Novel natural immunogenic peptides from Numb1 and Notch1 proteins for CD8+ cells in ovarian ascites

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Abstract. Notch is a plasma membrane receptor involved in the control of cell fate specification and in the maintenance of the balance between proliferation and differentiation in many cell lineages. Disruption of Notch has been implicated in a variety of hematological and solid cancers. Numb is also expressed in many adult mammalian cells. Adult cells divide symmetrically, and Numb is symmetrically partitioned at mitosis. The Numb-mediated regulation of Notch is believed to play a causative role in naturally occurring breast cancers. Reduction of Numb levels in breast tumors is regulated by proteasomal degradation. We reasoned that if the disregulated negative control of Notch by Numb protein is the consequence of Numb proteasomal degradation, then degradation of Numb can generate peptides which are transported, presented by MHC-I molecules. Surprisingly we found few candidate naturally processed peptides from Notch1, Notch2, and Numb1. CD8+ T cells expressing TCRs which specifically recognized peptides Notch1 (2112-2120) and Numb1 (87-95) were presented in the ascites of ovarian cancer patients. Many of these cells were differentiated and expressed high levels of Perforin. The natural immunogenicity of Notch1 and particularly of Numb1 suggests a mechanism of immunosurveillance which is overcome during tumor progression. Immunotherapy with tumor antigens from Notch and Numb should be important for treatment of cancer patients.

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Introduction

Notch is a plasma membrane receptor involved in the control of cell fate specification and in the maintenance of the balance between proliferation and differentiation in many cell lineages (1,2). Notch signaling is important in regulating numerous physiological processes, disruption of Notch has been implicated in a variety of hematological and solid cancers.

The best-studied example is the link between mutations of Notch1 and T-cell acute lymphoblastic leukemia and lymphoma (T-ALL). In a subset of T-ALL tumor cells, a t (7; 9) chromosomal translocation fuses the 3' portion of Notch1 to the T-cell receptor Jß locus. This results in a truncated Notch1 protein, which is constitutively active and aberrantly expressed (3). In addition, activating mutations in Notch1 independent of the t (7; 9) translocation have been found in >50% of human T-ALL cases (4).

Abnormal Notch signaling has also been reported in solid tumors, including cancers of the breast, pancreas, prostate, liver, stomach and colon cancer, although without evidence of genetic lesions (5-7). Notch may play either an oncogenic or a tumor-suppressive role, depending on the cancer type, other signaling pathways present and the identity of Notch receptor activated.

However, in a large majority of cases including breast cancer, Notch signaling promotes tumor growth (8). One mechanism for the oncogenic role of Notch may derive from its ability to prevent differentiation and maintain the stem cell phenotype. Stem cells and tumor cells share common characteristics, such as unlimited proliferation and undifferentiation. Further, self-renewal in stem cells and tumor cells are regulated by similar pathways, including sonic hedgehog, Wnt and Notch. It is possible that tumor cells may derive from normal stem cells or that cancers may harbor 'cancer stem cells' that are resistant to treatment (9).

There is a single Notch receptor and two ligands (Delta and Serrate) in *Drosophila*. In mammals, there are four receptors and five ligands, which are the focus of this review. Notch1-4 are homologues of *Drosophila* Notch; Delta-like-1, -3 and -4 (Dll1, Dll3, Dll4) are homologues of Delta; Jagged1 and Jagged2 (Jag1 and Jag2) are homologues of Serrate.

Each Notch receptor is synthesized as a full-length precursor protein consisting of extracellular, transmembrane and intracellular domains. Notch signaling is normally activated by ligand-receptor binding between two neighboring cells. This interaction induces a conformational change in the receptor, exposing a cleavage site, S2, in its extracellular domain. After cleavage by the metalloprotease TNF-α converting enzyme (TACE) and/or Kuzbanian, Notch receptor undergoes intramembrane proteolysis at cleavage site S3. This cleavage, mediated by the γ-secretase complex, liberates the Notch intracellular domain (N-ICD), which then translocates into the nucleus to activate Notch target genes. Inhibiting γ-secretase function prevents the final cleavage of the Notch receptor, blocking Notch signal transduction. In the absence of N-ICD cleavage, transcription of Notch target genes is inhibited by a repressor complex mediated by the Suppressor of Hairless [re-combination-signal binding protein jκ (RBP-jκ) homologue] in *Drosophila*.

Recent studies in *Drosophila* have suggested that Notch can signal independently of the canonical Suppressor of Hairless pathway. However, it is unclear if this is the case in vertebrates. Some early evidence from myogenic cell lines and the developing avian neural crest suggests that Notch signaling can occur in the presence of dominant negative Suppressor of Hairless, but additional characterization is needed to establish alternative downstream pathways in vertebrates (10).

During asymmetric cell division in embryogenesis, the activity of Notch is biologically antagonized by the cell fate determinant Numb (11,12). The asymmetric cell division consists in division of a stem cell in a differentiated and in a non-differentiated daughter. Numb is also expressed in many adult mammalian cells (13). Adult cells divide symmetrically, and Numb is symmetrically partitioned at mitosis. The symmetric partitions suggest that either Numb is inactive or has additional functions. The Numb/Notch antagonism is relevant to control of the division of the normal mammary parenchyma. The normal breast parenchyma invariably expresses intense and homogeneous Numb staining. In contrast, tumors display marked heterogeneity and in many cases complete absence of Numb immunoreactivity (14,15).

Based on this and additional information it is believed that subversion (by blocking or inhibition) of the Numb-mediated regulation of Notch plays a causative role in naturally occurring breast cancers. Eighty percent of breast tumors show Numb immunoreactivity in 50% of the tumor cells. Thus, almost one half of all breast tumors have reduced levels of Numb. A strong inverse correlation was found between Numb expression levels and tumor grade and Ki67 labeling index, which are known indicators of aggressive disease (14). The low Numb levels were reported to be restored to high levels by treatment with proteasome inhibitors such as MG132 (14). Reduction of Numb levels in breast tumors studied did not appear to be the consequence of a generally increased proteasomal activity, as the basal levels of other cellular proteins also regulated by proteasomal degradation, were not affected under the same experimental conditions, although this matter requires further investigation.

We reasoned that if the disregulated negative control of Notch by Numb protein is the consequence of Numb proteasomal degradation, then degradation of Numb can generate peptides which are transported by Transporter associated with antigen processing (TAP) and presented by MHC-I molecules. It is possible that T cells which recognize these MHC-I Numb peptide complexes are tolerized or eliminated in healthy individuals. Furthermore, if degradation of Notch is required for its signaling, then cytoplasmic degradation of the N-ICD should also generate Notch peptides. If some of the Notch fragments are degraded by the proteasome, they may be also presented by MHC-I molecules. If Notch and Numb peptides are not tolerogenic then activated $CD8^+$ T cells bearing receptors for such peptides should be detected *in vivo*, in cancer patients. The current study was performed to address these hypotheses.

Materials and methods

Identification of candidate MHC-I binding peptides with predictive algorithms. We used the following programs to identify peptides which can bind HLA-A, B, C and HLA-DR molecules: i) BIMAS (Informatics and Molecular Analysis Section) to predict peptides binding to HLA-A, B, C. (http://bimas.cit.nih.gov/molbio/hla_bind) (16); ii) *PAPROC* (Prediction Algorithm for Proteasomal Cleavages), a prediction tool for cleavage by human and yeast 20S proteasomes, based on experimental cleavage data (http://www.paproc2.de/paproc1/paproc1.html); and iii) *TEPITOPE* program for prediction of MHC-II binding peptides. This program was available from Dr Jurgen Hammer (Roche) (www.vaccinome.com) (17,18).

To identify the predicted proteasome-generated and MHC-I binding peptides, we downloaded the amino acid sequences of Notch1, Notch2 and Numb1 from NCBI. Their accession numbers are: Notch1 (NM_017617), Notch2 (NM_024408), and Numb1 (P49757), respectively. We identified the peptides produced by the human proteasomes wild-type 1, 2, and 3.

The tridimensional protein structure models of the Notch1 and Numb1 areas containing the peptide candidate $CD8^+$ cells' epitopes were down-loaded using the Swiss Model Program. The Swiss Model Program is a fully automated protein structure homology-modeling program, accessible via the ExPASy web server (http://swissmodel.expasy.org/repository/) or from the program DeepView (Swiss Pdb-Viewer, http://swissmodel.expasy.org/spdbv/) (19). The molecular models of the Notch1 and Numb1 regions where the peptides are located are shown in Fig. 1 (20-22).

Cell lines. We used the human breast cancer cell line MCF7, human ovarian cancer cell line SK-OV-3, and human leiomyosarcoma cell line SK-LMS-1 obtained from the American Type Culture Collection (Rockville, MD). All cell lines were grown in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in monolayers to a confluency of 80% before treatment.

Lymphocyte culture. Lymphocytes were isolated by Ficoll-gradient centrifugation from heparinized ascites from $HLA\text{-}A2^+$ ovarian cancer patients. After separation, we cultured lymphocytes with RPMI-1640 medium with 10% FCS and 300 IU of

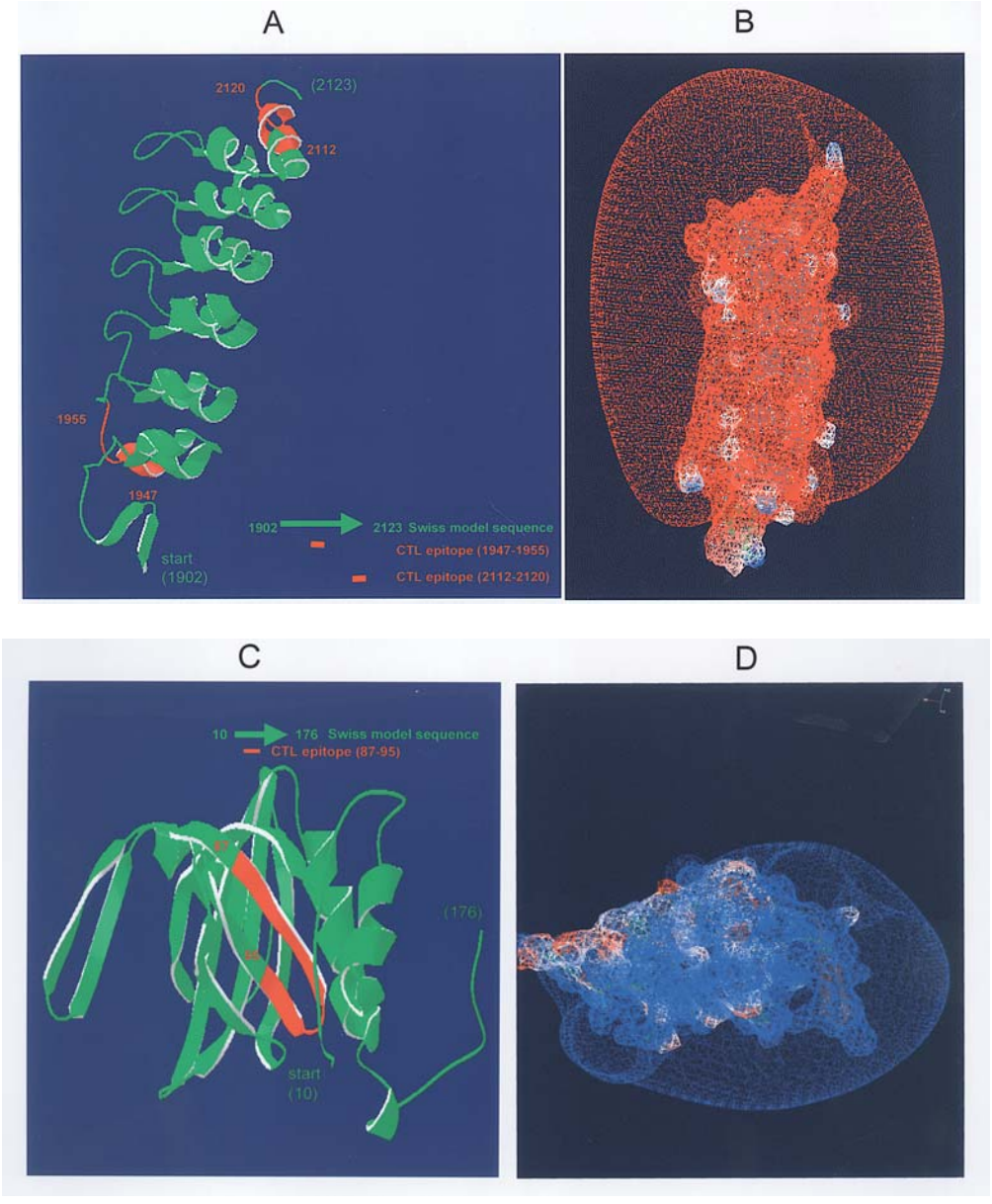

Figure 1. Molecular models of Notch1 C-terminal domain amino acids 1902-2143 (A and B) and Numb1 phosphotyrosine-binding domain (PTB) (C and D). B and D show the charges of these molecules, red indicates a positive charge, blue indicates a negative charge. The positions of Notch1-1947, Notch1-2112, and Numb1-87 peptides are shown in A and C.

IL-2 (Biosource Camarillo, CA) for 1 week, as we described (23,24).

Synthetic peptides. The following peptides were used in this study: Notch1 (1947-1955, RLLEASADA), Notch1 (2112-2120, RLLDEYNLV), Numb1 (87-95, VLWVSADGL), Gli1 (580-588, GLMPAQHYL) and AES1 (128-137, LPLTPLPVGL). All these peptides were synthesized by Dr Martin Campbell at the Synthetic Antigen Core Facility, of the University of Texas M.D. Anderson Cancer Center. Amino acids were coupled in sequential format from the -COOH terminus using standard *N*-(9-fluorenyl) methoxy-carbonyl peptide chemistry on a Rainin Symphony Automated Peptide Synthesizer and purified by high-performance liquid chromatography. The purity of the peptides ranged from 95% to 97%. Peptides were dissolved in PBS with 10% DMSO and stored at -20˚C as aliquots of 1 mg/ml until use as we described (23).

Flow cytometry. To examine the expression of Notch1 molecules on tumor cell lines, cells that were pre-treated by BD Cytofix/Cytoperm and washed by BD Perm/Wash (BD Bioscience Pharmingen, San Diego, CA) for intracellular staining were stained with anti-Notch1 monoclonal antibody PE (phycoerythrin)-labelled and PE-conjugated mouse monoclonal isotype control antibody (BD Bioscience Pharmingen) were analyzed using a Becton Dickinson FACS Caliber with Cell Quest software (Becton Dickinson, NJ) and the Flow-Jo Program (Mac version 8.11 Tree Star, Inc, OR) (25).

We identified cells expressing high concentrations/numbers of T cell receptors (TCRs) reactive with each peptide to evaluate the role of TCR density in CTL differentiation upon *in vivo* stimulation with the same ligands. The TCR$^+$ population, which usually includes cells staining with antigen-tetramers/dimers with a mean fluorescence intensity (MFI) higher than 10^1, was divided in three populations, one staining

Table I. Proteasome generated Notch1, Notch2 and Numb1 peptides.[a]

HLA-	Start position	Sequence	Digestion type[b]	Digestion product[c]	Length
Notch1					
A2.1	1947	RLLEASADA	1	*AAK***R/LLEASAD/A**	7
A2.1, 2.5	2112	RLLDEYNLV	1	*V***R/LLDEYNLV**	8
A24	1938	RYSRSDAAK	1	**RYSRSD/AAK***R*	6
A33	274	DGVNTYNCR	3	**DGVNTYNC/R**	8
Cw4	None	N/A		N/A	
Notch2					
A2.1	None	N/A		N/A	N/A
A2.5	7	ALLWALLAL	1, 2	*MPALRP***ALLWALLAL***WLCCA*	21
A24, 2.5	1940	RMNDGTTPL	3	**RMNDGTTPL***I*	10
A33	1995	LLLKNGANR	1	*EATL***LL/LKNGANR**	7
A33	277	DGVNTYNCR	2	**DGVNTYNCR***CPPQWTG*	16
	277	DGVNTYNCR	3	*NGGVCV***DGVNTYNC/R**	14
Cw4	None	N/A		N/A	
Numb1					
A2.1	87	VLWVSADGL	1	**V/LWVSADGL**	8
A2.1. 2.5	443	WLEEVSKSV	2	***R*WLEEVSKSV***RA*	12
A2.5	139	WICHCFMAV	1	***R*WICHCFMAV**K*D*	12
	139	WICHCFMAV	2	*CRDGTTRR***WICHCFMAV***KD*	19
A24	None	N/A		N/A	N/A
A33	594	DGRLASADR	1	*VD***DGRLASADR***HTEV*	15
Cw4	None	N/A		N/A	N/A

[a]The predicted proteasome generated peptides which can bind MHC-I were identified with the program *PAPROC* (http://www.paproc2.de/paproc1/paproc1.html). [b]Digestion type indicate the proteolytic specificities, designated as 1, 2, and 3 by the program *PAPROC*. [c]The positions of digestion of peptide and the resulting product. N/A, not applicable no peptides binding to.

with antigen-pulsed HLA-A2/IgG dimers (dimers) with an MFI (TCR) between 10^1 and 10^2, and another which stained with antigen-pulsed dimers with an MFI (TCR) between 10^2 and 10^3, and the other which stained with antigen-pulsed dimers with an MFI (TCR) between 10^3 and 10^4. These populations were designated as TCR^{lo}, TCR^{med}, and TCR^{hi}, respectively as described (26).

T cell: peptide-HLA-A2-IgG dimer interaction. Expression of TCRs specific for peptides Notch1 (1940-1948), Notch1 (2112-2120), Numb1 (87-95), Gli1 (580-588) and AES1 (128-137) was determined using HLA-A2-IgG-dimmers (BD Bioscience Pharmingen). The peptide loaded dimers were prepared as previously described (23). Staining of lymphocyte with dimers was performed as described previously (24,27,28).

The same cells were also stained for the expression of CD8 antigen and the presence of Perforin (effector pore forming enzyme) using specific antibodies conjugated to distinct fluorochromes than the dimers: fluorescein isothiocianate (FITC), allophycocyanin (APC) and PE. Cells reacting with the corresponding peptide-loaded dimers are designated as Notch1-1940-TCR^+, Notch1-2112-TCR^+, Numb1-87-TCR^+, and Gli1-87-TCR^+ cells, respectively. Cells reacted with control HLA-A2-IgG dimers not loaded with peptide are designated as dNP-TCR^+ cells.

Results

Selection of proteasome processed peptides. A preliminary analysis of the candidate immunogenic Numb and Notch peptides indentified the peptides from Notch1, Notch2, and Numb1 which, based on the HLA-A, B, C binding-prediction algorithm would bind to HLA-A, B, C molecules. Results show a very large number of peptides, which are potential binders to several MHC-I. The very large number of MHC-I binding peptides made peptide selection difficult. We searched and identified the peptides with potential to bind to: a) HLA-A2, which is more frequently expressed in Caucasians and Chinese, b) HLA-A24, which is more frequently expressed in Japanese, and c) HLA-A33, and HLA-Cw4, which were reported to be associated with T cell responses to HIV in African-Americans (29). We also investigated the potential binders to HLA-A2.5 which is more frequent (25%) in $HLA\text{-}A2^+$ African-Americans than in other HLA-A2 populations (30).

Table II. Repair of proteasome generated peptides by modification of flanking residues of the core peptide.

Peptide	Flank	Core	Flank	Proteasome digestion product
Notch1				
Wild-type	RMHHDI	**VRLLDEYNLV**	RSPQL	*RMHHD*/*I*/*VR*/**LLDEYNLV**/*RSPQL*
A. Replace N-terminal flanking sequence with the Her-2 E75 peptide N-terminal flanking sequence NIQEAFAGCL				
N-flank-modified	NIQEAFAGC	**LRLLDEYNLV**	RSPQL	*NIQEAFAGC*/*L*/**RLLDEYNLV**/*RSPQL*
B. Replace N-terminal flanking sequence with NIQEAFAGCL and then replace in the core: R^2 with K				
	NIQEAFAGC	**L*K*LLDEYNLV**	RSPQL	*NIQEAFAGC*/*L*/***K***LLDEYNLV/*RSPQL*
Numb1				
Wild-type	GKTGKKAVKA	**VLWVSADGL**	RVVDEKTK	GKTGKKA/V/KA/V/**LWVSADGL**/RVVDEKTK
Substitutions[b]				
	A→P			GKTGKKA/V/K/***P***VLWVSADGL/RVVDEKTK
	KA→LFK			GKTGKKA/V/***LF***/***K***VLWVSADGL/RVVDEKTK
Replace the N and C-terminal flanking residues with RMHHDI and RSPQL respectively plus insert R before the start of the minimal epitope[a]				
	*RMHHDI*AV*R*	**VLWVSADG**L	*RSPQL*	*RMHHDI*/AV/*R*/**VLWVSADGL**/*RSPQL*

[a]RMHHDI and RSPQL are the flanking residues of the Notch1 peptide above. [b]All resulting peptides have very low affinity for HLA-A2. HLA-A2 binding scores are: 147.697 (9 mer), 0.075 (10 mer) and 11.861 (10 mer). Bold and italicized letters indicate substitutions in the sequence.

The immunodominance of self-/tumor-antigens (TAs) is not always determined by the binding affinity of the antigen to MHC-I. In fact some of the immunogenic peptides, (C85, MART-1) are very weak binders to HLA-A2. To improve our chances of selection of immunogenic peptides, which are endogenously processed, we performed proteasome-digestion prediction analysis (18). Results in Table I show that only very few Notch1, Notch2, and Numb1 peptides of the ones predicted to bind any of the HLA-molecules can be also generated by proteasomal digestion of internal proteins. In fact, only two peptides from Notch1, and one from Numb1 were similar to their MHC-I-predicted to bind counterparts.

Results in Table I show that peptides Notch1 (2112-2120) and Notch1 (274-282) are processed by the proteasome and presented as octamers, by HLA-A2 and HLA-A33, respectively. Based on the position of N- and C-terminal anchor motifs, only Notch1 (2112-2120) can form a complex with HLA-A2. Of interest, Notch1 (2112-2120) can also bind A2.5 although with lower affinity than HLA-A2.1. Therefore Notch1 (2112-2120) can be a common/shared epitope for Caucasian and African-American populations, which express A2.1 and A2.5 respectively.

Completely different results were obtained for Notch2 peptides. Only the peptide Notch2 (1940-1948) can be digested by the proteasome and presented as a decamer by HLA-A24. This peptide and all other Notch2 peptides cannot be presented by HLA-A2 or any of the histocompatibility gene products associated with responses in African-American populations. However, Notch2 (1940-1948), can be generated by proteasome and presented by HLA-A2.5. Therefore Notch2 (1940-1948) can be presented by tumors in association with both HLA-A24 and HLA-A2.5. It should be also emphasized that Notch2 (1940-1948) differs in sequence from Notch1 (1947-1955).

Results were surprising for Numb. The Numb1 peptide (87-95) can be digested by the proteasome and presented as an octamer by HLA-A2.1. The Numb peptide 443-451 can be presented by HLA-A2.1 and HLA-A2.5 as a dodecamer, thus its immunogenicity may depend on trimming by exopeptidase.

Detection of naturally immunogenic peptides. To address whether the peptides imperfectly digested by the proteasome can be repaired we engineered new candidate immunogens. Peptides which exceed the 9-amino acid length such as Notch2 (1940-1948) and Numb (443-451) can be trimmed at N- and C-terminal ends before presentation. To engineer repairs, we kept the same minimal 9-amino acid epitope and modified the flanking residues. Modification was made by replacing the Notch/Numb flanking residues with the flanking residues from other proteins (e.g. HER-2 protein) which allows presentation of the minimal CTL epitope, E75, associated with HLA-A2. Results show that only the HLA-A2 binding peptides from Notch1 and Numb1 could be presented after proteasome digestion (Table I).

To identify which of these proteins is antigenic *in vivo*, we determined the presence of $CD8^+$ T cells expressing TCRs which can specifically recognize peptides Notch1 (1947-1955),

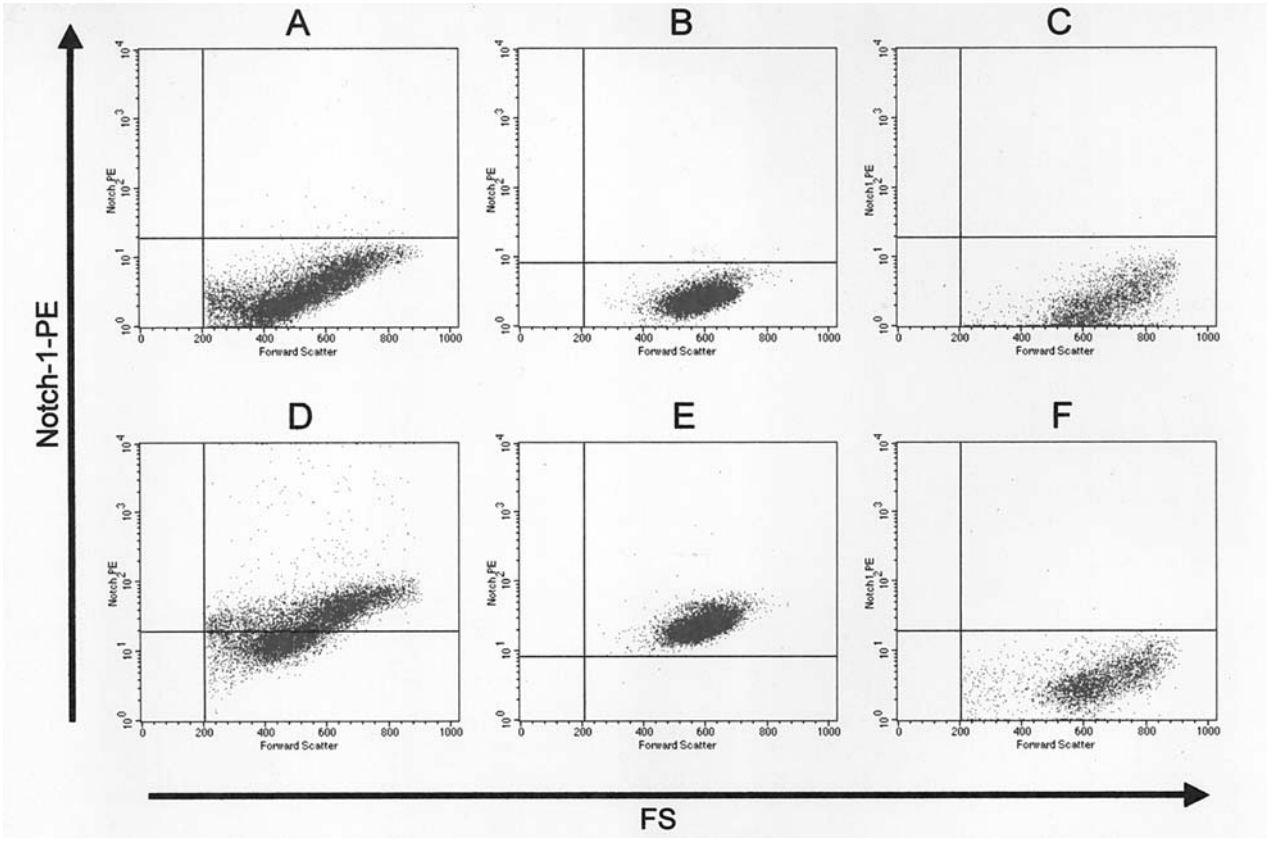

Figure 2. Expression of Notch1 on breast MCF7 and ovarian SK-OV-3 tumor cell lines. (A-C) Cells stained with isotype control antibody. (D-F) Cells stained with antibody against Notch1. MCF7 (A and D), SK-OV-3 (B and E), and SK-LMS-1 leiomyosarcoma (C and F).

Notch1 (2112-2120), and Numb1 (87-95). The AES1 peptide (128-137) which is known to be generated by proteasomal digestion was used as negative control for *in vivo* immunogenicity. The Gli1 peptide (580-588) which is not generated by proteasomal digestion was used as a negative control. The base line TCR^+ cell numbers, were determined with dNP-dimers. We investigated the presence of $CD8^+$ cells bearing TCRs with high, medium and low affinity in ovarian tumor-associated lymphocytes from patients with advanced disease.

The significance of the presence of Notch and Numb proteins and ligands in ovarian cancer, due to the fact that Notch and Numb are expressed in a subset of ovarian vessels during oncogenesis, including both mature ovarian vasculature as well as angiogenic neovessels (31). Their expression in the ovary was found in both endothelial and vascular associated mural cells (32). +Tumor angiogenesis involves many of the same pathways as physiological angiogenesis, including Notch. This has been shown in both human tumor samples and mouse xenografts. Measured by *in situ* hybridization and quantitative polymerase chain reaction (qPCR), Dll4 mRNA was undetectable in normal kidney or breast samples, but highly expressed in the vasculature of human clear-cell renal cell carcinomas and breast cancers. Among the tumor samples, Dll4 expression positively correlated with VEGF expression at the mRNA level (33). In a xenograft study, the human MCF7 cell line, which does not express Dll4, resulted in tumors expressing high levels of mouse Dll4 within their vasculature (34). Currently, the study of Dll4 expression in tumors is hampered by the lack of a good monoclonal antibody. Work is underway to develop antibodies that allow measurement of Dll4 protein levels by immunohistochemistry.

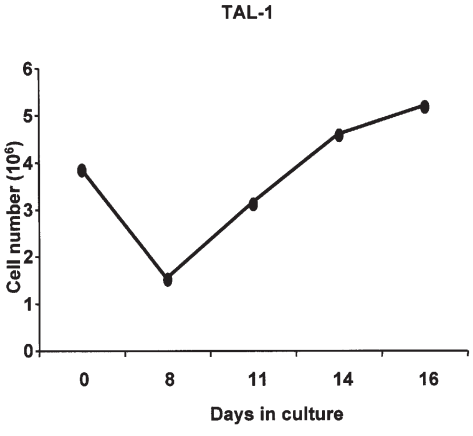

Figure 3. Kinetics of proliferation of TAL-1. Freshly isolated TAL-1 were cultured with 150 IU/ml IL-S. Most cells died in low concentration of IL-2 in the fist 8 days. Surviving cells increased in number afterwards.

Elements of the Notch pathway that regulate differentiation are expressed more frequently in adenocarcinomas whereas Deltex, and Mastermind were more frequent in adenomas (35). qPCR revealed decreased Notch1 mRNA in ovarian adenocarcinomas compared with adenomas. The expression of Notch1-extracellular protein was similar in benign and

Table III. The Notch1 and Numb1-TCR$^+$CD8$^+$ populations based on the density of the specific TCR.

Patient	TCR-density	% TCR$^+$ cells for HLA-A2: peptide				
		NP	Notch1-1947	Notch1-2112	Numb1-87	AES1
1	High	0.19	ND	0.26	0.64[a]	0.19
	Medium	0.27	ND	0.28	0.66[a]	0.23
	Low	0.43	ND	0.24	0.51	0.23
2	High	0.10	0.10	0.17	0.16	ND
	Medium	0.30	0.32	0.35	0.46	ND
	Low	0.85	0.99	2.09[a]	2.76[a]	ND
3	High	0.09	0.10	0.08	0.09	ND
	Medium	0.22	0.24	0.28	0.21	ND
	Low	0.51	0.65	0.43	0.50	ND
4	High	0.11	0.22	0.08	0.22	ND
	Medium	0.13	0.26	0.34[a]	0.26	ND
	Low	0.84	0.53	0.88	0.53	ND
5	High	0.11	0.14	0.17	0.27[a]	ND
	Medium	0.22	0.26	0.36	0.27	ND
	Low	1.98	1.98	2.52	1.84	ND

[a]Significantly higher (2-fold) than the percentage of positive cells reactive with base line control dNP and higher than the specificity control Notch1(1947)-TCR$^+$ cells. Ovarian TALs were cultured for 1 week in medium containing with 300 IU IL-2.

malignant tumors (35). HES-1 protein was strongly expressed in 18/19 ovarian cancers and borderline tumors but not in adenomas. Thus, some of the Notch pathway elements are differentially expressed between adenomas and carcinomas (36).

In separate experiments we found that AES1 is strongly expressed in SK-OV-3 (ovarian cancer cells) and SKBR3 (breast cancer cells). To examine the expression of Notch1 on tumor cell, we stained SK-OV-3, MCF7, and SK-LMS-1 malignant leiomyosarcoma cells with antibodies against Notch1 and corresponding isotype controls. Results in Fig. 2 show that SK-OV-3 and MCF7 express Notch1, but SK-LMS-1 does not express Notch1.

We cultured ovarian ascites with low concentrations of IL-2 to avoid expansion of non-activated clones. Fig. 3 shows the kinetics of growth of tumor associated lymphocyte (TAL). We found that CD8$^+$ Numb1-87-TCR$^+$ cells were present in cultured ascites from patient 1, in higher numbers than the Notch1-2112-TCR$^+$, and AES1-128-TCR$^+$ cells (Fig. 4B-D). Numb-TCR$^+$ CD8$^+$ cells expressed Perforin indicating that these cells were differentiated *in vivo* (Fig. 4G). It should be mentioned that expression of Perforin is controlled by two main signals: one from TCR and the other from IL-2. Since T cells of all specificities were cultured in the same amount of IL-2, our results indicate that differences in Perforin expression were due to activation by antigen.

To address whether Notch1-TCR$^+$ and Numb-TCR$^+$ cells, are present in ascites from other patients we repeated the experiment with ovarian-TAL from four additional HLA-A2$^+$ patients. Table III, and Fig. 5 show that ascites from patients 2, 4, and 5 contained Notch1-2112-TCR$^+$, and Numb1-87-TCR$^+$ CD8$^+$, cells. Notch1-2112-TCR$^+$ and Numb1-87-TCR$^+$ cells were no longer detected in the cultured ascites from patient 3 after two weeks of culture with IL-2, (Table III), indicating that these cells either did not expand or they were diluted because of outgrowth of other T cell populations.

To characterize the CD8$^+$ populations based on the density of the specific TCR, we investigated the presence of TCR^{hi}, TCR^{med}, and TCR^{lo} cells. Fig. 5D and H show the presence of a significant number of Numb1-87-TCR^{lo} CD8$^+$ cells in patient 2, compared with controls, cells interacting with base-line control, empty dimers (dNP-TCR$^+$ cells) and cells interacting with HLA-A2 dimers pulsed with negative control, Notch1-1947 peptide. There was also a small increase in Notch1-2112-TCR$^+$ cells (Fig. 5C and G). These results were confirmed at a separate analysis of CD8$^+$ cells, in the large blast-size population (Fig. 5G and H). The large blast-size T cells are lymphocytes with active cellular synthesis and divide. Similar results were observed with patient 5, with the difference that in this patient Numb1-87-TCR^{hi} CD8$^+$ cells were 2.45-times more than cells reacting with control, dNP-HLA-A2-IgG dimers. Notch1-2112-TCR^{med} cells were also 1.63-times more than cells reacting with the base-line control, dNP (Table III). In patient 4 we found 2.61-times more Notch1-2112-TCR^{med} cells compared with cells interacting with the base-line NP dimers (Table III). These results show that all ascites from all four ovarian patients contained cells bearing TCR for Notch1-2112 and/or for Numb1-87 peptides.

Therefore peptides Notch1-2112 and Numb1-87 not only are generated *in vivo*, but also activate CD8$^+$ cells *in vivo* in the ascites of ovarian cancer patients.

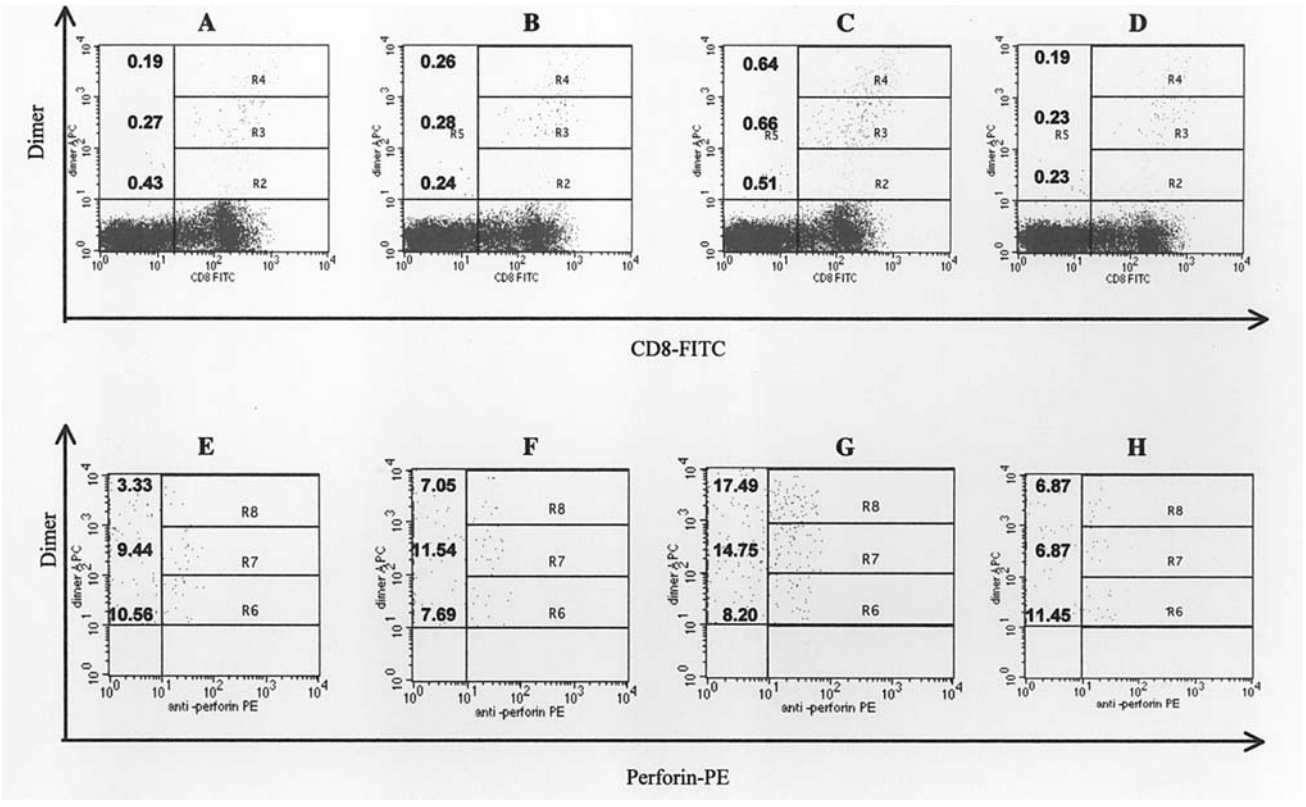

Figure 4. (A) TAL-1 stained with HLA-A2-IgG dimer not pulsed with peptide (*dNP*) was used as a negative dimer control. (B) TAL-1 stained with Notch1-2112 peptide HLA-A2-IgG dimer (*dNotch1-2112*). (C) TAL-1 stained with Numb1-87-HLA-A2 peptide dimer (*dNumb1-87*). Note a 3.3-fold increase in the numbers of TCR^{hi} Per^{hi} cells compared with B. (D) TAL-1 stained with AES1-HLA-A2-IgG peptide dimer. (E-H) TAL-1 stained with antibody against Perforin. (G) Numb1-87-TCR^{+} cells have the highest amount of Perforin.

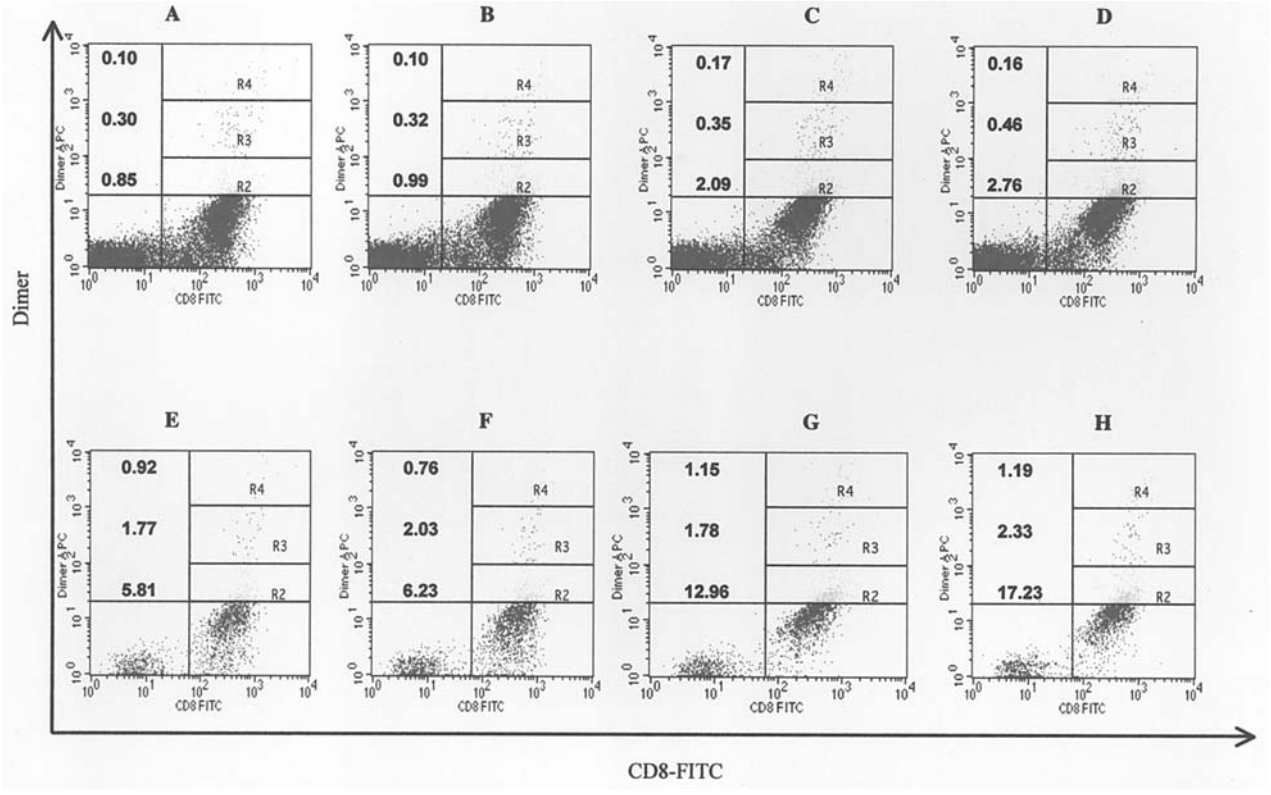

Figure 5. (A-D) Analysis of all gated in TAL-2. (A) TAL-2 stained with HLA-A2-IgG dimer not pulsed with peptide (dNP) was used as a negative dimer control; (B) TAL-2 stained with Notch1-1947 peptide HLA-A2-IgG dimer (dNotch1-1947); (C) TAL-2 stained with Notch1-2112-HLA-A2-IgG dimer (dNotch2112); (D) TAL-2 stained with Numb1-87-HLA-A2-IgG peptide dimer (dNumb1-87). (E-H) Analysis of large-size lymphocytes TAL-2. (E) dNP; (F) Notch1-1947; (G) Notch1-2112; (H) Numb1-87 increases 3-fold the numbers of TCR^{lo}.

Discussion

In this study, we identified candidate peptides from Notch and Numb, which are natural immunogens *in vivo* for $CD8^+$ cells in ovarian cancer patients. The candidate peptides were selected based on their binding motifs to the HLA-A2, HLA-A24, HLA-A33, and HLA-Cw4 molecules. As an additional parameter of stringency we identified the candidate naturally immunogenic peptides produced by the proteasome. Of the peptides identified to be produced by the proteasome we selected only the 'reparable' peptides. Only 'reparable' peptides can be expressed by DNA and RNA vectors which deliver the precursor of tumor Ag in APC.

Surprisingly, we found very few naturally immunogenic peptides from each protein and only one each to be presented in association with HLA-A2. The naturally immunogenic peptides were identified by a novel and sensitive method. We used TA/peptide loaded HLA-A2-IgG dimers, and we determined the specificity of recognition of the ovarian TAL by comparing the staining with negative control dimers which were not loaded with peptides. Differentiation of these lymphocytes was determined by measuring expression of Perforin and the amount of Perforin (as MFI) per cell. We found that two of five patients had activated $CD8^+$ $Perforin^+$ cells expressing TCR specific for the Notch1-2112 peptide and three of five had activated $CD8^+$ $Perforin^+$ cells expressing TCR specific for the Notch1-87 peptide. These $CD8^+$ cells expressed a higher density of TCRs than the known low TCR density of T cells recognizing tumors. Our results predict the use of Notch1-2112 peptide and Numb1-87 peptide for ovarian cancer immunotherapy.

Notch and Numb are expressed not only in ovarian cancer cells but also in breast, pancreas, liver, stomach and colon cancers (5-7,37). Specific immunotherapy targeting these molecules can be effective in elimination of tumors which express those antigens. Recently, Notch and Numb were shown to control differentiation and the metastatic potential of cancer cells. It is possible that immunotherapy targeting Notch and Numb will become a therapeutic choice for cancers of the liver and pancreas which are chemotherapy resistant and rapidly result in the death of patients.

Results of this study also indicate a selectivity of immunogenic TA towards the HLA-A2 system. The HLA-A2 supertype includes in addition to HLA-A2 (subtypes 1-7), HLA-A68.2, and HLA-A69.1. However, when the results of proteasome digestion were compared with the affinity for HLA-A2 subtypes, only HLA-A2.5 could present the same peptide with HLA-A2.1. HLA-A2.5 is considered an ancestral allele, associated with human origins. However Numb1 peptides which can be presented by HLA-A2.5 do not appear to confer protection to cancer. Only Notch2 peptides associated with HLA-A2.5 and HLA-A24 may confer some protection. Is then Notch2 significant for cancer prevention in some African-Americans, while Notch1 is significant for prevention in Caucasians?

The association of Notch1 and Numb1 with HLA-A2.1 may be significant for cancer prevention in Caucasians and Hispanics. Is then protection from liver and pancreatic cancer due to the 'redundancy' of the immunosurveillance first by Numb1 and then by Notch1.

Peptides binding to HLA-A24 were negatively selected for presentation. We found only the decamer Notch2 (1940-1949), as both potentially binding to HLA-A24 and produced by proteasome digestion. None of the Notch1 and Numb1 peptides associated with HLA-A24 were positively selected. The HLA-A24 product is frequently preset in South-East Asian, especially it is most frequent in Japan (38).

There are clear differences in cancer incidences among different ethnic groups. For example, there is at least a 25-fold variation in occurrence of colorectal cancer worldwide. The highest incidence rates are in North America, Australia/New Zealand, Western Europe and, in men especially, Japan (49.3 per 100,000); incidence tends to be low in Africa and Asia (e.g. P.R. China, 13.6 per 100,000 in men) and intermediate in southern parts of South America. For gastric cancer, geographical distribution of stomach cancer is characterized by wide international variations; high-risk areas include East Asia (e.g., Japan; age standardized rate, 62.1), Eastern Europe, and parts of Central and South America. Incidence rates are low in men in Southern Asia, North and East Africa, North America (e.g., age standardized rate of only 7.4), and Australia and New Zealand. The incidence of pancreatic cancer is highest among USA and Japan (11.8 and 10.9 per 100,000 respectively), while it is lowest in Africa and P.R. China (2.1 and 6.3 per 100,000, respectively). Many factors could have contributed to the wide variation, e.g. diet, environment, habits (smoking and drinking history), and genetics. Immunogenetics could certainly be one of the contributing factors (39).

Such factors may include the composition of the diet, and at the same nominal composition of the diet, the presence in the diet of compounds which interfere with metabolic or tissue regeneration pathways. Development of immunotherapy against Notch1 and Numb with peptide vaccines may be useful for populations at high risk of developing rapidly deadly cancers.

When this manuscript was prepared for publication, Park *et al* reported that Notch-3 is overexpressed in ovarian cancer (37). We found 6 Notch-3 peptides that bind to HLA-A2 molecules and are digested by proteasome type I enzymatic activity, but few or none digested by protesome type II, or type III. Notch-3 peptides may be good targets for cancer immunotherapy.

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