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Dynamic Regulation of Notch 1 and Notch 2 Surface Expression during T Cell Development and Activation Revealed by Novel Monoclonal Antibodies

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It is well established that Notch signaling plays a critical role at multiple stages of T cell development and activation. However, detailed analysis of the cellular and molecular events associated with Notch signaling in T cells is hampered by the lack of reagents that can unambiguously measure cell surface Notch receptor expression. Using novel rat mAbs directed against the extracellular domains of Notch1 and Notch2, we find that Notch1 is already highly expressed on common lymphoid precursors in the bone marrow and remains at high levels during intrathymic maturation of CD4+CD8+ thymocytes. Notch1 is progressively down-regulated at the CD4+CD8+ and mature CD4+ or CD8+ thymic stages and is expressed at low levels on peripheral T cells. Immunofluorescence staining of thymus cryosections further revealed a localization of Notch1+CD25+ cells adjacent to the thymus capsule. Notch1 was up-regulated on peripheral T cells following activation in vitro with anti-CD3 mAbs or infection in vivo with lymphocytic choriomeningitis virus or Leishmania major. In contrast to Notch1, Notch2 was expressed at intermediate levels on common lymphoid precursors and CD117+ early intrathymic subsets, but disappeared completely at subsequent stages of T cell development. However, transient up-regulation of Notch2 was also observed on peripheral T cells following anti-CD3 stimulation. Collectively our novel mAbs reveal a dynamic regulation of Notch1 and Notch2 surface expression during T cell development and activation. Furthermore they provide an important resource for future analysis of Notch receptors in various tissues including the hematopoietic system. The Journal of Immunology, 2009, 183: 7212–7222.

The highly conserved Notch signaling pathway is instrumental in cell fate determination in various tissues of multiple species. To activate the Notch pathway in mammals, one of four cell surface receptors (Notch 1–4) must bind to one of five ligands (Jagged 1–2, Delta-like 1, 3, 4). Following receptor: ligand binding a series of directed proteolytic cleavages liberates the Notch intracellular domain, which translocates to the nucleus and forms a complex with the transcriptional repressor RBP-J and other co-factors to initiate transcription of lineage- and tissue-specific target genes. This process ultimately influences cell fate by up-regulating genes that promote one specific lineage while repressing the expression of genes that could potentially promote alternative lineages (1–3).

In the hematopoietic system the best-characterized role of Notch signaling is in the specification of T cell fate in the thymus. Notch1 (N1)4 expressed on multipotent T cell progenitors must interact with Delta-like 4 (DL4) expressed on thymic epithelial cells to initiate early stages of intrathymic T cell development and suppress other alternative cell fates (including B cell development) (4–6). N1 is also required at a later stage of intrathymic development to promote rearrangement of the TCRβ-chain and allow formation of a pre-TCR complex that permits further expansion and differentiation of the developing T cells (7).

Despite the overwhelming genetic evidence implicating N1 in T cell development and fate determination many questions remain concerning the precise cellular differentiation pathways and molecular events associated with this process. One reason for this controversy is the lack of reagents that can unambiguously measure cell surface expression of N1 (and other Notch receptors) during hematopoietic development. Historically Notch receptor expression in hematopoietic cells has been largely assessed using RT-PCR or Western blot analysis with polyclonal antisera (3, 8, 9). These techniques are usually only semiquantitative and (most importantly) do not measure Notch receptor expression at the cell surface or at the single cell level. More recently, fusion proteins of Notch ligands (particularly DL4-Fc) have been employed to visualize Notch receptor expression on T cells by flow cytometry (10–12); however, this technique does not allow identification of the specific Notch receptor involved due to the redundancy of Notch

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4 Abbreviations used in this paper: N1, Notch1; DL, Delta-like; LCMV, lymphocytic choriomeningitis virus; BM, bone marrow; DN, double negative; ISP, immature single positive; iIEL, intestinal intraepithelial lymphocytes; DP, double positive; CLP, common lymphoid precursor; K5, keratin 5; DAPI, 4′,6-diamidino-2-phenylindole; EGF, epidermal growth factor-like.

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receptor-ligand binding. To overcome these problems we have generated novel rat mAbs against the extracellular domain of N1 or N2. Here we describe the regulation of expression of N1 and N2 during T cell development and activation.

Materials and Methods

Production of Notch receptor chimeric fusion proteins and mAbs

The murine cDNA encoding the extracellular 12 epidermal growth factor-like (EGF) repeats of N1 (amino acid residues Met1–Glu488) or N2 (amino acid residues Met1–Glu494) were cloned via EcoRI/Sall or HindIII/Sall, respectively, into the P S21 expression vector (13) to generate N1- and N2-huGlo fusion proteins (N1- and N2-Fc). These biologically active fusion proteins were used to produce mAbs in adult rats against the extracellular domains of N1 and N2 as previously described for mAbs generated against DL1 and DL4 (14). After screening by ELISA and then by FACS, three anti-Notch1 hybridomas (22E5.5, 18E11.2, and 1B3.7) and one anti-N2 mAb (16F11) were identified and subcloned.

Full-length N1 and N2 cDNAs were cloned in frame into the pEGFP expression vector (Clontech). 293T cells were transiently transfected with 5 μg of N1- or N2-EGFP expression vectors, respectively, with or without 5 μg of the Lunatic fringe expression vector as previously described (11). Twenty-four hours later cells were directly stained with anti-N1 or anti-N2 biotinylated mAbs and revealed with streptavidin aliphycocyanin (Molecular Probes).

DL1- and DL4-Fc fusion proteins were generated as previously described (11). Mouse β2m and soluble CD1d from the pEAK8 vector (gift from Dr. A. Donda, University of Lausanne, Epalinges, Switzerland) were cloned into the PS S21 expression vector (see above) to generate CD1d-Fc fusion proteins. The corresponding expression vectors were transfected into 293T cells using the calcium-phosphate method, and IgG fusion proteins were subsequently purified over protein A columns according to the manufacturer’s instructions (Hitrap Protein A FF; GE Healthcare). Purity of the fusion proteins was verified by Coomassie blue staining and Western blot analysis using standard methods.

N1- and N2-Fc fusion protein staining and blocking

OP9 stromal cells engineered to express the GFP and the mouse DL1 gene (OP9-DL1) or GFP and the mouse DL4 gene (OP9-DL4) (11) were indirectly stained with different concentrations of N1- or N2-Fc (from 1 ng to 0.5 μg in 50 μl for 0.25 × 10^6 cells). Binding of the fusion proteins to the Notch ligands was performed in HBSS/5% FCS staining buffer and detected using donkey anti-human IgG-PE polyclonal ab (Jackson Immuno-Research). For blocking experiments, 0.25 × 10^6 CTL2-2 or BAF3-3 cells were pre-incubated with 1 μg of DL4-Fc or unconjugated anti-N1 or anti-N2 mAbs, and then stained with anti-N1 and anti-N2 biotinylated mAbs followed by streptavidin-PE (eBioscience). Alternatively, 0.25 × 10^6 CTL2-2 or BAF3-3 were pre-incubated with 1 μg of unconjugated anti-N1 or anti-N2 mAbs, respectively, and then stained with different concentrations of DL4-Fc (1 ng to 1 μg) followed by Donkey anti-human IgG-PE (Jackson ImmunoResearch).

Mice

C57BL/6 males and females (Harlan Olac) were used at E16 or 6–8 wk of age. Mx-cre;Notch1lox/lox (4), CD4-cre;Notch1lox/lox (15), Mx-cre;Notch2lox/lox (11), and P14 TCRtg (16) have all been previously described (12). Thymocyte and spleen suspensions were prepared by pressing organs through a 70-μm mesh before centrifugation. Common lymphoid precursors (CLPs) were identified by gating on lineage negative CD27^− CD135^− CD127^+ BM as described (20). Thymic CD4 Treg cells were identified in FoxP3GFP mice (17) by gating on CD4^+ CD8^- thymocytes, then on the CD25^+ GFP^- subset. Intestinal intraepithelial lymphocytes (IELs) were isolated as described previously (21). Briefly, the small intestine was removed, cleaned, and flushed of fecal content. Peyer’s patches were excised, and the small intestine was opened longitudinally and cut into 1 cm long pieces. Then the specimens were washed twice in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin. The pieces were then stirred at 37°C in prewarmed DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FCS for 30 min, and then supernatants were separated on a 40–70% Percoll density gradient (Amersham Biosciences). The cells that layered between the 40 and 70% fractions were collected as IELs.

mAb conjugates and flow cytometry

The following mAb conjugates were used: TCRγδ (GL3)-FITC, TCRβγ (536)-FITC, TCRβ (H57)-FITC, -PE-Cy5.5, and -allophycocyanin-Alexa 750; CD3 (17A2)-FITC and -PE; CD4 (GK1.5)-FITC, (RM4-5)-PE-Cy5.5, -PE-Cy7, -Alexa 647, and -allophycocyanin-Alexa 750; CD8α (53.6.7)-FITC, -PE-PE-Cy7, and -allophycocyanin-Alexa 750; CD8β (H5)-Alexa 647; CD11b (M170)-FITC, -PE-Cy7, and -allophycocyanin-Alexa 750; CD11c (HL3)-FITC; CD24 (M1/69)-PE-Cy5; CD25 (PC61)-PE and -Alexa 700; CD27 (LG.79)-FITC; CD44 (IM7)-Pacific blue-PE, -Cy7, and -allophycocyanin-Alexa 750; CD45 (50-F11)-PE-Cy5.5; CD45.1 (A20)-PE-Cy5.5; B220 (RA3.6B2)-FITC; –PE-Red, -PE-Cy7, and -allophycocyanin-Alexa 750; CD62L (Mel-14)-FITC; CD117 (B28)-PE-Cy5; -Alexa 700, and -PE-Cy7; CD127 (A7R34)-PE-Cy5; CD135 (A2F10)-PE; CD161 (NK1.1/PK136)-FITC and PerCy-Cy5.5; F4/40-FLT; Gr1 (RB6.8C5)-PE-Cy7 and -allophycocyanin-Alexa 750, Sca-1 (D7)-PE-Cy7; Ter119-PE-Cy7, and -allophycocyanin-Alexa 750. All conjugates were purchased from eBioscience with the exception of CD161 and TCRγδ, which came from BD Biosciences, and all FITC and biotin conjugates were prepared in this laboratory using standard protocols. CD3 and CD8 was prepared using the Prozyme PE conjugation kit (Europa Bioproducts) and Alexa 647 conjugates with the Alexa 647 protein-labeling kit (Molecular Probes). N1- (22E5.5) and N2- (16F11) biotin were revealed with Qdot-605 or allophycocyanin-streptavidin conjugates (Invitrogen) or PE-PE-Cy5.5. All conjugates were prepared using a FACSCalibur, a FACSCan, or a SORP LSRII (BD Biosciences) flow cytometers. Data were analyzed using FlowJo (TreeStar).

Immunofluorescence microscopy

Unfixed thymi were embedded in OCT compound (Sakura), cut, fixed in acetone, and stained as previously described (22). The primary Abs used were rat anti-N1 (clone 22E5.5), isotype-matched rat anti-DL1 (30B11.1) (14), rabbit anti-keratin 5 (K5) (Covance), and biotinylated rat anti-CD25 (PC61). Secondary reagents used were HRP-conjugated donkey anti-rat IgG (Jackson ImmunoResearch), Alexa 488-streptavidin (Molecular Probes) and Alexa 647-conjugated donkey anti-rabbit IgG (Molecular Probes). Sections were first labeled with anti-N1 mAb followed by the anti-rat IgG HRP, then blocked for 1 h using 4% normal rat serum before starting the CD25 and K5 labeling. HRP was visualized using a tyramide-Cy3 signal amplification system. Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using a Zeiss AxioImager Z1 microscope and processed using Adobe Photoshop (brightness and contrast were adjusted similarly). The color of the K5 staining was altered for better visualization.

In vitro activation

Freshly isolated splenocytes were cultured in IMDM supplemented with 10 mM HEPEs, penicillin-streptomycin-neomycin (all obtained from Invitrogen), 50 μM 2-mercaptoethanol (Invitrogen), and 10% FCS (Sigma-Aldrich). Splenocytes (5 × 10^6) C57BL/6 in 3 ml were seeded into 12-well Costar plates and cultured with IL-2 (30 U/ml, EL-4 supernatant produced in our Institute), with immobilized anti-CD3ε mAb (145-2C11) at 5 μg/ml, or with 1 μM GP33 peptide (KAVYNFATC).

Lymphocytic choriomeningitis virus (LCMV) infection

C57BL/6 mice were infected with 200 PFU LCMV-WE strain via retro-orbital injection as previously described (23). Mice were sacrificed 8 days after infection at the peak of the CD8 response. LN cells from infected mice were stained with GP33–41 Db tetramers (produced in our Institute) and cut into 1 cm long pieces. Then the specimens were washed twice in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin. The pieces were then stirred at 37°C in prewarmed DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FCS for 30 min, and then supernatants were separated on a 40–70% Percoll density gradient (Amersham Biosciences). The cells that layered between the 40 and 70% fractions were collected as IELs.
Leishmania major infection and in vitro restimulation of draining LN cells

Leishmania major LV 39 parasites (MRHO/Sv/59/P strain) were maintained in vivo in DBA/2 mice and then grown in vitro. C57BL/6 mice (3 per group) were infected s.c. in the hind footpad with \(3 \times 10^6\) stationary phase promastigotes as previously described (24). Sixty days after infection, draining popliteal LN cells were analyzed directly ex vivo or cultured in 96-well plates, at a final concentration of \(5 \times 10^6\) cells/ml, in the presence of UV-irradiated \(L.\) major stationary phase promastigotes (\(L.\) major: cells ratio 1:5) and analyzed 16 h later by FACS.

Results

Production of rat mAbs against the extracellular domain of mouse N1 and N2

We have recently described the production of rat mAbs against mouse DL1 and DL4, two Notch ligands belonging to the DL family (14). These mAbs were produced by immunization of rats with dimeric fusion proteins containing the extracellular domain of mouse DL1 or DL4 fused to the Fc domain of human IgG1. Presumably the presence of human Fc increased the immunogenicity of the fusion proteins and helped to overcome the high degree of homology (98–99%) between mouse and rat DL proteins.

Using a similar strategy we therefore attempted to produce rat mAbs against the mouse Notch receptors N1 and N2. Given the extremely large size of the extracellular domain of N1 and N2 (containing 36 EGF repeats) we decided to use truncated N1 and N2 molecules containing only the 12 N-terminal EGF repeats that are essential for ligand binding. These truncated N1 and N2 proteins were fused to the Fc domain of human IgG1. Presumably the presence of human Fc increased the immunogenicity of the fusion proteins and helped to overcome the high degree of homology (98–99%) between mouse and rat DL proteins.

We then used these biologically active N1-Fc and N2-Fc fusion proteins as immunogens to produce mAbs against the extracellular domains of N1 and N2. Adult rats were immunized and boosted with N1-Fc and N2-Fc as previously described (14), and immune rat spleen cells were fused with mouse myeloma cells to generate hybridomas producing anti-N1 and anti-N2 mAbs. After a two-step screening procedure (first by ELISA and then by FACS; see Materials and Methods for details) three hybridomas producing anti-N1 mAbs and one hybridoma producing anti-N2 mAbs were identified and subcloned. Since all three anti-N1 mAbs had the same isotype and exhibited complete cross blocking in competition binding experiments (data not shown) only one mAb (clone 22E5.5; rat IgG2a) was selected for further study, together with the single anti-N2 mAb (clone 16F11, rat IgG1). As shown in Fig. 2A, anti-N1 and anti-N2 mAbs bound specifically to human 293T cells transfected with N1-GFP or N2-GFP, respectively. This binding was not affected when Notch-expressing 293T cells were co-transfected with lunatic fringe (Fig. 2A), a glycosyltransferase that modifies Notch receptors and alters ligand binding (11, 25). Moreover the binding of anti-N1 and anti-N2 mAbs to cell lines that exclusively express N1 (CTLL2, Fig. 2B) or N2 (BAF3, Fig. 2C) could not be inhibited by pre-incubation with DL4-Fc. In reciprocal experiments anti-N1 or anti-N2 mAbs were unable to block binding of DL4-Fc to CTLL2 (Fig. 2B) or BAF3 (Fig. 2C) cell lines, even at limiting DL4-Fc concentration. Taken together these experiments establish that our anti-N1 and anti-N2 mAbs bind N1 and N2 independently of lunatic Fringe modification. In addition they indicate that the mAbs bind to N1 and N2 at a site that does not competitively inhibit binding of DL4 ligand.

Expression of N1 and N2 on developing thymocytes

Since N1 (but not N2) is essential for early stages of intrathymic T cell development we examined the expression of both N1 and N2 on immature and mature thymus subsets. Immature thymocytes are CD4–CD8– (DN) and can be divided into 4 stages based on the differential expression of CD44, CD25 and CD117. The most immature DN1 subset (also referred to as early thymic progenitors) expresses CD44 and CD117 but not CD25. DN1 cells differentiate successively through DN2 (CD44+CD25−CD117−), DN3 (CD44+CD25+CD117−) and DN4 (CD44+CD25+CD117+)
stages (26). Subsequently DN4 cells acquire expression of CD8 immature single positive (ISP) and then CD4^+ CD8^- (DP) before undergoing positive and negative selection to become CD4^- CD8^- (CD4 SP) and CD4^- CD8^- (CD8 SP) mature thymocytes. Interestingly, the most immature DN1 and DN2 thymus subsets expressed high levels of N1 and intermediate levels of N2 (Fig. 3A). During subsequent intrathymic differentiation N1 levels remained high until the ISP stage and then decreased progressively through the DP and SP stages (Fig. 3A). In contrast N2 expression was already undetectable at the DN3 stage and remained at background levels thereafter (Fig. 3A).

Analysis of the embryonic thymus (E16) largely confirmed data obtained for the adult, with high levels of N1 expressed on DN1, DN2, DN3, DN4, and ISP subsets (Fig. 3A). Of note, however, was the fact that N2 could not be detected at the most immature (DN1/DN2) stages of E16 thymus development, in contrast to the adult.
FIGURE 3. Expression of Notch receptors during T cell development. A. N1 (solid line), N2 (dotted line), and negative control (shaded histogram) on adult and embryonic (E16) thymocyte subsets from C57BL/6 wild-type or from adult N2-deficient (N2^ΔMx) mice 6 wk post-deletion. DN1, double negative 1 (CD117^+CD25^-CD44^-); DN2 (CD117^+CD25^-CD44^-); DN3 (CD117^-CD25^-CD44^-); DN4 (CD117^-CD25^-CD44^-); ISP, immature single positive (CD4^-CD8^-TCR^β^-); DP, double positive (CD4^+CD8^-). B. N1 (solid line), N2 (dotted line), and negative controls (shaded histograms) on CLPs (common lymphoid precursors, lin-CD27^-CD135^-CD127^-) from littermate controls (LM), N1-deficient (N1^ΔMx) or N2-deficient (N2^ΔMx) bone marrow. C. N1 expression (solid line) and negative control (shaded histogram) on adult thymocyte subsets from N1 deficient (N1^ΔCD4) or littermate controls (LM). D. Co-expression of N1 or N2 with DL4-Fc on CLPs from mice described in B. E. Reverse gating of N1 high thymocytes. Top panel: co-expression of N1 and DL4-Fc (right) and second step alone controls (left) on total thymocytes. Gate shows 2% N1^high. Middle panel: CD8 vs CD4 expression on total thymocytes (left) and 2% N1^high thymocytes (right). Note the relative enrichment of immature DNs and the complete absence of CD4^-CD8^- thymocytes in the N1^high gate. Bottom left panel: surface TCRβ expression on the CD4^-CD8^- gate from total thymus (dotted line) and N1^high thymocytes (solid line). Bottom right panel: Forward scatter (FSC) profiles of the DP gate from total thymus (dotted line) and N1^high thymocytes (solid line). All data shown are representative FACS plots from three to six independent experiments.
Specificity of staining of the anti-N1 and anti-N2 mAbs on CLPs was confirmed in N1/H9004Mx and N2/H9004Mx mice, respectively (Fig. 3B), whereas specificity of the anti-N2 staining on DN1 and DN2 thymocytes was verified in N2/H9004Mx mice (Fig. 3A). Furthermore, N1 staining of thymocyte subsets was undetectable from the DP stage onwards in N1/H9004CD4mice (Fig. 3C), as expected from previous analyses of these mice (15).

**Specificity of Notch:Delta interactions on hematopoietic cells**

Since our anti-N1 and anti-N2 mAbs did not interfere with the binding of DL4-Fc to N1 and N2 in control cell lines (Fig. 2, B and C), we decided to directly investigate the relationship between Notch receptor expression and ligand binding on hematopoietic cells. For this purpose we chose to examine CLPs since this population expresses both N1 and N2. Multicolor staining of gated CLPs in BM with DL4-Fc and either N1 or N2 demonstrated that both the mAbs and the fusion protein could bind simultaneously, as expected from the results obtained with transfected cells. Interestingly however the pattern of double staining was different depending upon the Notch receptor analyzed (Fig. 3D). In particular N1:DL4-Fc staining of CLPs resulted in a predominantly diagonal staining pattern characteristic of 2 reagents binding to the same molecular complex. On the other hand N2:DL4-Fc staining was much more diffuse, with no clear correlation between the 2 reagents among double positive cells. We reasoned that the failure to observe a correlation between N2 and DL4-Fc staining on CLPs was most likely due to the fact that N2 receptors are present at much lower numbers than N1 receptors on these cells (10 fold lower staining with mAbs, Fig. 3B). To test this hypothesis directly we examined N2:DL4-Fc staining on CLPs from N1-deficient BM cells. As shown in Fig. 3D, a clear diagonal staining pattern was observed for N2 and DL4-Fc in the absence of N1. In parallel experiments N1:DL4-Fc staining was even more clearly correlated in N2-deficient CLPs. As expected N1 and N2 staining disappeared in N1/Ac38 and N2/Ac38 CLPs, respectively (Fig. 3D). Taken together these data indicate that double staining for Notch receptors and DL4-Fc fusion proteins can provide additional information regarding the hierarchy of expression of Notch receptors on a given cell subset.

**Identification of brightly staining N1high thymocyte subsets by “reverse gating”**

Taking advantage of the increased sensitivity achieved by using a combination of N1 and DL4-Fc staining (Fig. 3D) we attempted to directly identify the brightest N1-expressing thymocytes via a “reverse gating” strategy. In particular we stained total thymocytes with N1 and DL4-Fc and gated on the brightest 2% of thymocytes in the diagonal profile. As shown in Fig. 3E, the N1high gate was highly enriched for DN and ISP (CD4+CD8−TCRβ−) subsets as expected and did not contain any detectable mature CD4+CD8−TCRβ+ or CD4+CD8−TCRβ+ thymocytes. Interestingly however a significant fraction (~40%) of N1high thymocytes was concentrated within the outer cortical area of the thymus and include CD25+ DN2/3 thymocytes.
were DP. Further analysis of forward scatter among N1\textsuperscript{high} DP thymocytes clearly demonstrated that they were all large blasts, which normally comprise only a small fraction (~5\%) of the DP subset (Fig. 3E). These reverse gating data thus reveal that a very minor subset of large (presumably rapidly cycling) DP thymocytes continue to express high levels of N1. These N1\textsuperscript{high} DP cells are most likely the immediate progeny of ISP thymocytes, which are also N1\textsuperscript{high} and have similar forward scatter properties (data not shown). A similar reverse gating strategy for N2 was not possible due to the low frequency and weak staining intensity of N2\textsuperscript{+} thymocytes.

**N1 Expression on thymus cryo-sections**

Since N1 was preferentially expressed in immature thymus subsets we examined the localization of N1-expressing cells on frozen sections in both the embryonic and adult thymus. As shown in Fig. 4A, N1 was expressed relatively homogeneously and at high levels in the E16 thymus, as expected from the fact that most thymocytes are DN or ISP at this stage. Nevertheless counterstaining with Keratin 5 (K5) (which detects small pockets of embryonic epithelial cells destined to develop into medulla) revealed much lower N1 staining in the K5\textsuperscript{+} regions of E16 thymus. In contrast to the embryo N1 staining in the neonatal and adult thymus was less homogeneous and largely confined to the cortical region, with a clear enrichment of N1-expressing cells in the outer cortex adjacent to the capsule. Double staining for CD25 and N1 confirmed that most CD25\textsuperscript{−} (DN2/DN3) thymocytes co-expressed N1 in both the embryonic and adult thymus (Fig. 4B). Interestingly a large fraction of brightly staining N1\textsuperscript{+} thymocytes in the subcapsular region of the adult thymus did not co-express CD25 (Fig. 4C). Taken together with the ‘reverse gating’ results for N1 (Fig. 3E) these data strongly suggest that DN4, ISP and early DP stages preferentially localize to this zone.

**N1 and N2 up-regulation during peripheral T cell activation**

In addition to its well-defined role in T cell development Notch signaling has recently been implicated in peripheral T cell activation and in the acquisition of functional activities by activated peripheral T cells (reviewed in (27, 28)). We therefore examined N1 and N2 expression in peripheral T cells before and after activation by various stimuli. In general the expression of N1 was lower on CD4\textsuperscript{+} and CD8\textsuperscript{+} splenic T cells than on mature thymic CD4 SP and CD8 SP subsets while N2 expression remained undetectable (Figs. 5B, 3A, respectively). Following in vitro activation by immobilized anti-CD3 mAbs N1 was rapidly up-regulated (within 4 h) on both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and remained at high levels for at least 48 h (Fig. 5A). In contrast N2 up-regulation was not detectable until 24 h and decreased already by 48 h. Up-regulation of both N1 and N2 appeared to depend upon TCR triggering, since it could also be induced by stimulation of P14 TCR transgenic CD8\textsuperscript{+} T cells with the cognate GP33 peptide in vitro (Fig. 5A), although in this instance both N1 and N2 expression decreased slightly between 24 and 48 h. Furthermore addition of IL-2 alone as a survival factor only slightly increased N1 or N2 expression (Fig. 5A).

To evaluate a possible regulation of N1 or N2 expression on peripheral T cells under more physiological conditions we infected mice with LCMV and analyzed N1 and N2 expression directly ex vivo on peripheral T cells at the peak of the CD8 response (day 8). As shown in Fig. 5B, N1 (but not N2) was clearly up-regulated on activated (CD44\textsuperscript{high}) but not naive (CD44\textsuperscript{low}) CD8\textsuperscript{+} T cells following LCMV infection. Direct gating of LCMV-specific CD8\textsuperscript{+} T cells using GP33-Db tetramers confirmed that N1 was up-regulated as a result of a specific immune response (Fig. 5B). These results were compared with another well-established infection model using the intracellular parasite *Leishmania major*. In this system CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells isolated directly ex vivo in draining LNs from mice infected 60 days before with *L. major* exhibited only a very slight increase in N1 expression (Fig. 5C). However a much stronger increase in N1 (but not N2) could be seen following in vitro overnight stimulation of CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells with UV-irradiated *L. major* (Fig. 5C), again confirming the Ag specificity of N1 up-regulation.

In contrast to the thymus (Fig. 3E) reverse gating of N1\textsuperscript{high} peripheral T cells was not feasible since only rare activated T cells from immunized mice expressed low levels of N1.

**N1 and N2 expression by unconventional T cells**

Finally we also examined expression of N1 and N2 on several subsets of unconventional T cells. In the adult thymus TCRγδ cells (Fig. 6A) as well as CD1d-restricted NKT cells at various developmental stages (Fig. 6B) and regulatory T cells (Fig. 6C) all expressed detectable levels of N1 but not N2. In the intestinal epithelium all T cell subsets examined (including TCRγδ cells as well as CD8αβ, CD4, and CD8αε subsets of TCRαβ cells) expressed very low levels of N1 and undetectable N2 (Fig. 6D). Finally Vγ3\textsuperscript{+} cells in E16 thymus (which are precursors of dendritic epidermal T cells in the skin) as well as other fetal Vγ3\textsuperscript{−} γδT cells expressed intermediate levels of N1 and very low (but detectable) levels of N2 (Fig. 6A). Thus in general expression of N1 and N2 by unconventional T cell subsets was similar to that observed for conventional T cells present in the same tissue.

**Discussion**

In this report we have analyzed cell surface Notch receptor expression on T cells during development and activation using novel rat mAbs directed against N1 and N2. It is clear from conditional loss-of-function studies that N1 is critical for intrathymic T cell development at the earliest stage, whereas N2 is totally dispensable (4, 11, 29). Our results indicate that N1 is already highly expressed in CLPs (the putatively most proximal BM precursors of thymic DN1 cells (20)) and remains expressed at high levels during early intrathymic development (DN1–4) up to the ISP (or even early DP) stage. Subsequently N1 is progressively down-regulated in DP and SP thymocyte subsets and expressed at relatively low levels on peripheral CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. In general our results confirm and extend two previous studies conducted with polyclonal rabbit antisera directed against N1 (30, 31). Both of these studies concluded that the DN thymocyte subset expressed higher levels of N1 compared with more mature DP and SP thymocytes. In one study (31) it was further claimed that N1 expression was very low in the DN1 subset and up-regulated in DN2, in contrast to our results (Fig. 3A). It is noteworthy however that DN1 cells were not further gated as CD117\textsuperscript{+} in this earlier study (31) and thus probably contained a majority of contaminating thymic CD44\textsuperscript{+} cells unrelated to the T cell lineage. In other related reports, strong binding of DL4-Fc to CLPs and to DN thymus subsets was observed (10, 11), consistent with our finding that these populations preferentially express N1.

The surface expression pattern of N1 in immature thymocyte subsets is of considerable interest in view of two recent reports indicating a negative regulatory role for pre-TCR signaling in N1 transcription (32, 33). Using quantitative RT-PCR, both of these studies demonstrate that N1 mRNA expression peaks at the DN3 (or DN3a) stage and decreases dramatically thereafter, a finding that we ourselves have confirmed (unpublished data). Surprisingly however, levels of surface N1 remain at equally high levels from the DN3 stage until the ISP stage and only decrease subsequently
in DP thymocytes. Maintenance of N1 surface expression between the DN3 and ISP stages cannot be explained by a long half-life of the N1 protein, since it is estimated that thymocytes undergo 8–10 rounds of cell division during this period. Moreover, N1 expressed by DN4 and ISP subsets is potentially functional since it binds similar levels of DL4-Fc as compared with the DN3 stage (11).

Collectively, these findings suggest that the decreases in N1 transcription and N1 target gene expression observed subsequent to the pre-TCR checkpoint in thymus development (32, 33) may occur independently of any regulation of N1 surface expression or ligand

**FIGURE 5.** Notch expression on resting and activated mature T cells. *A*, Left panel, N1 or N2 expression on CD4⁺ or CD8⁺ single positive splenocytes from C57BL/6 wild-type mice after 4, 24, or 48 h in culture with IL-2 (dotted line), anti-CD3 on plastic (solid line), or medium alone as a negative control (shaded histogram). Right panel, N1 or N2 expression on CD8⁺ splenocytes from P14 tg mice (see Materials and Methods) after 4, 24, or 48 h culture with IL-2 (dotted line), gp33 peptide (solid line) or medium alone as a negative control (shaded histogram). *B*, N1 (solid lines) and N2 (dotted lines) expression on naive (CD44low, CD62Lhigh) or activated (CD44high, CD62Llow) LN cells from non-infected (left) or LCMV-infected (right) C57BL/6 mice. *C*, N1 (solid lines) or N2 (dotted lines) expression on CD4⁺ (top panels) or CD8⁺ (bottom panels) lymphocytes obtained from draining LNs of C57BL/6 mice 60 days after in vivo infection with L. major (see Materials and Methods). Cells were analyzed directly ex vivo or after overnight (16 h) stimulation with UV-irradiated L. major. Data shown are representative of one to three experiments in *A*, 3 mice in *B*, and 3–12 mice in *C.*
binding capacity. Additional experiments will be required to clarify this important issue.

In contrast to N1 we find that N2 is expressed at intermediate levels on CLPs and on the most immature DN1 and DN2 thymic subsets. Subsequently, N2 expression is lost at the DN3 stage and remains undetectable during further thymic differentiation and on mature peripheral T cells. These results significantly extend those of an earlier study (9) where N2/H11001 cells were detected at a very low frequency in DN and CD8/H11001 CD4/H11002 thymocyte subsets using a polyclonal rabbit Ab.

The expression pattern of N2 in BM and thymus is interesting for several reasons. First the similarity between levels of N2 (as well as N1) in CLPs and early intrathymic precursors (DN1 and DN2) is further evidence in favor of CLPs as being the most proximal BM precursors of the T cell lineage (20). Second the expression of significant levels of N2 on CLPs and other BM precursor populations including HSCs (data not shown) provides a simple explanation for our previous observation (11) that N1-deficient BM precursors could give rise to immature T cell populations both in vitro (on OP9-DL1 stromal cells) and in vivo (in the spleen after short-term transplantation). Moreover, the expression of N2 on DN1 and DN2 (but not DN3) thymic subsets is consistent with the observation that N1-deficient (presumably N2-dependent) T cell development on OP9-DL1 stromal cells is arrested at the CD25/H11001 stage in the absence of significant TCR/H9252 expression (11). Importantly however N1-deficient BM precursors were unable to differentiate into CD25/H11001 immature T cells in vitro on OP9-DL4 stromal cells (and possibly in vivo in the spleen after short-term BM transplantation, although the ligand in this case remains to be determined). These results are particularly surprising in view of the fact that N2-Fc interacts efficiently with both OP9-DL1 and OP9-DL4 stromal cells as assessed by direct in vitro binding, and conversely, both DL4-Fc and DL1-Fc bind similarly to N2-expressing cells such as BAF3 (unpublished data). The reason(s) for the failure of N2:DL4 interactions to induce T cell development in vitro or in vivo remains obscure. In this regard it should be noted that N1:DL1 interactions, which cannot be detected via binding of the relevant fusion proteins, efficiently induce T cell development in vitro in the OP9 system (34). A minimal conclusion of these studies is that the relative avidity of Notch receptor:ligand interactions (at least as assessed by binding of dimeric fusion proteins) does not necessarily correlate with Notch signaling via the same receptor:ligand pairs either in vitro or in vivo.

Immunofluorescent staining of frozen thymus sections with anti-N1 mAbs provided additional information concerning the localization of cells expressing high levels of N1. In the embryonic thymus (E16) N1 was expressed quite homogeneously, as would be expected since most thymocytes at this stage are immature cells (DN or ISP) that express high levels of N1 as assessed by flow microfluorometry. Nevertheless counterstaining with K5 revealed that N1 expression was less prominent in small K5/H11001 regions of the E16 thymus that are destined to

**FIGURE 6.** Notch receptor expression on unconventional T cell subsets from C57BL/6 mice. A, N1 (solid line), N2 (dotted line), and negative control (shaded histogram), on adult and E16 embryonic thymocyte γδ T cell subsets. B, N1 (solid line), N2 (dotted line) or negative control (shaded histogram) on thymic NK T subsets (all CD1d dimer "TCRβ") at various developmental stages. ST1 (CD44/H11001 NK1.1/H11001), ST2 (CD44/H9252 NK1.1/H11001), ST3 (CD44/H11001 NK1.1/H11001). C, N1 (solid line), N2 (dotted line) or negative control (shaded histogram) on thymic regulatory T cells (Treg) from Foxp3-GFP mice. D, N1 (solid line), N2 (dotted line), or negative control (shaded histogram) on intestinal intraepithelial lymphocyte (iIEL) subsets. Data shown are representative of three independent experiments for each panel.
we observed only very modest up-regulation of N1 on CD4 receptor involved was not directly addressed. Increased amounts of DL4-Fc (12) but the identity of the Notch Previous studies have demonstrated that activated T cells bind in-
creased at 24h and returns to lower levels at 48h. In contrast N2 subsets and remains at high levels for at least 48h. In contrast N2 is transiently up-regulated at 24h and returns to lower levels at 48h. Previous studies have demonstrated that activated T cells bind increased amounts of DL4-Fc (12) but the identity of the Notch receptor involved was not directly addressed.

In contrast to primary activation with anti-CD3 or Ag in vitro we observed only very modest up-regulation of N1 on CD4+ or CD8+ T cells isolated directly ex vivo following infection with L. major or LCMV. Nevertheless re-stimulation of T cells from L. major infected animals with cognate Ag for 16h in vitro induced strong up-regulation of N1 on both CD4+ and CD8+ subsets, confirming that N1 expression can be regulated by TCR signaling. Finally it should be noted that hamster mAbs directed against mouse N1 and N2 have recently been described by another group (36). Although thymus and BM were not examined in this other study it was reported that splenic T cells expressed low levels of N1, in agreement with our results. Surprisingly however N2 expression appeared to be higher than N1 on splenic T cells using this hamster mAb, whereas we found no detectable N2 on peripheral naive T cells. There are several possible explanations for this apparent discrepancy. First, it is very likely that the hamster anti-N2 mAb described recognizes a different epitope than our rat anti-N2 mAb, since the hamster mAb blocks binding of N2-Fc to DL1-transfected CHO cells whereas our rat mAb fails to block binding of N2-Fc to OIP9 cells transfected with either DL1 or DL4. Second, it is known that Notch receptors can be modified by glycosylation mediated by the Fringe family of glycosyltransferases, a process that can alter binding of N1 and N2 to DL or Jagged ligands (25). In this context we have confirmed that our rat anti-N1 and anti-N2 mAbs bind equally well to human 293T cells transfected with N1 or N2 in the presence or absence of Lunatic Fringe, a member of the Fringe family that influences development of T cells and marginal zone B cells (25, 37). Thus it appears that our mAbs do not detect epitopes on N1 or N2 that are modified by Fringe glycosylation. Whether the presence of high levels of Fringe may influence the binding or other anti-Notch mAbs (such as the hamster anti-N2 described in (36)) remains to be determined. Whatever the explanation for this discrepancy the availability of novel mAbs capable of measuring surface expression of Notch receptors should facilitate future studies of the development and activation of cells in numerous tissues including the lymphoid system.

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