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

Takashi Mine,

Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

Departments of Immunology and Surgery, Kurume University School of Medicine, Kurume, Japan

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

Satoko Matsueda,

Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

Yufeng Li,

Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

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

Hiroshi Tokumitsu,

Department of Cell Signaling, Faculty of Medicine, Kagawa University, Kagawa, Japan

Hui Gao,

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

Cristopher Danes,

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

Kwong-Kwok Wong,

Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

Xinhui Wang,

Departments of Surgery, Immunology and Pathology, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15123, USA

Soldano Ferrone, and

Departments of Surgery, Immunology and Pathology, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15123, USA

Constantin G. Ioannides

Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

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

Abstract

Cancer stem cells (CSC) are resistant to chemoand 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-Xuorouracil (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]-Ser²⁶⁵ were similar in GEM^{Res} and GEM^{Sens} MCF7 cells. Only the levels of Numb-L (long)-Ser²⁹⁵ 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 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 iso-forms, which differ by 1 kDa, and are diYcult 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 Ser²⁹⁵ and migrates to cytoplasm. In cytoplasm Numb-[P]-Ser²⁹⁵ 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-Xuorouracil (5-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-Xuorourcil (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 Bio-

technology, Inc., Santa Cruz, CA), anti-NICD, anti-Numb, and anti-Bcl-2 mAb (Abcam). mAb against rat Numb [P]-Ser²⁶⁴ and Numb [P]-Ser²⁸³ = Human Numb-L-P-Ser²⁶⁵ 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]-Ser²⁶⁴ and [P]-Ser²⁸³ of rat Numb differs among iso-forms. In human Numb-S is [P]-Ser²⁶⁵ and [P]-Ser²⁸⁴ [Numb-3 (*NP_003735*) and Numb-4 (*NP_0010057450*)]. Rat [P]-Ser²⁷⁶ is [P]-Ser²⁹⁵ in human Numb-L [Numb-1 (*NP_001005743*) and Numb-2 (*NP_001005744*)]. Rat [P]-Ser²⁸³ corresponds to Ser²⁹⁵ in human Numb-L. We used the designations [P]-Ser²⁶⁵ and [P]-Ser²⁹⁵ since they are close to the [P]-Ser positions reported in the recent literature.

IC₅₀ of anticancer drugs

The 50% inhibitory concentration (IC₅₀) of GEM, PTX and 5-FU was determined after a 72-h incubation with drugs as described [13].

Flow cytometry

Cells (25–30 × 10⁶) were cultured with drugs at 2 × IC₅₀ for 4 days followed by drug at 0.5–1.0 × IC₅₀ for the next 3–6 days. Surviving cells (2 × 10⁵) were incubated with 20 μg of human IgG (Sigma) for 1 h on ice to inhibit nonspecific binding of specific mAb during staining. Analysis was performed using a Becton Dickinson FACS^{Calibur} with CellQuest 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 × 10⁵) were co-cultured in six-well plates with 3.0 × 10⁵ 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 Xow-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 IC₅₀ of GEM, 5-FU and PTX for MCF7 and SKOV-3 cells. The IC₅₀ (GEM) for MCF7 cells was 430 nM and the IC₅₀ (GEM) for SK-OV-3 cells was 16.1 nM. The IC₅₀ (5-FU) for MCF7 cells was 1,302 nM. The IC₅₀ (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 IC₅₀ (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 Ala¹⁷¹⁰ and Val¹⁷¹⁷ to generate the Notch-extracellular truncated domain (NEXT). NEXT is further cleaved at Val¹⁷⁴⁴ 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 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 Ser²⁹⁵, in both untreated and GEM^{Res} cells. The amount of Numb-[P]-Ser²⁹⁵ decreased by 30%, in GEM^{Res} cells. The amount of Numb-L and Numb-S-[P]-Ser²⁶⁵ did not change in GEM^{Res} cells compared with untreated cells (Fig. 3b). Our results indicate differences in Ser²⁹⁵-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]-Ser²⁹⁵ to Numb-L/S than MCF7 cells and ratios of Numb-L/S-[P]-Ser²⁶⁵ 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 μ M, whereas Notch-1 did so at a concentration of 5 μ M.

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 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 Xuoescence 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 allogeneic PBMC eliminate cells with 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^{−/lo} 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^{−/lo} 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 effector-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 G₁ phase [36]. These 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 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-phosphorylated, [P]-lated at Ser²⁶⁵, [P]-lated at Ser²⁹⁵ and probably phosphorylated at both positions. Numb-[P]-Ser²⁹⁵ decreased. Since Numb-1 presentation required [P]-lation it is likely that the Numb-1 peptide derived from Numb-[P]-Ser²⁹⁵.

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]-Ser²⁹⁵ 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.

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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-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*)

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 Ser²⁶⁵ and Ser²⁹⁵ in GEM^{Sens} and GEM^{Res} cells. MCF7 cells contain significantly less Numb-S than SKOV3 cells. **c** Numb is more [P]-lated at Ser²⁶⁵ than at Ser²⁹⁵ 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

Fig. 4.

Peptides Notch-1 and Numb-1 expanded antigen-specific CD8⁺ cells. **a** Notch-1-TCR⁺ CD8⁺ cells expanded by incubation with 5 μ g Notch-1 (2112–212) and **b** Numb-1-TCR⁺ CD8⁺ cells expanded by incubation with 1 μ g 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-1 peptide 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*

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 PBMC. **d** Notch-1 peptide-activated PBMC. **e** Numb-1 peptide-activated PBMC. 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

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