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Involvement of Notch in Activation and Effector Functions of γδ T cells

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Notch signaling plays a pivotal role in cell fate decision and lineage commitment of lymphocytes. Although the role of Notch in CD4+ and CD8+ αβ T cells is well documented, there are no reports on how Notch signaling regulates effector functions of γδ T cells. γδ T cells are a minor fraction in the peripheral blood but are known to play a major role in defense against pathogens and tumors. In this study, we show that Notch receptors (mRNA and protein) are expressed in peripheral γδ T cells. Inhibition of Notch signaling by γ-secretase inhibitor inhibited the proliferation and IFN-γ secretion of γδ T cells in response to stimulation with phosphoantigens and anti-CD3 mAb. In the presence of γ-secretase inhibitor, the antitumor cytolytic ability of γδ T cells was inhibited with a decreased CD107a expression. Knockdown of Notch1 and Notch2 genes in γδ T cells using small interfering RNA inhibited their antitumor cytotoxic potential. Our study describes for the first time, to our knowledge, the role of Notch as an additional signal contributing to Ag-specific effector functions of γδ T cells. The Journal of Immunology, 2014, 192: 000–000.

C ompared with αβ T cells (>90%), γδ T cells are a minor fraction of T lymphocytes in the peripheral blood (<10%). γδ T cells differ from classical αβ T cells with respect to Ag recognition, tissue localization, and the use of TCR gene repertoire (1). Vγ9Vα2 represents the dominant subset of the peripheral blood in humans (2). γδ T lymphocytes play a major role in defense against pathogens and tumors (3–5). γδ T lymphocytes are activated by phosphoantigens-isopentyl pyrophosphate (IPP) or 4-hydroxy-3-methyl-but-2-enyl pyrophosphate, which is produced through the mevalonate pathway in mammalian cells or nonmevalonate/rohmer pathway in nonmammalian cells, respectively (6). Aminobisphosphonates are synthetic analogs of inorganic pyrophosphates and are widely used in the treatment of skeletal disorders (7). Nitrogen-containing bisphosphonates such as risedronate and zoledronate inhibit farnesyl pyrophosphate synthase, a key enzyme of the mevalonate pathway leading to accumulation of IPP pool in the cells (8). Upon activation, γδ T cells release copious amounts of IFN-γ and TNF-α (2, 5, 9). Earlier data from our own laboratory and others have shown that γδ T cells isolated from cancer patients can mediate potent antitumor immunity (10–12). Tumor cells treated with bisphosphonate zoledronate are actively lysed by activated γδ T cells (13, 14).

The Notch signaling pathway, originally described in drosophila, controls the development and activation of a variety of immune cells (15). Notch signaling is suggested to play a role in cell fate decisions and has been implicated in γδ versus αβ lineage decisions (16, 17). The molecular events triggering T cell development (γδ versus αβ lineage) are essentially different in human and mice. In mice, it was reported that the development of γδ T cells from γδ TCR-expressing T cell progenitors requires the absence of Notch ligand interaction (16–18). In contrast, there is an opposing role for Notch signal in human αβ/γδ lineage decision. The induction of γδ-lineage precursors to split off from the αβ T cell program by Notch1 activity was observed in humans (19). It was also reported that high level of Notch activation generates T lineage precursors and γδ T cells but inhibits differentiation toward αβ lineage (20).

The role of Notch in regulating effector functions of CD8+ T cells and NK cells have been described (21, 22). Signaling through the TCR in both CD4+ and CD8+ T cells induces the activation of Notch1 (22, 23). However, the role of Notch in regulating the effector functions of human γδ T cells has not been reported earlier.

Notch proteins are single-pass transmembrane receptors that require multiple enzymatic cleavages to produce the full-length heterodimer expressed on the cell surface. In mammals, there are four Notch receptors (Notch1-4) and five Notch ligands, three delta-like (Dil1, Dil3, and Dil4) and two Jagged (Jag1 and Jag2) (24). The interaction between Notch receptor and ligand pair cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and γ-secretase, respectively (25). The γ-secretase–induced cleavage generates Notch intracellular domain (NICD), which translocates to the nucleus. In the nucleus, NICD binds to cofactors including CBF-1/suppressor of hairless/Lag1, mastermind like1, and p300/CBP to create a complex that acts as a transcriptional coactivator. Notch signaling then induces the expression of target genes, for example, HES1 (hair and enhancer of split1), HES-related repressor protein, and so on (26).

In this report, we describe the expression of Notch receptors on human γδ T cells in the peripheral blood of healthy individuals and further demonstrate the importance of Notch pathway in Ag-specific responses of γδ T cells. Our data demonstrate that Notch pathway also regulates the cytotoxic effector functions of γδ T cells against tumor cells. These results suggest that Notch signaling can be viewed as an additional mechanism regulating antitumor effector functions of γδ T cells.

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Abbreviations used in this article: BrHPP, bromohydrin pyrophosphate; Dil, delta-like; GSI-X, γ-secretase inhibitor-X; HES1, hairy and enhancer of split-1; IPP, isopentyl pyrophosphate; Jag, Jagged; LAMP1, lysosome-associated membrane protein-1; NICD, Notch intracellular domain; NICD, Notch1 intracellular domain; PI, propidium iodide; siRNA, small interfering RNA.

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Materials and Methods

γδ T cell expansion and purification

Blood samples were collected from healthy individuals. The study was approved by the institutional Ethics Committee, and written informed consent was obtained from the donor before collection of blood samples. PBMCs were isolated by differential density gradient centrifugation (Ficoll Hypaque; Sigma-Aldrich, St. Louis, MO), and γδ T cells were enriched from peripheral blood using plate-bound anti-CD3 (OKT3) mAb and rIL-2 (Peprotech, Rocky Hill, NJ). In brief, lymphocytes were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum plus rIL-2 (100 U/ml), 2 mM glutamine, and antibiotics. Five milliliters of cell suspension (1 × 10^6/ml) was added to 25-cm² culture flasks (Nunc, Roskilde, Denmark) precoated with 1 mg/ml anti-CD3 mAb, detection system (Amersham Pharmacia). Ab at a dilution of 1:1000 and developed with ECL plus Western blot spectrophotometrically. Immunostaining was performed using appropriate secondary 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue), vortexed to conduct according to the manufacturer’s instructions. The purity of separated cells was >95% as determined by flow cytometry (BD Biosciences, San Jose, CA).

Quantitative RT-PCR

RNA was extracted from immunomagnetically purified γδ T cells using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY) in agreement with the manufacturer’s instructions. Quantitative RT-PCR for different Notch receptor isoforms, ligands, and its target genes was performed with PRISM 7700 (PE Applied Biosystems, Foster City, CA). Samples were analyzed using TaqMan primer sets purchased from Applied Bio-systems (NOTCH1 Hs01061201_m1, NOTCH2 Hs01057019_m1, NOTCH3 Hs00112851_m1, NOTCH4 Hs00270200_m1, DLL1 Hs01094599_m1, DLL3 Hs00213561_m1, DLL4 Hs00718902_m1, JAG1 Hs01070036_m1, JAG2 Hs00171432_m1, HES1 Hs01072878_m1, NF-kB Hs00765730_m1, ACTB [β-actin] Hs99999903_m1). All values were normalized to the expression of the housekeeping gene β-actin.

Western blotting

A total of 1 × 10^6 γδ T cells was incubated with rIL-2 (100 U/ml; Peprotech) and bromohydry pyrophosphate (BrHPP/IPP101), which was kindly provided by Innate Pharma (Marseille, France) at a concentration of 200 nM for 24 h. These cells were pretreated for 30 min at 37°C with γ-secretase inhibitor-X, L-685,438 (GSI-X) (Calbiochem, La Jolla, CA) at a concentration of 15 μM, or left untreated, before stimulation. The expression of Notch1 intracellular domain (N1ICD) and c-Myc were analyzed by Western blot analysis. Whole-cell lysates (1 mg/well) were prepared in SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue), vortexed to reduce sample viscosity, denatured by boiling, and then cooled on ice. Samples were resolved on 8% SDS-PAGE gels, transferred onto Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The primary Abs to N1ICD (R&D Systems, Minneapolis, MN), c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma-Aldrich) as loading control were added at 1:5000, 1:1000 and 1:1000 dilution, respectively. Immunostaining was performed using appropriate secondary Ab at a dilution of 1:1000 and developed with ECL plus Western blot detection system (Amersham Pharmacia).

Flow cytometry

Purified γδ T cells were rested overnight at 37°C. Next day, these cells were rinsed in cold PBS and fixed in 1% paraformaldehyde in PBS for 10 min at 4°C. The cells were washed and permeabilized for 15 min with 0.1% BSA in PBS. Cells were stained with allophycocyanin labeled mouse anti-human γδ TCR Ab (BD Bioscience, San Diego, CA), sheep anti-human N1ICD Ab, or goat anti-human Notch2 intracellular domain Ab (R&D Systems) for 45 min at 4°C. Thereafter, cells were washed and incubated with FITC-labeled donkey anti-sheep IgG or FITC-labeled rabbit anti-goat IgG, respectively, for another 45 min at 4°C. For cell-surface markers, nonpermeabilized cells were stained with labeled Abs in PBS DLS Hs00184092_m1, CD19-PE, CD3-PECy7, and CD56-FITC (BD Biosciences, San Diego, CA). γδ T cells were also stained with rabbit anti-human Dll1 and Jag1 ligands (Calbiochem) for 45 min at 4°C. Thereafter, cells were washed and incubated with FITC-labeled goat anti-rabbit IgG (Sigma Aldrich) for another 45 min at 4°C. Appropriate isotype controls were used. The Annexin propidium iodide (PI) staining was performed to determine the effect of GSI-X on the cell viability. In brief, γδ T cells were left untreated or were stimulated with rIL-2 alone or rIL-2 and BrHPP for 48 h at 37°C in round-bottomed, 96-well plates (Nunc). GSI-X was added as described earlier. Cells were then harvested, suspended in binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.25 mM CaCl2), and incubated with PI and FITC-conjugated Annexin V (BD Biosciences) in dark for 15 min at room temperature. After incubation, 400 μl binding buffer was added and cells were analyzed. For analyzing cell-surface expression of activation markers, γδ T cells were left untreated or treated with rIL-2 and BrHPP for 24 h with GSI-X or left untreated as previously described. Cells were then incubated with FITC-conjugated CD69 or PE-conjugated CD25 (BD Biosciences) for 45 min in dark and subsequently washed with FACS buffer (0.01 M PBS pH 7.4, 1% FCS, 0.01% sodium azide), fixed with 1% paraformaldehyde, and the intensity of fluorescence was measured using flow cytometer (FACSAria; BD Biosciences).

For degranulation assay, purified γδ T cells were incubated alone or with rIL-2 (0.1 U; Peprotech) overnight at 37°C in round-bottom, 96-well plates (Nunc) and were taken as effector cells. The target cells were oral cancer cell line, AW13516 (28), and were treated for 18 h with zoladecol (100 μM; Panacea Biotech, New Delhi, India). Cells were cocultured at an E/T ratio of 4:1 in the presence of monensin (5 μg/ml; Sigma-Aldrich). Anti-CD107a-PE Ab (BD Biosciences) was added at the start of coculture assay. After 4 h, cells were washed and γδ T cells were then stained using anti-human TCR-γδ FITC Ab (BD Biosciences), and were acquired and analyzed on a flow cytometer for the expression of CD107a on γδ T cells. For these experiments, γδ T cells were gated on CD3-γδ T cells. The area of forward and side scatter characteristics and the fluorescence intensity was measured. Cells were analyzed using FlowJo software (Tree Star, Ashland, OR).

Proliferation assays and cytokine ELISA

Proliferation of γδ T cells was assayed by [3H]thymidine uptake assay. A total of 5 × 10³ γδ T cells was pretreated for 30 min at 37°C with GSI-X (Calbiochem, La Jolla, CA) at different concentration ranging from 2.5 to 15 μM, or left untreated, before cells were stimulated in round-bottom, 96-well tissue culture plates with rIL-2 (0.1 U/ml; Peprotech) and plate-bound anti-CD3 mAb (1 μg/well; BD Biosciences) for 72 h. Similarly, γδ T cells were also incubated in round-bottom, 96-well tissue culture plates with rIL-2 (0.1 U/ml; Peprotech) plus BrHPP (200 nM; Innate Pharma) or IPP (40 μM; Sigma-Aldrich) or 20 nM c-HDMAPP (IPH1201/picostim; Innate Pharma) for 72 h. For experiments using GSI-X, cells were pretreated with 15 μM GSI-X. The cultures were pulsed with 1 μl [3H]thymidine (Board of Radiation and Isotope Technology, Mumbai, India) during the last 18 h of the assay. The radioactivity incorporated in the DNA was measured in a liquid beta scintillation counter (Packard, Meriden, CT).

For cytokine ELISA, γδ T cells were stimulated with anti-CD3 mAb or without GSI-X as described earlier. Likewise, γδ T cells were treated with different phosphoantigens (IPP, BrHPP, and c-HDMAPP). After 24 h, supernatants were collected, and IFN-γ concentration was assayed with an ELISA-based assay using anti-IFN-γ purchased from BD Biosciences.

Cytotoxic assay

[51Cr] release assay was used to measure the cytotoxicity of γδ T cells against oral cancer cell line (AW13516) as target cells. γδ T cells were left alone or treated with rIL-2 (0.1 U; Peprotech) overnight at 37°C, and AW13516 cells were treated with zoladecol (100 μM; Panacea Biotech). For experiments using GSI-X, cells were treated as described earlier. Standard 4 h [51Cr] release assay was performed as previously described (13). AW13516 cells were labeled with [51Cr] for 90 min at 37°C. Labeled target cells (AW13516) were incubated with effector cells (γδ T cells) at 40:1 E/T ratio at 37°C in 5% CO₂ for 4 h. After incubation, plates were centrifuged, supernatants were collected, and radioactive chromium release was measured using 1470 Wallac automated gamma counter (Perkin-Elmer, Downs Grove, IL). Spontaneous release was determined by incubating the target cells with medium alone, and maximum release was determined by incubating target cells with 10% Triton X-100. The percent specific lysis was calculated as [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.

Small interfering RNA

γδ T cells isolated by MACS column (as described earlier) were transfected with small interfering RNA (siRNA) specific for NOTCH1, NOTCH2
genes, and fluorescent oligonucleotide SiGLO (transfection indicator; Thermo Fisher Scientific, Waltham, MA). siRNA oligos were transfected at a concentration of 50 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). The inhibition of Notch1 and Notch2 expression were assessed at 48 h.

Statistical analysis
Statistical analyses were performed using GraphPad Prism version 5.0. The Student t test was used as the test of significance.

Results
Expression of Notch receptors and ligands in human peripheral blood γδ T cells
The mRNA expression of Notch genes (Notch1–4) and its ligands Dll1, Dll3, and Dll4 and Jag1 and Jag2 were quantitated in ex vivo expanded and purified γδ T cells. The mRNA expression of Notch2 gene was higher than Notch1 (Fig. 1A). Relatively very low expression of Dll1 and Jag1 mRNA was observed. mRNA of Notch3, Notch4, Dll3, Dll4, and Jag2 was not detected in γδ T cells. After Notch activation, NICD enters the nucleus and then regulates the expression of target genes (25). Flow cytometry showed that Notch receptors (Notch1 and Notch2) are dominantly expressed on γδ T cells (Fig. 1B), although a low-level expression of ligands Dll1 and Jag1 are observed. The purity of isolated γδ T cells was analyzed by flow cytometry (95 ± 5%) and further, the contamination of non–T cell was ruled out by staining isolated γδ T cells for CD14, CD15, CD19, CD33, and CD56 markers (Fig. 1C). Cells were positive for CD56, which is a marker expressed by γδ T cells. The expression of NICD in γδ T cells further confirmed that Notch signaling may be active in γδ T cells.

Disruption of Notch signaling in activated γδ T cells reduces expression of Notch receptor and target genes
The release of NICD mediated by γ-secretase activity is required by all Notch receptors (Notch1–4) to initiate downstream signaling (29). In this study, we used GSI-X to block γ-secretase activity in γδ T cells (30). γδ T cells were stimulated with rIL-2 and BrHPP in the presence and absence of GSI-X. Stimulation of γδ T cells with BrHPP and rIL-2 triggered the activation of Notch signaling, which can be observed by abundant release of N1ICD by Western blot analysis (Fig. 2A). Treatment of BrHPP and rIL-2 activated γδ T cells with GSI-X decreased release of processed Notch1 (decreased NICD expression) compared with γδ T cells stimulated with BrHPP and rIL-2 alone (Fig. 2A). Stimulation of the Notch signaling pathway leads to the induction of c-Myc expression (31). Finally, we assessed whether activation of γδ T cells with BrHPP and rIL-2 was necessary for the Notch-mediated induction of cell-cycle regulator, c-Myc. We observed that Notch1 activation governs the downstream induction of c-Myc expression, which was abrogated upon GSI-X treatment (Fig. 2A). Simultaneously, we also monitored expression of mRNA for Notch receptors (1–4) and Notch ligands (Dll1, Dll3, Dll4, Jag1, and Jag2) in γδ T cells stimulated with BrHPP and rIL-2 in the presence and absence of GSI-X (Fig. 2B). After treatment with

FIGURE 1. Notch receptors are expressed on peripheral γδ T cells. γδ T cells were MACS purified from the peripheral blood. (A) Real-time quantitative PCR showed mRNA expression of Notch1, Notch2, Dll1, and Jag1 on γδ T cells. Data were normalized to expression of GAPDH. Data represent mean of four independent experiments. (B) Expression of N1ICD, Notch2 intracellular domain, Dll1, and Jag1 on γδ T cells. The MFI in each case has been corrected for the MFI of the isotype control. (C) Purified γδ T cells were negative for CD14, CD15, CD19, and CD33 receptors, and were positive for CD56 marker. Filled histogram indicates the isotype control.
GSI-X, a marked decrease in the expression of mRNA for Notch1 receptor was observed in Ag-activated γδ T cells (Fig. 2B).

In addition to the well-known c anonical Notch signaling pathway, evidence has emerged that noncanonical Notch signaling may also play an important role in T cells (32). We therefore investigated the expression of two target genes Hes1 and NF-κB in γδ T cells, which are representatives of canonical and noncanonical Notch signaling pathway, respectively. Using real-time PCR, we demonstrated that relative expression of mRNA for Hes1 and NF-κB were reduced in BrHPP and rIL-2 activated γδ T cells upon treatment with GSI-X (Fig. 2C).

**FIGURE 2.** GSI-X treatment leads to decreased expression of Notch receptors and its target genes in Ag-activated γδ T cells. (A) Western blotting for detection of 120-kDa NICD and 67-kDa c-Myc in unstimulated (lane 1), BrHPP, and rIL-2–activated (24 h) γδ T cells in the presence (lane 3) or absence of GSI-X (lane 2), using Abs that recognize the cleaved active form of Notch1 (N1ICD) and c-Myc. (B) Real-time PCR was performed for all the notch receptor isoforms (Notch1–4) and its ligands (Dll1, Dll3, and Dll4; Jag1 and Jag2) on γδ T cells. (C) γδ T cells were analyzed for both canonical (HES1) and noncanonical (NF-κB) target gene expression. In both (B) and (C), total RNA was extracted from BrHPP and IL-2–stimulated γδ T cells (4 h) with or without GSI-X treatment. The expression of specific mRNA is relative to GAPDH and is normalized to that same ratio in unstimulated cells. The Student t test was used as the test of significance (*p < 0.05, **p < 0.005).

**Inhibition of Notch signaling blocks γδ T cell activation and proliferation**

To determine the functional consequences of Notch signal in activated γδ T cells, we assessed γδ TCR-mediated proliferation response by [3H]thymidine incorporation assay. Purified γδ T cells were pretreated with different concentrations of GSI-X (15, 10, 5, and 2.5 μM) or left untreated, before cells were activated with plate-bound anti-CD3 mAb and rIL-2. A marked increase in the proliferation of γδ T cells was observed in the presence of anti-CD3 mAb and rIL-2. However, blocking of Notch signal by GSI-X leads to decreased proliferative response.
in a concentration-dependent manner (Fig. 3A). We also used freshly isolated, as well as ex vivo–stimulated and expanded, γδ T cells to compare their ability to respond to phosphoantigens in the presence and absence of GSI-X (15 μM). We observed a significantly increased proliferative response of γδ T cells to phosphoantigens (BrHPP, IPP, and c-HDMAPP) in the presence of rIL-2 (Fig. 3B, 3C). Freshly isolated γδ T cells showed robust proliferative responses to Ags compared with ex vivo–expanded γδ T cells. However, in the presence of GSI-X, the proliferative responses of γδ T cells to Ags were significantly reduced in both sets of isolated γδ T cells (Fig. 3B, 3C).

The expression of early and late activation markers, CD69 and CD25, respectively, were analyzed on unstimulated and Ag-stimulated

**FIGURE 4.** GSI-X inhibits the expression of activation markers on γδ T cells but does not induce apoptotic or necrotic cell death in γδ T cells. (A) The effect of GSI-X on cell-surface expression of late (CD25) and early (CD69) activation markers on unstimulated and BrHPP-stimulated (24 h) γδ T cells was analyzed by flow cytometry. Blocking of Notch signaling by GSI-X inhibits the surface expression of activation markers. Data indicate the MFI of the activation markers. Dark shaded histogram indicates isotype control. (B) Annexin V and PI staining of unstimulated γδ T cells (γδ), rIL-2–activated γδ T cells (γδ+IL-2), and γδ T cells stimulated with BrHPP in the presence of rIL-2 (γδ+IL-2+BrHPP) without GSI-X (upper panel) or with GSI-X (lower panel). These cells were stained after culturing for 24 h and analyzed by flow cytometry. The unaffected, early apoptotic, late apoptotic, and necrotic cells are present in the lower left, lower right, upper right, and upper left quadrant, respectively. Dot plots show the mean percentage of positive cells. Results shown are representative of three independent experiments.
γδ T cells with or without GSI-X treatment. Ex vivo–expanded γδ T cells alone (unstimulated) or after stimulation with BrHPP and rIL-2 showed higher expression of CD25 compared with CD69. Treatment with GSI-X showed a moderate reduction in CD69 expression. A marked reduction in the expression of late activation marker CD25 was observed on both unstimulated and BrHPP-stimulated γδ T cells (Fig. 4A).

We then investigated whether treatment with GSI-X results in apoptotic/necrotic cell death of Ag-activated γδ T cells. To verify, we compared the effects of GSI-X on the frequency of apoptotic cells in unstimulated γδ T cells and after stimulation with rIL-2 alone or both rIL-2 and BrHPP. We did not observe differences in the frequency of early apoptotic (Annexin V+), late apoptotic (apoptotic V+PI+), and necrotic (PI+) γδ T cells in untreated compared with GSI-X–treated cells (Fig. 4B).

Notch regulates cytolytic potential of γδ T lymphocytes
Notch signaling has been reported to regulate cytotoxic responses in both CTL and NK cells (33, 34). The effect of Notch signaling in cytolytic potential of γδ T lymphocytes was examined. Purified γδ T cells were cocultured for 4 h with zoledronate-treated oral cancer cells. We evaluated CD107a (lysosome-associated membrane protein-1 [LAMP1]) expression, a marker of degranulation in unstimulated and rIL-2–stimulated γδ T cells in the presence and absence of GSI-X. Upon coincubation with the oral cancer cells, few γδ T cells showed surface expression of CD107a (18.4%). However, in the presence of rIL-2, the proportion of CD107a+ γδ T cells increased (40.9%; Fig. 5A). Addition of GSI-X leads to reduction in the percentage of CD107a+ in both unstimulated γδ T lymphocytes (13.1%) and rIL-2–stimulated γδ T cells (12.4%). The cytotoxic potential of γδ T cells against zoledronate-treated tumor cells (AW13516) was determined by

FIGURE 5. Notch signaling regulates cytolytic effector functions of γδ T cells. (A) Expression of CD107a or LAMP1 (marker for degranulation) on γδ T cells. Effector γδ T cells alone or in the presence of rIL-2 were cocultured with target oral cancer cell line (AW13516) at the ratio of 4:1 and incubated for 4 h. AW13516 were previously treated with zoledronate (100 μM) for 16 h. Decreased expression of CD107a was observed when the cells were treated with GSI-X (lower panel). Panel is representative of three experiments. (B) Zoledronate-treated AW13516 cells labeled with [51Cr] used as target cells were cocultured with γδ T cells at different E/T ratios in a standard 4-h [51Cr] release assay. Data represent percentage cytotoxicity as described in Materials and Methods. Representative data of an independent experiment. (C) Cytolytic ability of γδ T cells alone or in the presence of rIL-2 against zoledronate-treated AW13516 was determined by [51Cr] release assay at the ratio of 40:1. Treatment of γδ T cells with GSI-X blocked the target cell lysis. *p < 0.05 compared with percentage cytotoxicity of both γδ T cells and γδ T cells with rIL-2 without GSI-X. Results are mean percentage cytotoxicity of γδ T cells from five healthy individuals. (D) γδ cells were transfected with 50 nM control (SiGLO) or different Notch-specific SiRNA duplexes. Forty-eight hours posttransfection, cells were harvested and samples were subjected to Western blot analysis for Notch1 and Notch2 (top panel) or β-Actin (bottom panel). The latter was included as loading/normalization control. (E) γδ T cells were transfected with 50 nM control (SiGLO) or specific Notch1 and Notch2 siRNA duplexes, Forty-eight hours posttransfection, the cytolytic effector function of rIL-2–activated γδ T cells against zoledronate-treated AW13516 was determined by [51Cr] release assay at the ratio of 40:1. Silencing of either Notch1 or Notch2 inhibits target cell lysis by γδ T cells. **p < 0.005 compared with percentage cytotoxicity of Notch-specific siRNA with SiGLO-treated γδ T cells. The results are mean percentage cytotoxicity of γδ T cells from five healthy individuals.
titration at different E/T ratio ranging from 5:1 to 40:1. As seen in Fig. 5B, maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1. This ratio of E/T was used in further experiments where effect of GSI-X on cytolytic ability of γδ T cells was examined. At E/T ratio of 40:1, ex vivo–expanded γδ T cells in the presence of rIL-2 efficiently lysed zoledronate-treated oral tumor cells compared with untreated cells. Addition of GSI-X significantly reduced the cytotoxic ability of γδ T cells against zoledronate-treated tumor targets (Fig. 5C).

Because pharmacological inhibition of the γ-secretase complex may have nonspecific effects, we assessed the contribution of the Notch pathway in regulating the cytotoxic potential using siRNA-mediated knockdown of individual Notch receptors. The synthetic siRNA sequences targeting the Notch1 and Notch2 were transfected into the γδ T cells. γδ T cells were transfected with 50 nM control (SiGLO) or different Notch-specific siRNA duplexes. After 48 h, cells were harvested and lysates were prepared. Western blot analysis for Notch1 and Notch2 receptors showed that siRNA transfection led to the reduced expression of Notch1 and Notch2 receptors compared with fluorescent oligonucleotide SiGLO (Fig. 5D). Next, we compared antitumor cytotoxic ability of γδ T cells transfected with siRNA specific for Notch1 and Notch2 against zoledronate-treated tumor cells (AW13516). Cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 and consistent with GSI-X treatment data (Fig. 5C); silencing of both Notch1 and Notch2 led to significant reduction in cytotoxic potential of γδ T cells (Fig. 5E). This observation confirms that cytotoxic potential of γδ T cells is regulated by Notch signaling.

Notch signaling regulates cytokines production in activated γδ T cells

To address whether Notch signal has any role in the effector functions of activated γδ T cells, we examined the effect of GSI-X on cytokine production. The consequence of GSI-X treatment on the concentration of TH1 (IL-2, IFN-γ, and TNF-α), TH2 (IL-4, IL-6, and IL-10), and TH17 (IL-17) production by γδ T cells treated with rIL-2 alone or along with BrHPP was assayed. It was observed that inhibition of Notch signaling leads to marked reduction in TNF-α, IFN-γ, and IL-17 production by activated γδ T cells (data not shown). The inhibition was more pronounced in IFN-γ production. We therefore confirmed the effect of GSI-X on IFN-γ production by ELISA. Activated human γδ T cells secrete IFN-γ, and Notch signaling regulates IFN-γ secretion in activated CD4+ T cells (5, 23). γδ T cells were stimulated with c-HDMAPP, IPP, and BrHPP, which leads to increased production of IFN-γ. The amount of IFN-γ decreased when the cells were pretreated with GSI-X (Fig. 6A). Like phosphoantigens, anti-CD3 mAb stimulation of γδ T cells was also inhibited by GSI-X, which is depicted by dose-dependent decrease in IFN-γ production (Fig. 6B). Thus, a role of Notch in regulating IFN-γ production in γδ T cells is unravelled, which suggests that Notch plays a role in effector functions of γδ T cells.

Discussion

The role of Notch signaling in T cell differentiation, activation, and effector functions have been well documented in CD4 and CD8 T cells (22, 23). However, there is a lacuna of studies addressing the role of notch signaling in effector functions of human γδ T cells. A recent report showed that Hes1 is critically involved in the development of IL-17–producing γδ T cells (35). In these investigations, we report for the first time, to our knowledge, that peripheral human γδ T cells express notch receptors and notch signaling is required in Ag-specific responses of γδ T cells. We report Notch1 and Notch2 expression in γδ T cells at mRNA and protein level as analyzed by real-time PCR and flow cytometry. γδ T cells are known to be activated by phosphoantigens, for example, IPP and 4-hydroxy-3-methyl-but-2-ene1 pyrophosphatase, which are intermediates of the mevalonate pathway of cholesterol metabolism in eukaryotic cells and rohmer pathway in prokaryotic cells (6). Using BRHPP, a synthetic analog of IPP in the presence of pharmacological GSI-X, we show that loss of Notch signaling in γδ T cells results in downregulation of mRNA for Notch1 and Notch2 receptors. Reduction in the expression of NICD by GSI-X in BrHPP-stimulated γδ T cells further confirmed the involvement of Notch signaling in regulating Ag-specific responses of γδ T cells. Activation of the Notch signaling pathway leads to the induction of c-Myc expression in immune cells (36), which was also observed in γδ T cells. Recent reports suggest that Notch signaling involves both canonical and noncanonical pathways in T cells, and these interactions will influence cell fate decisions and functions (32, 37). We therefore analyzed the expression of

![FIGURE 6](https://www.jimmunol.org/)

**FIGURE 6.** Treatment with GSI-X blocks IFN-γ production by γδ T cells. (A) γδ T cells alone, γδ T cells with rIL-2, or along with three different phosphoantigens (BrHPP, IPP, and c-HDMAPP) were cultured in the presence or absence of GSI-X for 24 h in 96-well plates. (B) γδ T cells were either cultured alone, or in the presence of anti-CD3 mAb or with rIL2 and in the presence or absence of different concentration of GSI-X (15, 10, 5, and 2.5 μM) for 24 h in 96-well plates. Supernatants were collected, and level of IFN-γ was measured by sandwich ELISA. GSI-X treatment decreases IFN-γ production by both unstimulated and stimulated γδ T cells. Results shown are mean of three experiments. *p < 0.05, **p < 0.005, compared with IFN-γ production of the cells without GSI-X treatment. *p < 0.5, **p < 0.05, compared with cpm of cells treated with rIL-2 alone.
mRNA for Hes1 and NF-κB in BrHPP-stimulated γδ T cells after inhibiting the Notch signaling by GSI-X. A decrease in the Notch target gene NF-κB indicates that noncanonical Notch signaling pathway is active in activated γδ T cells.

Further, inhibiting the Notch signaling in anti-CD3 mAb-stimulated γδ T cells resulted in marked decrease in proliferation of γδ T cells, confirming TCR engagement as a key initiating event affected by GSI-X treatment. Similarly, γδ T cells (freshly isolated and ex vivo expanded) activated with BrHPP, IPP, and c-HDMAPP as Ags resulted in a significantly decreased proliferation of γδ T cells in response to these Ags. The observation that Notch signaling is involved in regulating Ag-specific proliferative responses of γδ T cells prompted us to look at the expression of early and late activation markers CD69 and CD25 on γδ T cells stimulated with the Ag (BrHPP) in the presence and absence of GSI-X. Reduction in CD69 expression and a marked decrease in CD25 (IL-2R) expression on Ag-stimulated γδ T cells was observed when Notch signaling was inhibited. Various reports have shown that Notch signaling induces the expression of CD25 in immune cells (38–40). NF-κB, which is downstream of Notch signaling, also regulates the expression of CD25 (41). γδ T cells are dependent on IL-2 for their growth and survival, and express high-affinity CD25 (42). It is therefore not surprising that CD25 expression in γδ T cells is also regulated by Notch signaling, and IL-2 stimulation will thereby enhance the proliferative response and cytokine production of γδ T cells via IL-2R (CD25).

The importance of Notch signaling in mediating cytotoxic responses in immune cells has been well documented. Earlier studies carried out in murine CD8+ T lymphocytes and NK cells have been reported. In these cells transduced with Jag2 (33), Notch signaling was shown to directly control CTL effector molecules (22, 33). Notch signaling was shown to directly regulate granzyme B expression in CD8+ cytotoxic T lymphocytes involved in the cytolytic activity of γδ T cells (22). Cho and colleagues (43) demonstrated that Notch1 regulates expression of eomesoderm, perforin, and granzyme B through direct binding to the promoter of these effector molecules. In CD8+ T cells, Notch2 signaling was shown to directly control CTL effector molecules including granzyme B, by integrating RBP-J and CREB1 (40). We observed that GSI-X treatment of γδ T cells alone or γδ T cells activated with IL-2 results in a reduction of CD107a expression in these cells. CD107a or LAMP1 is a marker of degranulation in cytotoxic T lymphocytes (44–46).

γδ T cells are known to secrete copious amounts of IFN-γ. IFN-γ plays a crucial role in protective immune response against certain pathogens and tumors (47–49). We showed that blocking Notch signaling with GSI-X inhibited the IFN-γ secretion by γδ T cells stimulated with phosphoantigens BrHPP, IPP, and c-HDMAPP. Likewise, we observed that GSI-X inhibits IFN-γ production by anti-CD3 mAb–activated γδ T cells. γδ T cells provide an early source of IFN-γ in tumor immunosurveillance and against viral challenge (47, 50). Epigenetic program that regulates IFN-γ gene transcription in γδ T cells is different from CD4+ and CD8+ T cells (51). Eomesoderm contributes to T–bet–independent IFN-γ production in γδ T cells (51). We observed that BrHPP-stimulated γδ T cells produced IL-17, and its secretion was inhibited in the presence of GSI-X (data not shown). γδ T cells have been reported to be a potent source of IL-17 (52, 53). It has been reported that DLL4 upregulates RORC expression in T cells, and both RORC and IL-17 gene promoters are direct transcriptional notch targets and enhance Th17 cell population (54).

In this study, we describe for the first time, to our knowledge, the role of Notch in regulating the effector functions of human γδ T cells. Notch signaling appears to play an important role in modulating the Ag-specific proliferation of γδ T cells, their ability to lyse tumor targets and secrete IFN-γ upon stimulation. Taken together, these studies identify Notch as an additional signal contributing to Ag-specific effector functions of γδ T cells. These studies may have important implications in clinical situations where new strategies for the clinical manipulation of γδ T cells for cancer immunotherapy are being investigated.

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Disclosures
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