Cancer Immunol Immunother (2009) 58:1185–1194
DOI 10.1007/s00262-008-0623-1

ORIGINAL ARTICLE

Breast cancer cells expressing stem cell markers CD44^{+} CD24^{lo} are eliminated by Numb-1 peptide-activated T cells

Takashi Mine · Satoko Matsueda · Yufeng Li · Hiroshi Tokumitsu · Hui Gao · Cristopher Danes · Kwong-Kwok Wong · Xinhui Wang · Soldano Ferrone · Constantin G. Ioannides

Received: 22 May 2008 / Accepted: 29 October 2008 / Published online: 2 December 2008

Abstract Cancer stem cells (CSC) are resistant to chemo- and radiotherapy. To eliminate cells with phenotypic markers of CSC-like we characterized: (1) expression of CD44, CD24, CD133 and MIC-A/B (NKG2 receptors) in breast (MCF7) and ovarian (SK-OV-3) cells resistant to gemcitabine (GEM), paclitaxel (PTX) and 5-fluorouracil (5-FU) and (2) their elimination by Numb- and Notch-peptide activated CTL. The number of cells in all populations with the luminal CSC phenotype [epithelial specific antigen^{+} (ESA) CD44^{hi} CD24^{lo}, CD44^{hi} CD133^{+}, and CD133^{+} CD24^{lo}] increased in drug-resistant MCF7 and SK-OV-3 cells. Similarly, the number of cells with expressed MIC-A/B increased 4 times in drug-resistant tumor cells compared with drug-sensitive cells. GEM^{Res} MCF7 cells had lower levels of the Notch-1-extracellular domain (NECD) and Notch trans-membrane intracellular domain (TMIC) than GEM^{Sens} MCF7. The levels of Numb, and Numb-L-[P]-Ser^{265} were similar in GEM^{Res} and GEM^{Sens} MCF7 cells. Only the levels of Numb-L (long)-Ser^{295} decreased slightly. This finding suggests that Notch-1 cleavage to TMIC is inhibited in GEM^{Res} MCF7 cells. PBMC activated by natural immunogenic peptides Notch-1 (2112–2120) and Numb-1 (87–95) eliminated NICD^{positive}, CD24^{hi} CD24^{lo} MCF7 cells. It is likely that the immunogenic Numb-1

Electronic supplementary material The online version of this article (doi:10.1007/s00262-008-0623-1) contains supplementary material, which is available to authorized users.

T. Mine · S. Matsueda · Y. Li · K.-K. Wong · C. G. Ioannides
Department of Gynecologic Oncology,
The University of Texas M. D. Anderson Cancer Center,
Houston, TX 77030, USA

T. Mine
Departments of Immunology and Surgery,
Kurume University School of Medicine, Kurume, Japan

H. Tokumitsu
Department of Cell Signaling, Faculty of Medicine,
Kagawa University, Kagawa, Japan

H. Gao
Department of Molecular Pathology,
The University of Texas M. D. Anderson Cancer Center,
Houston, TX 77030, USA

C. Danes
Department of Molecular and Cellular Oncology,
The University of Texas M. D. Anderson Cancer Center,
Houston, TX 77030, USA

Y. Li
Department of Melanoma Medical Oncology,
The University of Texas M. D. Anderson Cancer Center,
Houston, TX 77030, USA

X. Wang · S. Ferrone
Departments of Surgery, Immunology and Pathology,
University of Pittsburgh Cancer Institute,
Pittsburgh, PA 15123, USA

C. G. Ioannides
Department of Immunology,
The University of Texas M. D. Anderson Cancer Center,
Houston, TX 77030, USA

T. Mine (✉)
Multidisciplinary Treatment Center,
Kurume University School of Medicine,
67 Asahi-machi, Kurume 830-0011, Japan
e-mail: mine@med.kurume-u.ac.jp

peptide in MCF7 cells originated from Numb, [P]-lated by an unknown kinase, because staurosporine but not wortmannin and MAPK-inhibitors decreased peptide presentation. Numb and Notch are antagonistic proteins which degrade each other to stop and activate cell proliferation, respectively. Their peptides are presented alternatively. Targeting both antagonistic proteins should be useful to prevent metastases in patients whose tumors are resistant to conventional treatments.

Keywords Drug resistance · Breast/ovarian · Cancer stem cell · Notch · Numb · Peptide

Introduction

Renewal of embryonic (ESC) and adult stem cells (ASC) is regulated by signals from the surrounding environment. It is possible that cancer stem cells (CSC) originate from normal-ASC, or their intermediate progenitors (IP) which accumulate oncogenic mutations. More recently, they were shown to originate from ASC which activate embryonal differentiation programs. Regardless of their origin CSC renew after activation by Notch and amplification by Hedgehog and β-catenin (Wnt)-signals of Notch signals [2, 20, 23].

Breast cancer cells with high levels of CD44 ($CD44^{hi}$) and absent or low CD24 ($CD24^{neg/lo}$) expression have functional characteristics of CSC. Brain, colon and prostate cancer cells with CSC-characteristics express CD133 (prominin-1) [1, 3, 10, 28, 31].

The levels of CD44 (an adhesion molecule which binds to hyaluronate) directly correlate with metastasis in breast cancer. The levels of CD24 (an adhesion molecule that binds P-selectin) inversely correlate with survival of ovarian cancer patients. Studies in human mammary cells suggest that the most primitive mammary cells do not express estrogen-receptors, but are capable to differentiate to estrogen positive, luminal epithelial cells [6, 18, 24, 33].

CSC are more resistant to chemo- and radiotherapy than non-CSC [17, 25]. Histone-deacetylase (HDAC) and poly-A-ribose polymerase (PARP)-inhibitors are weak anti-CSC effectors. To avoid toxicity, it was proposed to lower doses of standard chemotherapy when used together with HDAC/PARP inhibitors [12, 26, 27].

A novel approach to cancer therapy is to eliminate CSC with immune effectors. We hypothesized that we can eliminate CSC by targeting peptides from the antagonistic Notch and Numb proteins with cytotoxic T lymphocytes (CTL). When CSC renew or become quiescent Notch and Numb induce degradation of each other, respectively. Notch and Numb are degraded to peptides by the proteasome. Peptides from NICD and Numb proteins are presented by HLA-class I molecules of cancer cells to T cells.

Hetero-dimeric Notch (hNotch) consists of an extracellular domain named NECD and a trans-membrane and intracellular domain, named NICD. NICD and NECD are linked by disulfide bonds. hNotch, located trans-membrane, is activated by its ligands. Human Notch-ligands belong to the Jagged (Jag) and Delta-like (DLL) families. Activation/inhibition of Notch can proceed in *Cis*- by Notch ligands expressed on the responding cell or in *Trans*-from Notch-ligand expressed by neighboring cells. Notch signals are transduced i by NICD, which activates gene transcription [14].

Numb has four isoforms. They form two groups Numb-Long (Numb-L) and Numb-Short (Numb-S) which differ in length by 5 kDa. Each group has two isoforms, which differ by 1 kDa, and are difficult to distinguish. Both Numb-L and Numb-S are present in cancer cells. However, most human cancer studies do not resolve Numb-S from Numb-L and Numb-like [19]. Non-phosphorylated Numb is adjacent to membrane. After receiving external signals Numb is [P]-lated at Ser^{295} and migrates to cytoplasm. In cytoplasm Numb-[P]-Ser^{295} cannot bind NICD to block cell-cycle activation. [P]-lation of Numb at other/additional sites directs its degradation by proteasome. The cells which degrade most Numb divide symmetrically, while the cells which do not degrade cytoplasmic Numb divide asymmetrically [4, 32].

Symmetric division of stem cells which lack Numb, double the number of "mother" stem cells. Asymmetric division of cells which contain both Notch and Numb results in one "mother" stem cell and one different cell [daughter/IP-stem cell [4, 32]]. Degradation of NICD by Numb inhibits activation of transcription by NICD and cells become quiescent [9, 11, 19].

The aim of this study was to determine whether: (1) the proportion of cells with CSC-phenotype/putative CSC increases in drug-resistant cancer lines, and (2) drug-resistant cells with CSC-phenotype can be eliminated by Notch- and Numb-peptide activated CTL.

We found higher numbers of $CD44^{hi}$ $CD24^{lo}$, $CD44^{hi}$ $CD133^{+}$ and $CD24^{lo}$ $CD133^{+}$ cells in gemcitabine (GEM^{Res}), paclitaxel (PTX^{Res}) and 5-fluorouracil ($5\text{-}FU^{Res}$) resistant breast and ovarian cancer lines compared with Drug sensitive ($Drug^{Sens}$) cells. Three-four times more Drug resistant ($Drug^{Res}$) cells expressed NKG2D receptors than $Drug^{Sens}$ cells. $NICD^{+}$ and $CD44^{hi}$ $CD24^{lo}$ cells were eliminated by Notch-1 (2112–2120) and Numb-1 (87–95) peptide-activated peripheral blood mononuclear cells (PBMC) and to a lesser extent by interleukin (IL)-2-activated PBMC.

Materials and methods

Cell lines and drugs

MCF7 and SK-OV-3 cell lines were from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 medium with 10% FBS, 100 U/L penicillin, and 100 μg/mL streptomycin. We used gemcitabine (Eli-Lilly Indianapolis, IN), paclitaxel (Bristol-Myers Squibb, Princeton, NJ), 5-fluorourcil (Sigma Chemical Co. St Louis, MO) and human recombinant DLL4 (rhDLL4, R&D Systems, Inc. Minneapolis, MN).

Antibodies

The mAb to human antigens included anti-ESA (Biomeda, Foster City, CA), anti-CD44 (BD Pharmingen, San Diego, CA), anti-CD44 (BD), anti-CD24 (BD), anti-CD24 (Abcam Inc., Cambridge, MA), anti-MHC class I chain-related gene A (MICA)/MHC class I chain-related gene B (MICB) (R&D Systems, Minneapolis, MN), anti-CD133/2 (Miltenyi Biotec Inc., Auburn, CA), and anti-Notch-1 (BD). Polyclonal Abs included anti-Notch-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-NICD, anti-Numb, and anti-Bcl-2 mAb (Abcam).

mAb against rat Numb [P]-Ser^{264} and Numb [P]-Ser^{283} = Human Numb-L-P-Ser^{265} and ^{295} were prepared by Dr. Hiroshi Tokumitsu [34, 35]. Numb has many N-terminal splice variants. The position of the corresponding residues to [P]-Ser^{264} and [P]-Ser^{283} of rat Numb differs among isoforms. In human Numb-S is [P]-Ser^{265} and [P]-Ser^{284} [Numb-3 (*NP_003735)* and Numb-4 (*NP_0010057450)*]. Rat [P]-Ser^{276} is [P]-Ser^{295} in human Numb-L [Numb-1 (*NP_001005743)* and Numb-2 *(NP_001005744)*]. Rat [P]-Ser^{283} corresponds to Ser^{295} in human Numb-L. We used the designations [P]-Ser^{265} and [P]-Ser^{295} since they are close to the [P]-Ser positions reported in the recent literature.

IC_{50} of anticancer drugs

The 50% inhibitory concentration (IC_{50}) of GEM, PTX and 5-FU was determined after a 72-h incubation with drugs as described [13].

Flow cytometry

Cells (25–30 × 10^6) were cultured with drugs at 2 × IC_{50} for 4 days followed by drug at 0.5–1.0 × IC_{50} for the next 3–6 days. Surviving cells (2 × 10^5) were incubated first with 20 μg of human IgG (Sigma) for 1 h on ice to inhibit non-specific binding of specific mAb during staining. Analysis was performed using a Becton Dickinson FACS^{Calibur} with Cell^{Quest} software (BD). Expression of CD24, CD44 and CD133 was quantified in gated ESA$^+$ cells as described [16].

CSC-like and their progenitors were characterized following the geometrical mean (x^2) of CD24 in CD44^{hi} cells. CD24^{negative} = MFI, 0–10, CD24^{lo} = MFI, 10–100, CD24^{hi} = MFI, 100–1,000 [16]. ESA$^+$ [(CD44^{hi} and CD24$^{lo/hi}$), and (CD133$^+$)] cells were quantified using the formula: (% of total ESA$^+$ cells) × (% of cells positive for both markers) × (number of cells in the culture).

Activation of GEM^{Res} MCF7 cells

Cells were stimulated for 24 h in serum free medium with 62.5 ng/mL rhDLL4 then cultured with or without GEM for 7 days. GEM^{Res} MCF7 cells were stimulated three times with DLL4 (62.5 ng/mL) weakly for 24 h.

Sensitivity of GEM^{Res} MCF7 cells to HLA-A2$^+$ PBMC activated by Notch- and Numb-peptides

Notch-1 = NICD peptide (2,112–2,120) and Numb-1 = Numb-1-PTB domain peptide (87–95) were previously identified by us as immunogenic [15]. Non-adherent PBMC were activated with peptide-pulsed autologous immature monocyte-derived dendritic cells (iDC), or T2 cells as we described [37]. In all activation procedures, we used IL-12 as co-factor followed by IL-2 48 h later.

Immunoselection

Because autologous T cells to MCF-7 are not available, we used allogeneic immunoselection. GEM^{Res} MCF7 cells (1.0 × 10^5) were co-cultured in six-well plates with 3.0 × 10^5 IL-2-activated, Notch-1-, or Numb-1-peptide-activated PBMC for 5 days. IL-2 was not added to cultures for 72 h before use. Residual IL-2 was washed out. Surviving MCF7 cells were analyzed by flow-cytometry.

Western blot

Cell lysates with nuclei removed were prepared from live MCF7, and SK-OV-3 cells. Immunoblotting and quantification of NECD, NICD, Numb, and Bcl-2 utilizing β-actin as a reference was performed in relation to actin in the same sample, as we described [8, 37].

Results

The number of ESA$^+$ [(CD44$^+$ CD24^{lo}), (CD44$^+$ CD133$^+$), and (CD24^{lo} CD133$^+$)] cells increase in drug-resistant MCF7 and SK-OV-3 cells

We quantified the IC_{50} of GEM, 5-FU and PTX for MCF7 and SKOV-3 cells. The IC_{50} (GEM) for MCF7 cells was

430 nM and the IC_{50} (GEM) for SK-OV-3 cells was 16.1 nM. The IC_{50} (5-FU) for MCF7 cells was 1,302 nM. The IC_{50} (5-FU) for SK-OV-3 cells was 3,560 nM. MCF7 cells were more sensitive to 5-FU than SK-OV-3 cells, and more resistant to GEM than SK-OV-3 cells. The IC_{50} (PTX) was similar for both cell lines = 4–5 nM.

To verify that ESA levels are the same in drug-resistant and sensitive cells we determined expression of ESA. The levels of ESA were the same in $Drug^{Res}$ and $Drug^{Sens}$ cells (Fig. 1). $Drug^{Res}$ MCF7 cells were luminal cells (Fig. 1 and Supplementary Table 1). The number of cells with CSC-markers increased five and tenfold in MCF7 and SK-OV-3 cells, respectively. 5-FU and PTX selected CSC-like cells with similar efficiency with GEM. The number of $CD133^+$, $CD133^+$ $CD44^{hi}$ and $CD133^+$ $CD24^{lo}$ cells increased by 3–5 fold in GEM^{Res} MCF7 cells and GEM^{Res} SK-OV-3 cells compared with the $Drug^{Sens}$ cells (Fig. 2d).

GEM^{Res} MCF7 cells have lower levels of NECD than GEM^{Sens} cells

We found differences in the expression of antagonistic proteins Notch and Numb, between $Drug^{Res}$ and $Drug^{Sens}$ cells. Reducing conditions break the disulfide bond between NECD and TMIC. TMIC is further cleaved between Ala^{1710} and Val^{1717} to generate the Notch-extracellular truncated domain (NEXT). NEXT is further cleaved at Val^{1744} to generate free NICD [5, 21, 30].

NECD levels decreased in GEM^{Res} MCF7 cells compared with GEM^{Sens} MCF7 cells. Longer ER-precursors of Notch-1 were present in similar amounts in GEM^{Res} and GEM^{Sens} MCF7 cells (Fig. 3a). TMIC levels also decreased in GEM^{Res} MCF7 cells. The amount of NICD was low and similar in GEM^{Res} and GEM^{Sens} cells. The decrease in Notch-1 suggested that Notch-1-NECD was degraded and not replaced, since the amount of its ER-precursor was

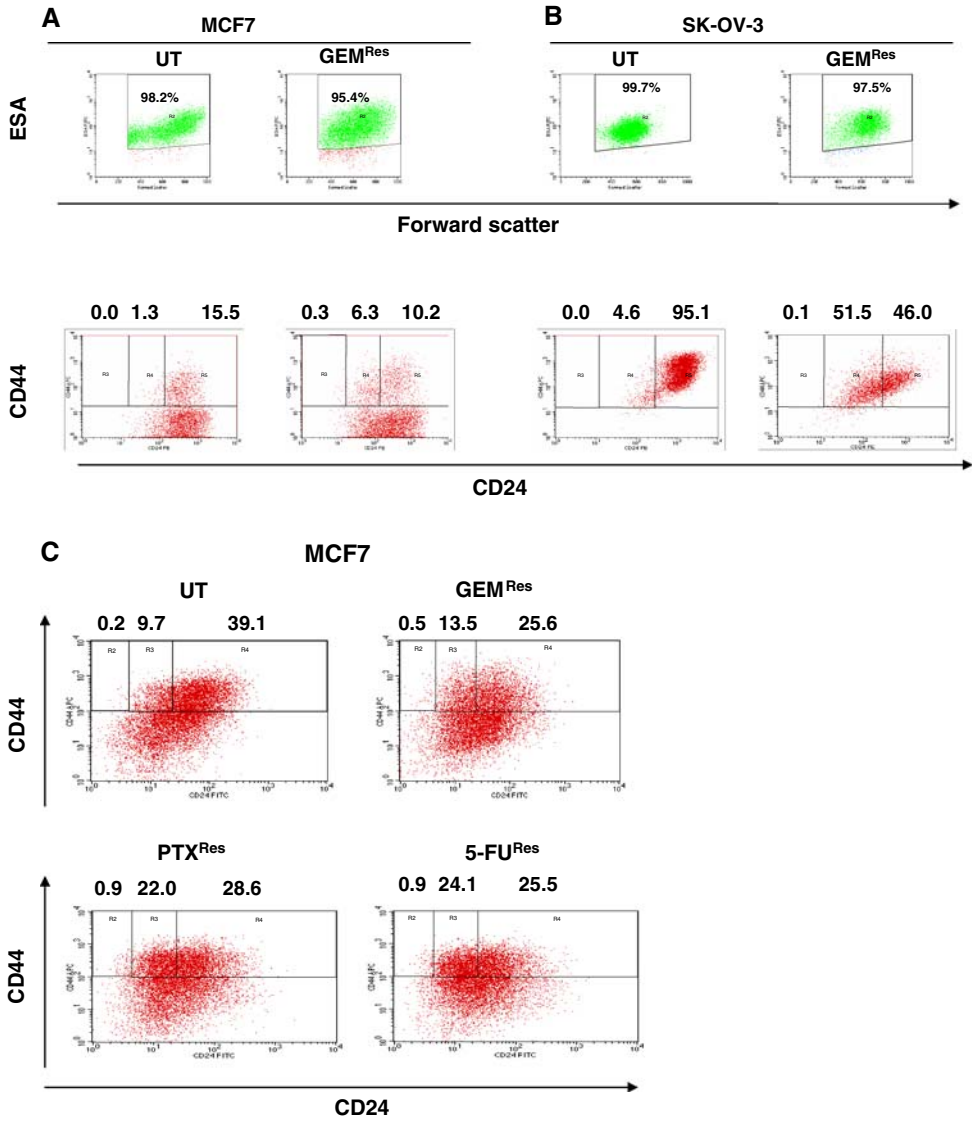


Fig. 1 **a**, **b** Increase in number of $CD44^+$ $CD24^{lo}$ cells in GEM^{Res} MCF7 and SK-OV-3 cells. MCF7 [MFI ($CD24^{lo}$)] cells range 10–100. **c** $CD24^{lo}$ MCF7 cells (MFI between 5 and 50) increase in number in $5\text{-}FU^{Res}$ and PTX^{Res} MCF7 cells. This finding was confirmed in two additional independently performed experiments

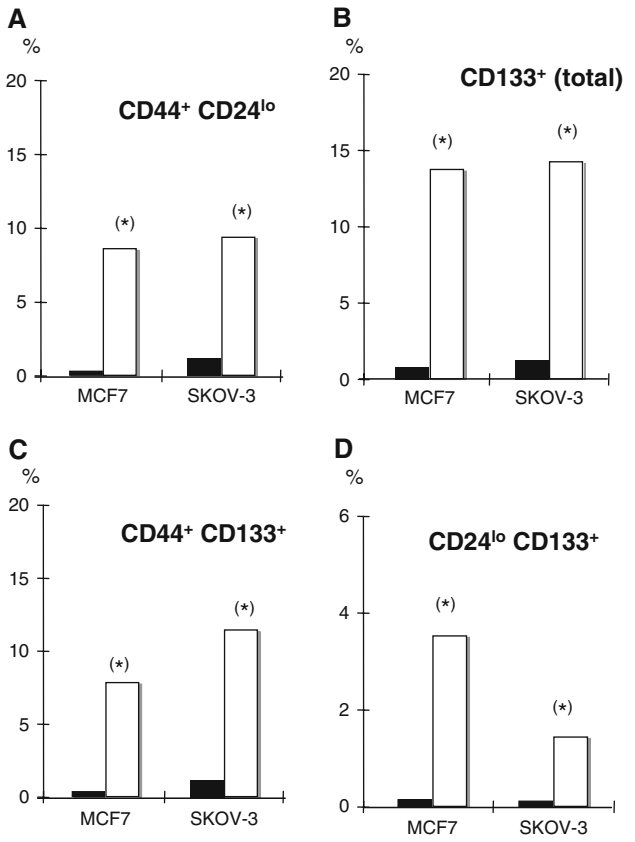


Fig. 2 CD133$^+$ cells increased in number in GEM^{Res} cells. (*) significant increase >twofold. Untreated cells (*black columns*); GEM^{Res} cells (*white columns*)

higher. This means that less of Notch-1-ER-precursor was cleaved to TMIC (Fig. 3a). The levels of TMIC were lower than in control cells. The levels of Numb were slightly lower in GEM^{Res} and untreated MCF7 cells (Fig. 3a). The ratio of NECD and TMIC (pre-NICD) to Numb-L and Numb-S decreased (see OD values). Therefore "quiescent" GEM^{Res} MCF7 cells have lower levels of Notch-NECD than "quiescent" GEM^{Sens} MCF7 cells. Control, dividing SK-OV-3 cells synthesized high amounts of Notch and expressed both TMIC (of 100 kDa) and NICD (of 80 kDa) (Fig. 3c).

Numb-L and Numb-S were [P]-lated at Ser^{295}, in both untreated and GEM^{Res} cells. The amount of Numb-[P]-Ser^{295} decreased by 30%, in GEM^{Res} cells. The amount of Numb-L and Numb-S-[P]-Ser^{265} did not change in GEM^{Res} cells compared with untreated cells (Fig. 3b). Our results indicate differences in Ser^{295}-phosphorylation between GEM^{Sens} and GEM^{Res} cells.

SK-OV-3 cells had more Numb-S than Numb-L. Furthermore SK-OV-3 cells had a lower ratio of Numb-L/S-[P]-Ser^{295} to Numb-L/S than MCF7 cells and ratios of Numb-L/S-[P]-Ser^{265} to Numb-L/S similar to those in MCF7 cells (Fig. 3c).

Soluble DLL4 expanded more CD24^{hi} cells from GEM^{Res} cells

The mRNA profile of Notch family members and of Notch ligands in MCF7 cells, untreated and treated with chemotherapeutics, has been reported. MCF7 has lower levels of Notch-ligands of the Delta-like family and higher levels of Notch-ligands of the Jagged-family [39]. MCF7 cells have similar levels of Notch-1 and Notch-2 with normal cells but significantly lower levels of Notch-3 and 4. We investigated the effects of Notch activation by its ligand, DLL4, on expansion of MCF7 cells. DLL4 expanded more CD24^{hi} than CD24^{lo} cells. DLL4 did not expand CD24^{hi} cells in the presence of GEM (Supplementary Table 2). Many rhDLL4-expanded cells were CD44^{lo} CD24^{lo} and CD44^{lo} CD24^{hi} (not shown). Therefore, rhDLL4 did not preferentially stimulate proliferation of CD24$^{neg/lo}$ cells.

Naturally immunogenic Notch and Numb peptides expand Ag-specific CD8$^+$ T cells

Our results show that a part of Numb-L/S is marked by [P]-lation. These findings suggest that cells with CSC-markers can be eliminated by HLA-A2-Notch-1, and Numb-1-peptide: complex-specific CTL.

Peptides Notch-1 and Numb-1 expanded Notch-1$^+$-TCR$^+$ CD8$^+$ and Numb-1$^+$-TCR$^+$ CD8$^+$ cells from the PBMC of a HLA-A2$^+$ healthy donor (Fig. 4a, b). The Numb-1 peptide was more immunogenic than the Notch-1 peptide because it activated more CD8$^+$ cells at 1 μM, whereas Notch-1 did so at a concentration of 5 μM.

Numb-1 peptide-activated PBMC produced IFN-γ when incubated with SK-OV-3.A2 cells

Numb-1 and NICD-1 peptide-activated PBMC produced similar amounts of IFN-γ, in the first 24 h of co-culture with SK-OV-3.A2 cells. The same levels of IFN-γ were produced by control peptide, Notch-1-1947, which is not generated by proteasome. The SK-OV-3.A2 cell line acquires expression of HLA-A2 following transfection with a HLA-A2 expression plasmid. IFN-γ produced by Numb-1-activated cells doubled at 48 h of co-culture. The amount of IFN-γ produced by Notch-1-activated cells did not increase and remained similar to the amount produced by IL-2 activated cells (Fig. 4c). Therefore, either SK-OV-3 cells presented more Numb-1 peptide than Notch-1 peptide to CD8$^+$ cells, or Numb-1-CD8$^+$ cells have higher functional avidity for HLA-A2-Numb-1 peptide complexes.

To identify whether Numb-degradation is activated by [P]-lation, we repeated the experiment with inhibitors of Ser–Thr-kinases Wortmanin did not inhibit presentation of the Numb-1 peptide, while SB-20380 had a marginal late

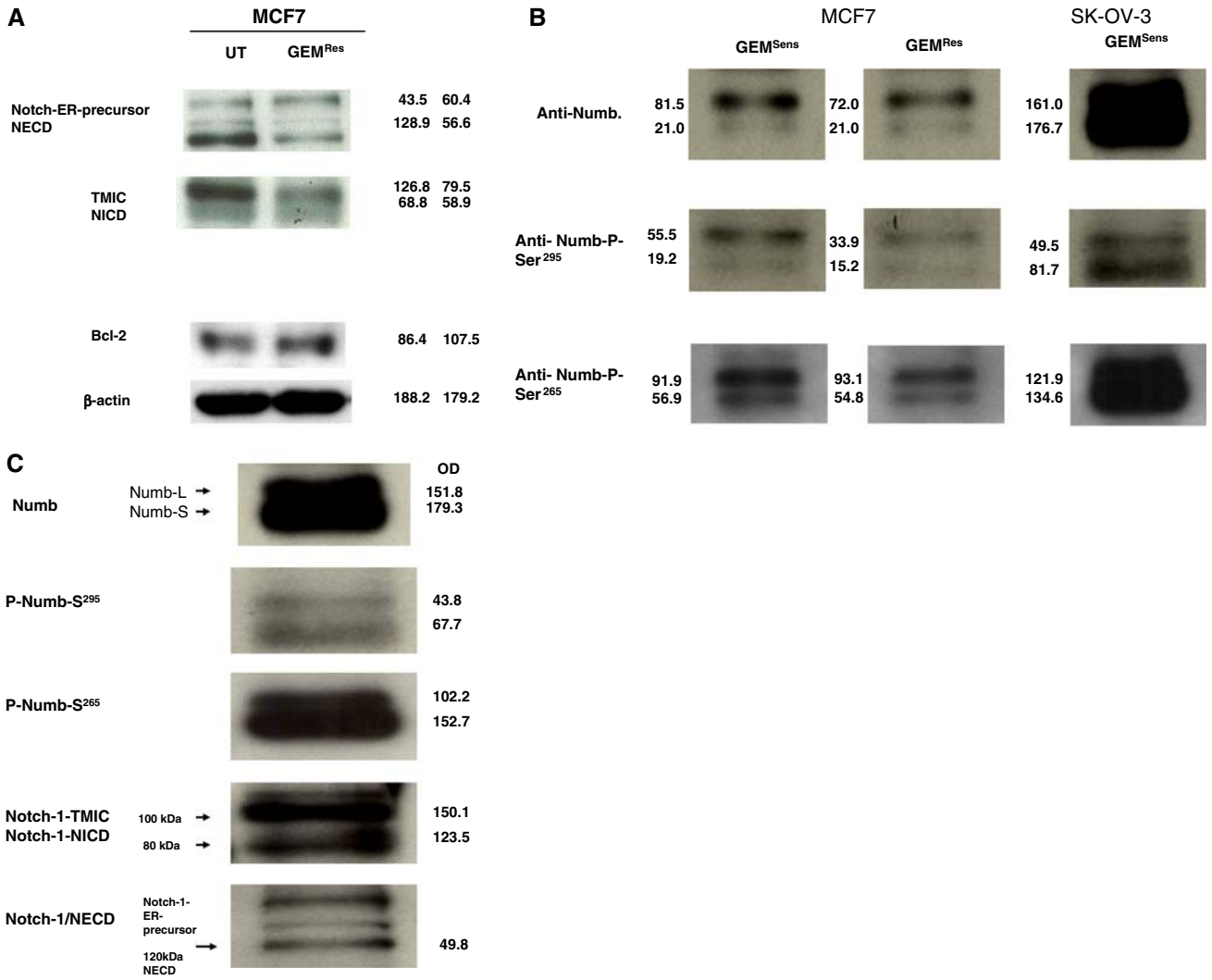


Fig. 3 **a** Decreased expression of Notch-1 (*NECD*) and Notch-1 (*TMIC*) in GEM^{Res} MCF7 cells compared with untreated GEM^{Sens} MCF7 cells. One of the two experiments is shown **b** Numb-L and Numb-S are [P]-lated at Ser^{265} and Ser^{295} in GEM^{Sens} and GEM^{Res} cells. MCF7 cells contain significantly less Numb-S than SKOV3 cells. **c** Numb is more [P]-lated at Ser^{265} than at Ser^{295} NECD was detected with mAbs-scc3275 (recognize the whole Notch molecule). H131 mAb detects two TMIC of 100 kDa and NICD of 80 kDa, respectively. One of two experiments is shown

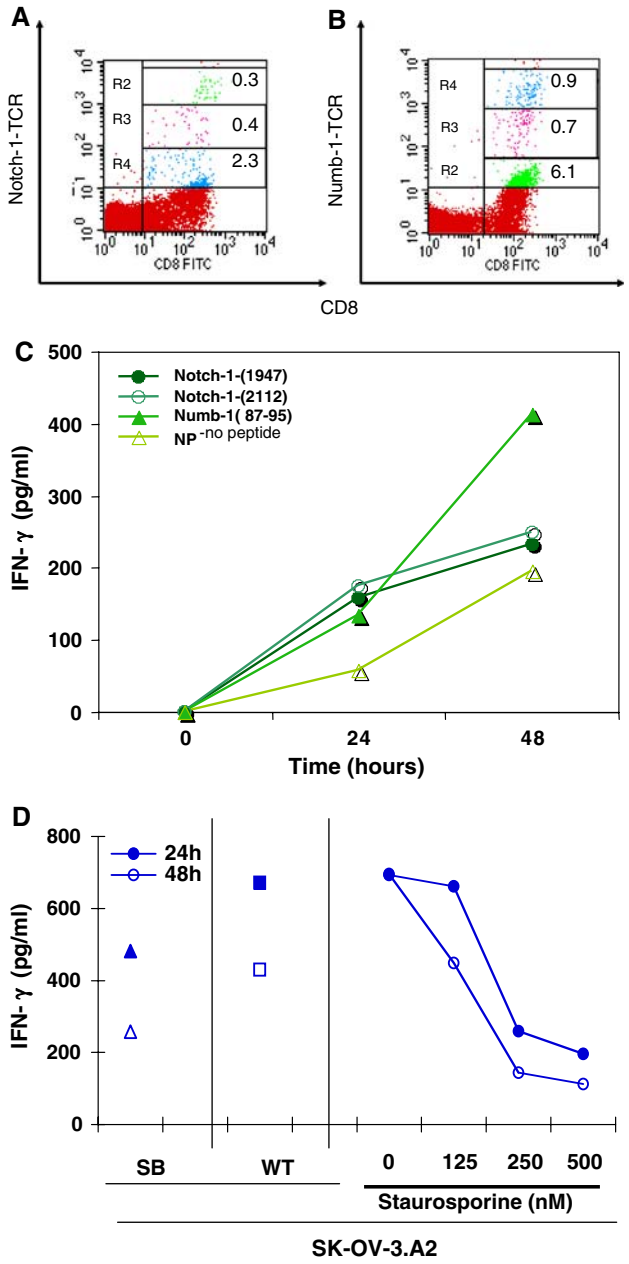
effect (Fig. 4d). The strongest inhibition of Numb-1 peptide presentation was mediated by staurosporine, a broad-spectrum inhibitor of protein–serine–threonine kinase family, indicating that an identified kinase is involved in Numb [P]-lation and degradation.

GEM^{Res} MCF7 cells express more NKG2D ligands than GEM^{Sens} MCF7 cells

To determine whether cells with CSC-markers are sensitive to cellular effectors, other than Ag-specific CD8$^+$ T cells, we quantified expression of MIC-A/-B in GEM^{Res}, PTX^{Res} and 5-FU^{Res} MCF7 cells. The percentage of MIC-A/B$^+$ cells increased by 4.5 fold (83.9%) in CD44^{hi} CD24^{lo} GEM^{Res} cells and by threefold (57.5%) in CD44^{hi} CD24^{lo} PTX^{Res} MCF7 cells (Fig. 5a). The percentage of MIC-A/B$^+$ CD133$^+$ cells increased from 0.22 in GEM^{Sens} to 6.34 in GEM^{Res} MCF7 cells (not shown). The mean fluorescence intensity values show that the density of MIC-A/B receptors per cell was similar in Drug^{Sens} and Drug^{Res} MCF7 cells. Therefore, more drug-resistant CSC-like cells will be sensitive to NK/NK-T cells than Drug^{Sens} cells. However, the sensitivity of each CSC-like cell to NK/NK-T cells is not expected to increase compared with Drug^{Sens} cells.

Allogeneic Notch and Numb peptide-activated PBMC eliminated cells with CSC-phenotype markers

We investigated whether IL-2-activated, Notch-activated, and Numb-activated allogenic PBMC eliminate cells with



◀ **Fig. 4** Peptides Notch-1 and Numb-1 expanded antigen-specific CD8$^+$ cells. **a** Notch-1-TCR$^+$ CD8$^+$ cells expanded by incubation with 5 μg Notch-1 (2112–212)and **b** Numb-1-TCR$^+$ CD8$^+$ cells expanded by incubation with 1 μg Numb-1 (87–95). TCR^{hi}, TCR^{med}, and TCR^{lo}, populations are shown in gates R2, R3 and R4. *Numbers* in each box indicate the percentage of Ag-specific cells in the entire population. In the absence of Ag, IL-2 induced expansion of Ag-specific cells, was: Notch-1-TCR$^+$ cells = (TCR^{hi}: 0.1%, TCR^{med}: 0.1%, TCR^{lo}: 1.0%). Numb-1-TCR$^+$ cells = (TCR^{hi}: 0.3%, TCR^{med}: 1.0%, and TCR^{lo}: 0.4%) in PBMC from the same donor cultured with IL-2. **c** SK-OV-3.A2 cells present Numb-1peptide to Numb-1 peptide-activated PBMC. **d** Presentation of Numb-1 peptide to Numb-1 peptide-activated cells is dependent on [P]-lation by protein-Ser/Thr-kinases PI3 K does not appear to be involved in peptide presentation as shown by lack of effect of wortmannin. The MAPK-kinase inhibitor SB20380 had a weak inhibitory effect. IFN-γ was quantified at 24 h (*Closed symbols*) and at 48 h (*Open symbols*). Numb-1 peptide-activated PBMC produced more IFN-γ than Notch peptide-activated PBMC. At 48 h the amount of IFN- γ produced by two Notch peptide-activated cell lines was similar with the amount produced by the IL-2-activated cell lines. Only Notch-1 peptide can be presented by HLA-A2 antigens after Notch digestion by proteasome according to the program *paproc.de*

CSC markers. To account for elimination of cells with CSC markers by allogeneic effectors we repeated the experiments, in the presence of IL-2-activated PBMC, and quantified each surviving population of CD44 CD24 cells. Therefore, in addition to allo-recognition of tumor cells by effectors, a significant recognition was due to Numb-1 peptide activated T cells. Forty-five percent of GEM^{Res} cells had detectable NICD (Fig. 5b). Notch-1-positive cells decreased by 68.5% (from 45.4 to 14.3%) after co-culture with Notch-1 peptide-activated PBMC (Fig. 5d). Numb-1 peptide-activated PBMC decreased the NICD$^+$ cells only by 25.3% (from 45.4 to 33.9%), whereas IL-2-activated non-specific PBMC had no significant effect (Fig. 5c, e). Therefore, Notch-1-specific CD8$^+$ T cells specifically eliminated NICD$^+$ cells.

To eliminate cells with CSC markers we repeated the experiments and quantified each surviving population of CD44 CD24 cells. To increase stringency of elimination, we used as target MCF7 cells because they expressed less Numb-L and ten times less Numb-S than SK-OV-3 cells.

Numb-1-activated T cells were more effective than Notch-1 and IL-2-activated effectors. Only 10% of the initially plated GEM^{Res} cells survived. The majority of surviving cells were CD44^{hi} CD24^{lo}. Numb-1-activated cells eliminated 2–3 times more CD44^{hi} CD24^{lo} MCF7 cells than other effectors (Fig. 5f. Columns All and CD44^{hi} CD24^{lo}). Per effecter-cell number, Numb-1-peptide activated cells were more effective than IL-2-activated cells, in eliminating CD44^{hi} CD24^{hi} cells (Fig. 5f, Column CD44^{hi} CD24^{hi}).

Discussion

We identified populations in breast and ovarian tumor cell lines which express the phenotypes of breast (ESA$^+$/CD44$^+$/CD24$^{-/lo}$) and brain, colon and prostate (CD133$^+$) CSC. Because their function was not yet characterized, we designated these populations as CSC-like. CSC-like cells increased in Drug^{Res} MCF7 and SK-OV-3 cells. Our novel findings are: (1) CD24^{lo} population increased in Drug^{Res} cells regardless of the mechanism of drug action; (2) The brain, colon and prostate CSC marker, CD133, is present on cells which express the breast CSC-markers; (3) Cells with CSC-markers/CSC-like cells in GEM^{Res} MCF7 cells were eliminated by Numb-1 peptide-activated PBMC.

GEM and 5-FU are inhibitors of DNA and RNA synthesis, which are incorporated in newly synthesized strands. Neither GEM nor 5-FU affects cells in G_1 phase [36]. These

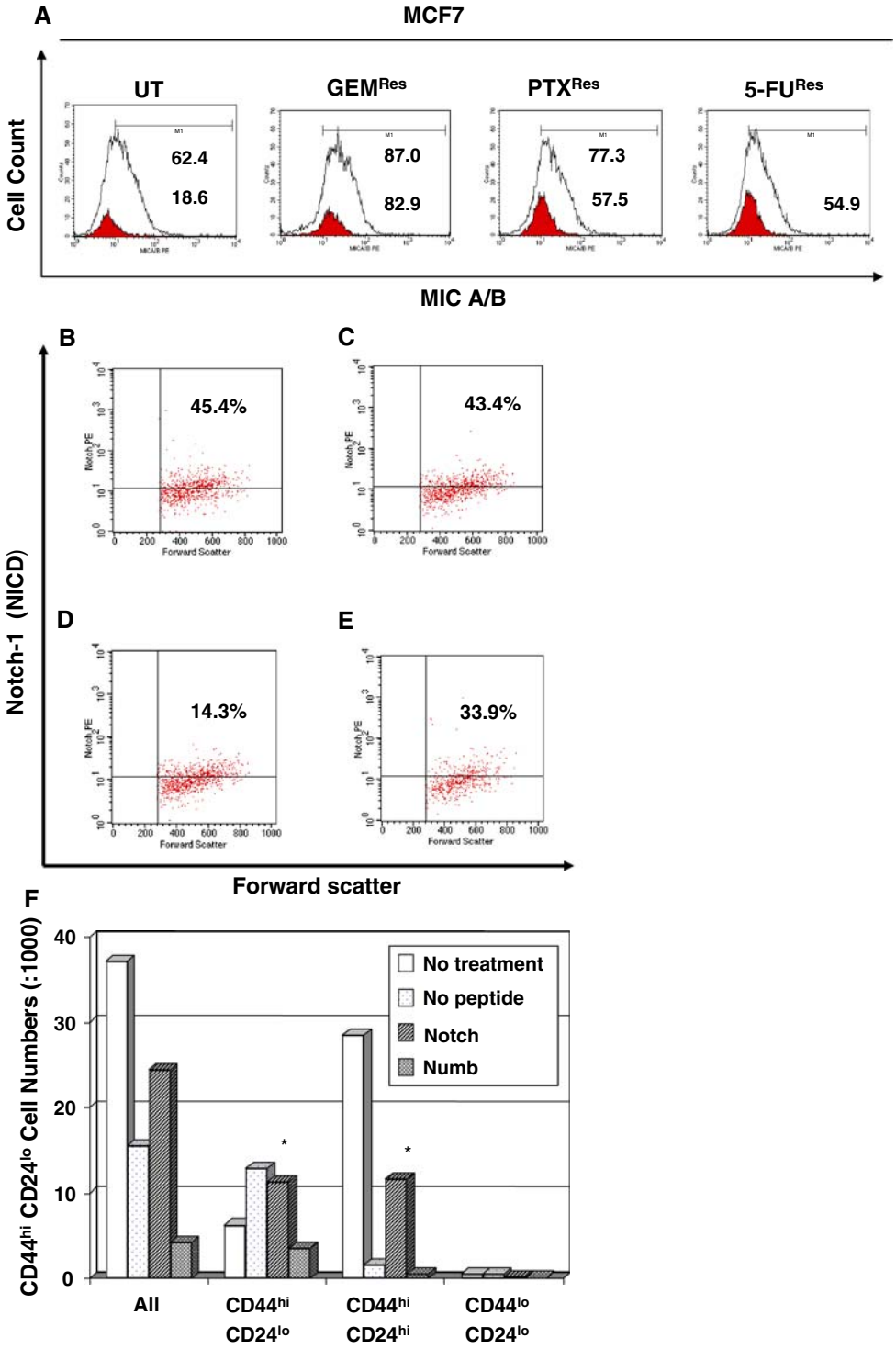


Fig. 5 **a** The number of MIC-A/-B$^+$ cells increased in drug-resistant MCF7. *White peaks* represent ESA$^+$ cells. *Black peaks* represent the MIC-A/B$^+$ CD44$^+$ CD24^{lo} cells. **b–e** Co-culture of GEM^{Res} MCF7 cells with Notch-1 peptide-activated PBMC decrease the NICD-Notch$^+$ cell numbers. Surviving NICD$^+$ MCF7 cells after co-culture with: **b** no effectors. **c** IL-2 activated PMBC. **d** Notch-1 peptide-activated PBMC. **e** Numb-1 peptide-activated PMBC. The % of NICD$^+$ cells is shown in the upper right quadrant. The decrease in NICD$^+$ cells in relation to the NICD$^+$ cells in panel (**b**) was: IL-2 activated PBMC, 4.4%; Notch-1-activated PBMC, 68.5%; and Numb-1-activated PBMC, 25.3%. Note: the amount of free NICD in GEM^{Res} MCF7 cells was lower than in UT-MCF7 cells (Fig. 3). **f** Numb-1 peptide-activated PBMC eliminate CD44$^+$ cells from UT-MCF7 cells. 50,000 GEM^{Res} MCF7 cells were cultured with indicated effectors for 5 days. Then live cells were collected and analyzed for expression of CD44 and CD24 in the same experiment. Analysis was performed in large tumor cells of similar cellularity (FS: 800–1000, side scatter 100–800). *All* indicate all live cells regardless of phenotype, *CD44^{hi} CD24^{lo}* indicate CSC-like cells, *CD44^{hi} CD24^{hi}* indicate potentially metastatic MCF7 cells, *CD44^{lo} CD24^{lo}* indicate non-invasive MCF7 cells. *Open columns* no effectors, *NP, dotted columns* MCF7 cultured with IL-2-activated PBMC, *NO, dashed columns left to right* MCF7 cultured with Notch-1-activated PBMC, *NU, textured columns* MCF7 cultured with Numb-1- activated PBMC

cells survive because their nucleic acid synthesis is minimal. In contrast, PTX acts by epigenetic mechanisms, by interfering with polymerization of actin.

We found only small differences in proliferation of CD24$^-$ and CD24$^+$ cells when stimulated with medium, and found differences when cells were stimulated with DLL4. Cell-cycle analysis indicates that most cells were in G1 phase and only a small fraction less than 5% were in G2 M phase (Supplemental Figure 1). We used stringent conditions for isolation of these cells by maintaining the drug in culture for several weeks. All non-adherent cells died in these conditions. In clinical practice, the drug given to patient starts decaying and only a small amount is present several days later. When MCF7 cells were cultured in

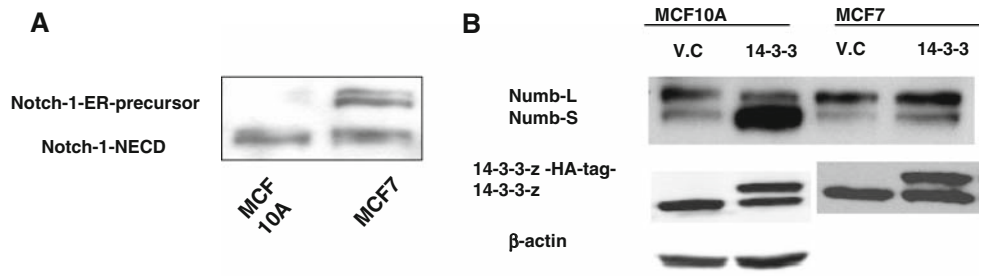


Fig. 6 Protein expression of Notch and Numb in MCF10A and MCF7. **a** Expression of Notch-1-NECD was not different between the normal breast cell line MCF10A and breast cancer cell line MCF7, but MCF7 cells expressed Notch-ER-precursor stronger than MCF10A cells. **b** The 14-3-3-ζ vector transfected MCF10A cells protected degradation of Numb-S but the 14-3-3-ζ transfected MCF7 cells did not. The band of 14-3-3-ζ HA-tag indicates expression of transfected 14-3-3-ζ protein. Cells which were transfected vector control have the band of 14-3-3-ζ protein by nature but not the band of 14-3-3-ζ HA tag

these conditions we counted that only 5–7 clones out of 50,000 drug-treated cells survived for one month and proliferated.

Therefore, we could not establish whether the cells which we analyzed are bona fide CSC, IP or drug-resistant tumor cells, which shared the phenotype of CSC.

$Drug^{Res}$ cells activated autonomous proliferation after drugs were removed. The Notch ligand, DLL4, expanded better $CD24^{hi}$ cells than $CD24^{lo}$ cells. Notch-ligands on neighboring cells activate Notch in *Trans*; by endocytosing NECD from responder cells. Free and plastic bound Notch-ligands are weak activators of Notch. Free Notch ligands, can stimulate or inhibit cells in *Cis* [23]. DLL4 delayed proliferation of $CD44^{lo}$ cells suggesting that it was inhibitory as described in other systems [14, 21].

We found that the balance between Notch and its antagonist, Numb, in $Drug^{Res}$ cancer cells, shifted in favor of Numb. The amount of NECD and TMIC-NICD decreased significantly. The amount of Numb did not change. Numb was present in four forms: non-phosporylated, [P]-lated at Ser^{265}, [P]-lated at Ser^{295} and probably phosphorylated at both positions. Numb-[P]-Ser^{295} decreased. Since Numb-1 presentation required [P]-lation it is likely that the Numb-1 peptide derived from Numb-[P]-Ser^{295}.

Numb interacts with the aPKC binding partner, PAR-3. Phosphorylation of Numb may inhibit cell migration by inhibiting integrin endocytosis [35]. [P]-Numb does not bind integrins when [P]-lated by atypical protein kinase-C (aPKC). Numb-[P]-Ser^{295} recruits 14-3-3 proteins, which inhibit the binding with AP-2 complex in vitro [22]. Although the 14-3-3-ζ vector rescued Numb-S from degradation in normal MCF-10A cells, Numb-S was not protected by 14-3-3-ζ from degradation in MCF7 cells (Fig. 6).

In normal cells, the Polo-kinase-1, Plk-1 regulates Numb asymmetry by [P]-lation of Pon [38]. The use of Polo- and Aurora-kinase inhibitors, in cancer treatment, raises the question whether more CSC will remain temporarily quiescent, or they will activate Notch when Numb decays and accelerate cancer progression. If this is the case, Numb-1 CTL can eliminate Plk-1-inhibitor-surviving cells. When this paper was prepared for publication two independent studies estimated the number of cells with CSC-characteristics in MCF7 to 2.4%. MCF7 cells formed mammospheres and required IL-6 to upregulate Notch-3 [7, 29].

In conclusion, we found that Numb-1 specific CTL can eliminate $CD44^{hi}$ $CD24^{neg/lo}$ cells. Based on our findings, adoptive and active immunotherapy (vaccines) with Notch and Numb should be effective in eliminating "quiescent CSC" in patients with breast and ovarian cancer.

References

1. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100:3983–3988
2. Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD (2006) Notch signaling regulates stem cell numbers in vitro and in vivo. Nature 442:823–826
3. Balic M, Lin H, Young L, Hawes D, Giuliano A, McNamara G, Datar RH, Cote RJ (2006) Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. Clin Cancer Res 12:5615–5621
4. Berdnik D, Torok T, Gonzalez-Gaitan M, Knoblich JA (2002) The endocytic protein a-adaptin is required for numb-mediated asymmetric cell division in *Drosophila*. Dev Cell 3:221–231
5. Blaumueller CM, Qi H, Zagouras P, Artavanis-Tsakonas S (1997) Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell 90(2):281–291
6. Bourguignon LY, Gilad E, Peyrollier K (2007) Heregulin-mediated ErbB2-ERK signaling activates hyaluronan synthases leading to CD44-dependent ovarian tumor cell growth and migration. J Biol Chem 282:19426–19441
7. Cariati M, Naderi A, Brown JP, Smalley MJ, Pinder SE, Caldas C, Purushotham AD (2008) ã6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. Int J Cancer 122(2):298–304
8. Castilleja A, Carter D, Efferson CL, Ward NE, Kawano K, Fisk B, Kudelka AP, Gershenson DM, Murray JL, O'Brian CA, Ioannides CG (2002) Induction of tumor-reactive CTL by C-side chain variants of the CTL epitope HER-2/neu proto-oncogene (369–377) selected by molecular modeling of the peptide: HLA-A2 complex. J Immunol 169:3545–3554

9. Chapman G, Liu L, Sahlgren C, Dahlqvist C, Lendahl U (2006) High levels of Notch signaling down-regulate Numb and Numb-like. J Cell Biol 175:535–540
10. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65:10946–10951
11. Di Marcotullio L, Ferretti E, Greco A, De Smaele E, Po A, Sico MA, Alimandi M, Giannini G, Maroder M, Screpanti I, Gulino A (2006) Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. Nat Cell Biol 8:1415–1423
12. Edwards SL, Brough R, Lord CJ, Natrajan R, Vatcheva R, Levine DA, Boyd J, Reis-Filho JS, Ashworth (2008) A resistance to therapy caused by intragenic deletion in BRCA2. Nature 451:1111–1115
13. Efferson CL, Tsuda N, Kawano K, Nistal-Villan E, Sellappan S, Yu D, Murray JL, Garcia-Sastre A, Ioannides CG (2006) Prostate tumor cells infected with a recombinant influenza virus expressing a truncated NS1 protein activate cytolytic CD8 + cells to recognize noninfected tumor cells. J Virol 80:383–394
14. Hurlbut GD, Kankel MW, Lake RJ, Artavanis-Tsakonas S (2007) Crossing paths with Notch in the hyper-network. Curr Opin Cell Biol 2:166–175
15. Ishiyama S, Matsueda S, Jones LA, Efferson C, Celestino J, Schmandt R, Ioannides CG, Tsuda N, Chang DZ (2007) Novel natural immunogenic peptides from Numb1 and Notch1 proteins for $CD8^+$ cells in ovarian ascites. Int J Oncol 30:889–898
16. Kawano K, Efferson CL, Peoples GE, Carter D, Tsuda N, Murray JL, Ioannides CG (2005) Sensitivity of undifferentiated, high-TCR density CD8 + cells to methylene groups appended to tumor antigen determines their differentiation or death. Cancer Res 65:2930–2937
17. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, Wong H, Rosen J, Chang JC (2008) Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 100(9):672–679
18. Liu S, Ginestier C, Charafe-Jauffret E, Foco H, Kleer CG, Merajver SD, Dontu G, Wicha MS (2008) BRCA1 regulates human mammary stem/progenitor cell fate. Proc Natl Acad Sci USA 105(5):1680–1685
19. McGill MA, McGlade CJ (2003) Mammalian Numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. J Biol Chem 278:23196–23203
20. Morrison SJ, Kimble J (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441:1068–1074
21. Mumm JS, Schroeter EH, Saxena MT, Griesemer A, Tian X, Pan DJ, Ray WJ, Kopan RA (2000) Ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol Cell 5(2):197–206
22. Nishimura T, Kaibuchi K (2007) Numb controls integrin endocytosis for directional cell migration with aPKC and PAR-3. Dev Cell 1:15–28
23. Orford KW, Scadden DT (2008) Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet 9:115–128
24. Ouhtit A, Abd Elmageed ZY, Abdraboh ME, Lioe TF, Raj MH (2007) In vivo evidence for the role of CD44 in promoting breast cancer metastasis to the liver. Am J Pathol 171(6):2033–2039
25. Phillips TM, McBride WH, Pajonk F (2006) The response of CD24(-/low)/CD44 + breast cancer-initiating cells to radiation. J Natl Cancer Inst 98(24):1777–1785
26. Piekarz RL, Sackett DL, Bates SE (2007) Histone deacetylase inhibitors and demethylating agents: clinical development of histone deacetylase inhibitors for cancer therapy. Cancer J 13(1):30–39
27. Ratnam K, Low JA (2007) Current development of clinical inhibitors of poly (ADP-ribose)-polymerase in oncology. Clin Cancer Res 13:1383–1388
28. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
29. Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M, Ceccarelli C, Santini D, Paterini P, Marcu KB, Chieco P, Bonafè M (2007) IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J Clin Invest 117:3988–4002
30. Schroeter EH, Kisslinger JA, Kopan R (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature 393(6683):382–386
31. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63:5821–5828
32. Smith CA, Lau KM, Rahmani Z, Dho SE, Brothers G, She YM, Berry DM, Bonneil E, Thibault P, Schweisguth F, Le Borgne R, McGlade CJ (2007) aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. EMBO J 26:468–480
33. Surowiak P, Materna V, Kaplenko I, Spaczyński M, Dietel M, Kristiansen G, Lage H, Zabel M (2006) Unfavorable prognostic value of CD24 expression in sections from primary and relapsed ovarian cancer tissue. Int J Gynecol Cancer 2:515–521
34. Tokumitsu H, Hatano N, Inuzuka H, Sueyoshi Y, Yokokura S, Ichimura T, Nozaki N, Kobayashi R (2005) Phosphorylation of Numb family proteins. Possible involvement of Ca2 +/calmodulin-dependent protein kinases. J Biol Chem 280:35108–35118
35. Tokumitsu H, Hatano N, Yokokura S, Sueyoshi Y, Nozaki N, Kobayashi R (2006) Phosphorylation of Numb regulates its interaction with the clathrin-associated adaptor AP-2. FEBS Lett 580(24):5797–5801
36. Tolis C, Peters GJ, Ferreira CG, Pinedo HM, Giaccone G (1999) Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur J Cancer 35:796–807
37. Tsuda N, Chang DZ, Mine T, Efferson C, García-Sastre A, Wang X, Ferrone S, Ioannides CG (2007) Taxol increases the amount and T cell activating ability of self-immune stimulatory multimolecular complexes found in ovarian cancer cells. Cancer Res 67:8378–8387
38. Wang H, Ouyang Y, Somers WG, Chia W, Lu B (2007) Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. Nature 449(7158):96–100
39. http://www.ncbi.nlm.nih.gov/geo/gds/profileGraph