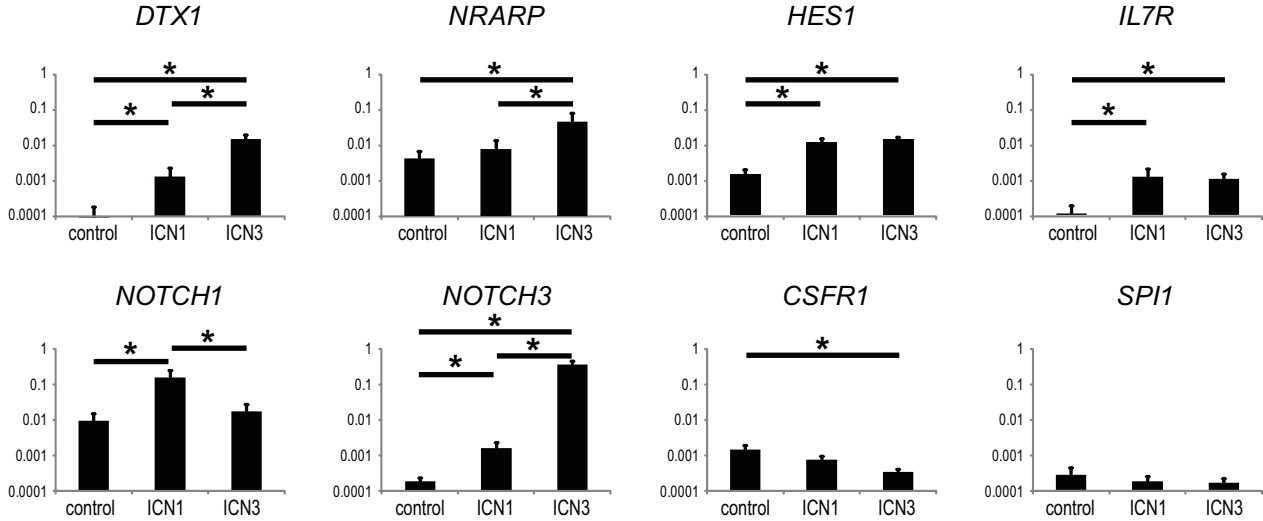

DTX1
NRARP
HES1
IL7R
NOTCH1
NOTCH3
CSFR1
SPI1
1
0.1
0.01
0.001
0.0001
control
ICN1
ICN3
*

Figure 4

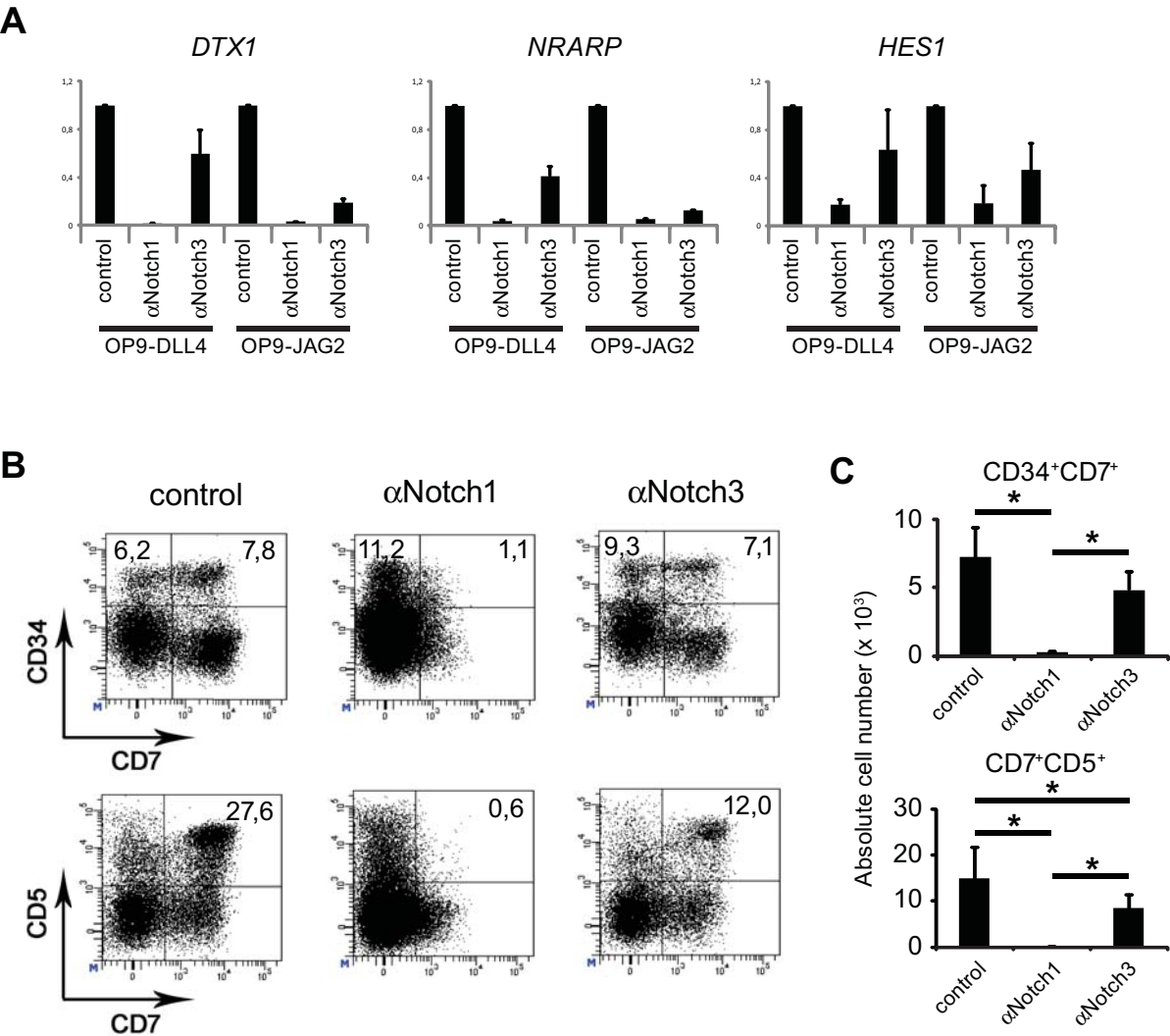

A
DTX1
NRARP
HES1
1,2
0,8
0,4
0
control
αNotch1
αNotch3
OP9-DLL4
OP9-JAG2
B
control
αNotch1
αNotch3
6,2
7,8
11,2
1,1
9,3
7,1
CD34
CD7
27,6
0,6
12,0
CD5
C
CD34+CD7+
CD7+CD5+
Absolute cell number (x 10³)
10
5
0
30
20
*

Figure 5

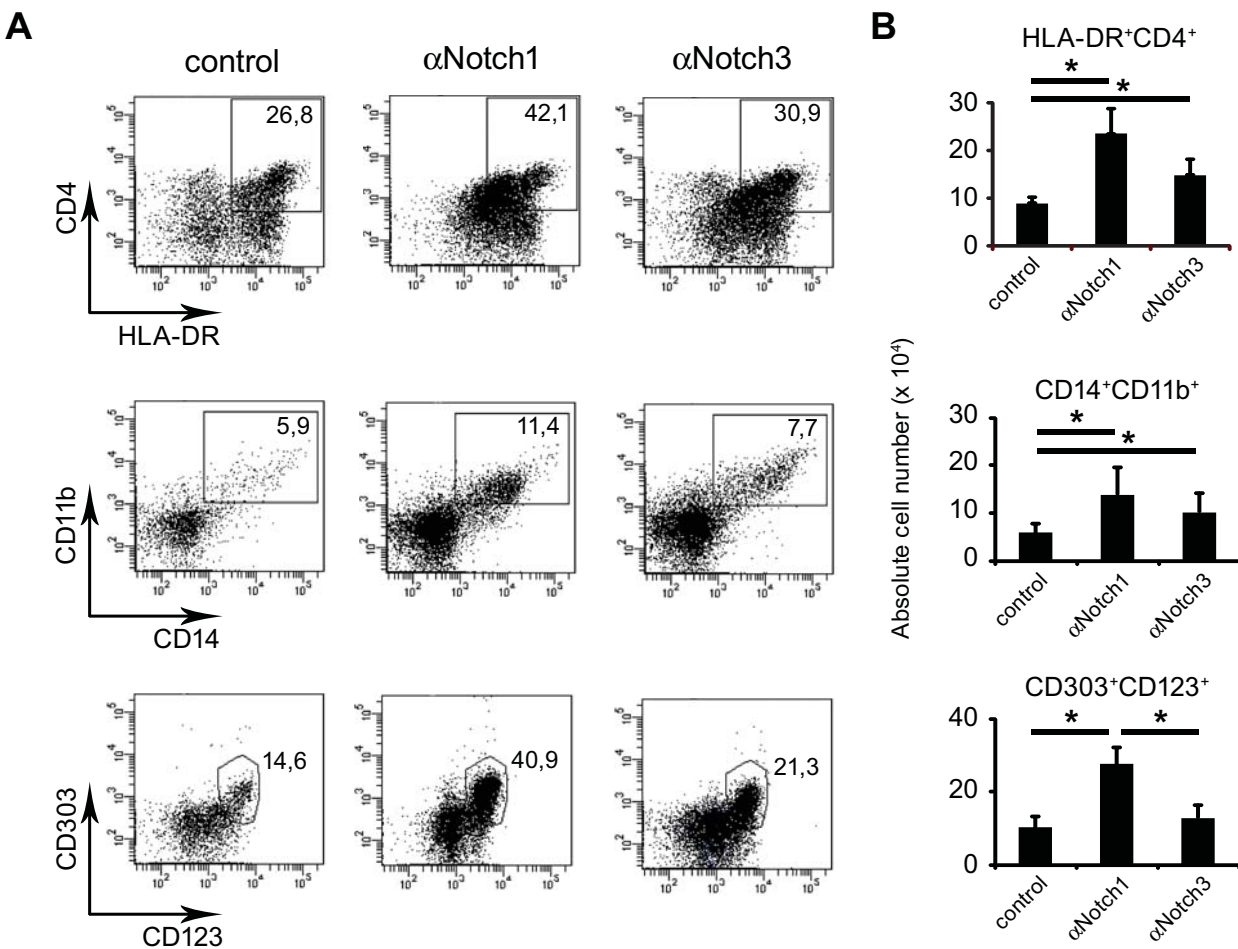
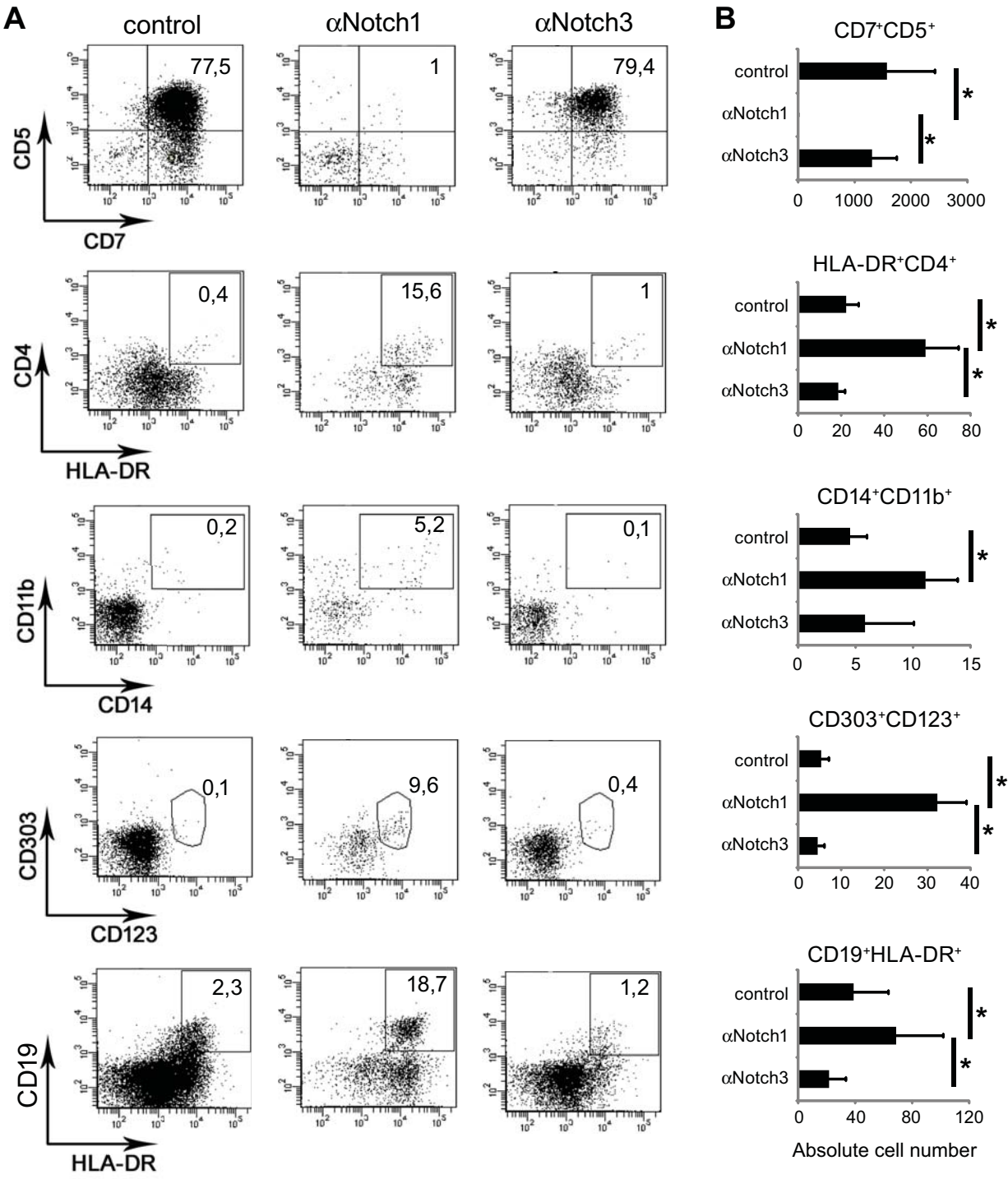

A
control
αNotch1
αNotch3
26,8
42,1
30,9
CD4
HLA-DR
5,9
11,4
7,7
CD11b
CD14
14,6
40,9
21,3
CD303
CD123
B
HLA-DR+CD4+
CD14+CD11b+
CD303+CD123+
Absolute cell number (x 10⁴)
control
αNotch1
αNotch3

Figure 6


A
control
αNotch1
αNotch3
77,5
1
79,4
CD5
CD7
0,4
15,6
1
CD4
HLA-DR
0,2
5,2
0,1
CD11b
CD14
0,1
9,6
0,4
CD303
CD123
2,3
18,7
1,2
CD19
HLA-DR
B
CD7+CD5+
control
αNotch1
αNotch3
0
1000
2000
3000
HLA-DR+CD4+
0
20
40
60
80
CD14+CD11b+
0
5
10
15
CD303+CD123+
0
10
20
30
40
CD19+HLA-DR+
0
40
80
120
Absolute cell number