# A therapeutic humanized anti-carcinoma monoclonal antibody (mAb) can enhance NK activity and target immunosuppressive regulatory T cells.

**Poster # 4534** **Massimo Fantini[1], M. Pia Morelli[2], Christina M. Annunziata[2], Philip M. Arlen[1] and Kwong Y. Tsang[1]**

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## Introduction

NEO-201 is a therapeutic IgG1 humanized mAb reactive against many different carcinomas, but not reactive against most normal tissues. No reactivity was observed with NEO-201 in subsets of hematopoietic cells except CD15+ granulocytes. Functional analysis revealed that NEO-201 is able to mediate ADCC and CDC to kill tumor cells. Previous studies showed that NEO-201 attenuates growth of human tumor xenografts in mice and demonstrates safety/tolerability in non-human primates with a transient decrease in neutrophils being the only adverse effect observed. A first in human clinical trial evaluating NEO-201 in adults with chemo–resistant solid tumors is ongoing at the NIH clinical Center. NEO-201 recognizes tumor-associated variants of CEACAM5 and 6. CEACAM1 is a potent inhibitor of natural killer (NK) cell function; binding between CEACAM1 on NK cells and CEACAM1 or CEACAM5 on tumor cells inhibits activation signaling by NKG2D, which prevents NK cell cytolysis and permits tumor cells to evade NK killing. Preclinical evaluation showed that NEO-201 reacts against human regulatory T cells (Tregs). This study was designed to assess NK enhancing pathway and further investigated the phenotypic and functional effects of NEO-201 on human Tregs *in vitro.*

## Experimental Design

Various human tumor cell lines were used as target cells and NK-92 cells (CEACAM1+/CD16-) were used as effectors to assess the ability of NEO-201 to block the interaction between CEACAM5 on tumor cells and CEACAM1 on NK cells in order to enhance the in vitro killing of tumor cells.

Peripheral blood mononuclear cells (PBMCs) were collected from 5 healthy donors and used for phenotypic and functional analysis.

The ability of NEO-201 to bind to human hematopoietic cells was evaluated by flow cytometry. The following markers were used to phenotype different immune-subsets: CD19 for B cells, CD4 for $CD4^+$ T cells, CD8 for $CD8^+$ T cells, CD56 for NK cells, CD14 for monocytes, CD15 for granulocytes. EasySep™ Human $CD4^+CD127^{low}CD25^+$ Regulatory T Cell Isolation kit and EasySep™ Human Biotin NEO-$201^+$ Selection Kit (biotin-labeled NEO-201 mAb) were used to isolate Tregs from PBMCs. Phenotypic analysis was conducted by flow cytometry for the following markers: CD4, CD25, CD127, FoxP3, CD15s, CD45RA, NEO-201 antigen, CEACAM5 and CEACAM6. The ability of NEO-201 to mediate killing of opsonized Tregs was evaluated using a CDC assay. NEO-201 positivity was defined as % positive ≥ 10%. Positivity was determined by using fluorescence-minus-one controls.

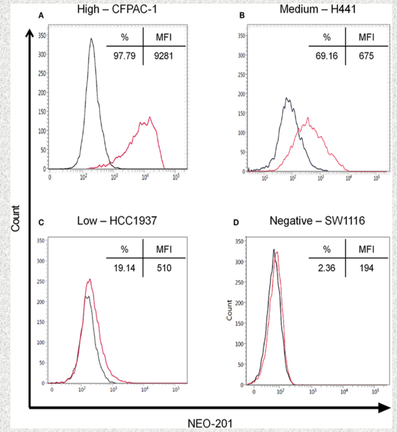
## Results

### 1. NEO-201 binds to various human carcinoma cell lines

#### Tumor Cell Line Flow Cytometry

| CELL LINE | TUMOR TYPE | % POSITIVE | MFI |
|---|---|---|---|
| **COLO 205** | **Colon** | **10.33** | **245** |
| **HT-29** | **Colon** | **38.40** | **352** |
| **LS174T** | **Colon** | **46.46** | **345** |
| SW1116 | Colon | 2.36 | 194 |
| SW1463 | Colon | 1.23 | 278 |
| SW480 | Colon | 1.70 | 575 |
| **ASPC-1** | **Pancreatic** | **79.26** | **8927** |
| **BxPC-3** | **Pancreatic** | **97.25** | **2584** |
| **CAPAN-2** | **Pancreatic** | **29.69** | **327** |
| **CFPAC-1** | **Pancreatic** | **97.79** | **9281** |
| PANC-1 | Pancreatic | 3.29 | 289 |
| **H441** | **NSCLC (adenocarcinoma)** | **69.16** | **675** |
| H522 | NSCLC (adenocarcinoma) | 1.38 | 238 |
| **HCC4006** | **NSCLC (adenocarcinoma)** | **99.27** | **9899** |
| **HCC827** | **NSCLC (adenocarcinoma)** | **77.46** | **692** |
| SK-LU-1 | NSCLC (adenocarcinoma) | 1.77 | 685 |
| CALU-1 | NSCLC (squamous) | 4.22 | 571 |
| H1703 | NSCLC (squamous) | 4.16 | 111 |
| H226 | NSCLC (squamous) | 4.83 | 209 |
| **H520** | **NSCLC (squamous)** | **61.78** | **443** |
| **AU-565** | **Breast (HER2+)** | **50.04** | **227** |
| **BT-474** | **Breast (PR+/HER2+)** | **68.79** | **591** |
| HCC1500 | Breast (ER+/PR+) | 1.53 | 597 |
| SK-BR-3 | Breast (HER2+) | 1.61 | 329 |
| T-47D | Breast (ER+/PR+) | 8.00 | 161 |
| **ZR-75-1** | **Breast (ER+/PR+/HER2+)** | **68.80** | **550** |
| BT-549 | Breast (ER-/PR-/HER2-) | 1.47 | 477 |
| **HCC1937** | **Breast (ER-/PR-/HER2-)** | **19.14** | **510** |
| HCC38 | Breast (ER-/PR-/HER2-) | 2.15 | 226 |
| MDA-MB-468 | Breast (ER-/PR-/HER2-) | 6.33 | 344 |

NEO-201 is reactive against a broad range of *in vitro* cultured tumor cell lines. NEO-201 positive cell lines appear in bold text. NEO-201 positivity was defined as % positive >10%.




Positivity was determined using fluorescence minus one (FMO) controls. Positive cell lines were ranked according to their quantified expression level (% positive × MFI), and then sorted into groups of low (<200), medium (200-1000), and high (<1000) expression.

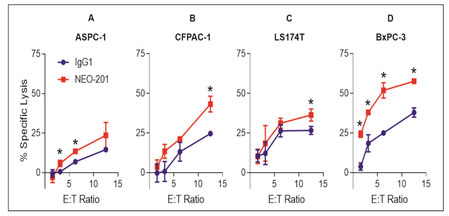
### 2. NEO-201 enhances NK-92 cell cytotoxicity against CEACAM5+ / NEO-201+ tumor cells

#### Tumor Cell Line Flow Cytometry

| *Cell line* | *CEACAM1+* | *CEACAM5+/NEO-201+* |
|---|---|---|
| | *% positive (MFI)* | |
| ASPC-1 | **61.15** (707) | 9.26 (869/9078) |
| BxPC-3 | 2.45 (1471) | **58.52** (1447/6420) |
| CFPAC-1 | **18.67** (1938) | **26.95** (1108/1728) |
| LS174T | 2.43 (3287) | **26.84** (1030/858) |

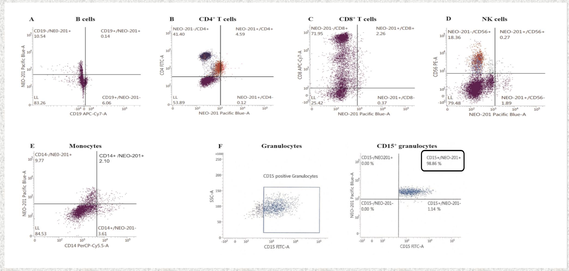
**ASPC-1, BxPC-3, CFPAC-1** are human pancreatic carcinoma cell lines. **LS174T** is a human colorectal carcinoma cell line.
Positive marker expression appears in bold text, where positivity was defined as % positive >10 %.

#### NK-92 killing assay




**NEO-201** and human IgG1 (negative control) were used at **10μg/mL**. **NK-92 cells** were used as effectors at the following **E:T ratios: 1.56:1, 3.12:1, 6.25:1, 12.5:1.** Asterisks denote statistical significance of NK-92 + NEO-201 vs NK-92 + IgG1 *p < 0.05.

### 3. NEO-201 binds only to granulocytes in human hematopoietic cells



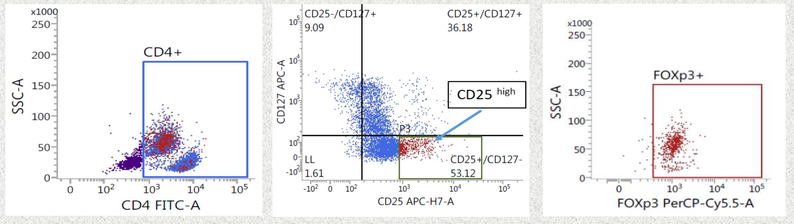

**NEO-201 doesn't react with other hematopoietic subsets (B cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, monocytes)**

### 4. NEO-201 binds to human regulatory T cells (Tregs)

#### Phenotypic analysis of isolated Tregs as determined by flow cytometry

**EasySep™ Human $CD4^+CD127^{low}CD25^+$ Regulatory T Cell Isolation kit**

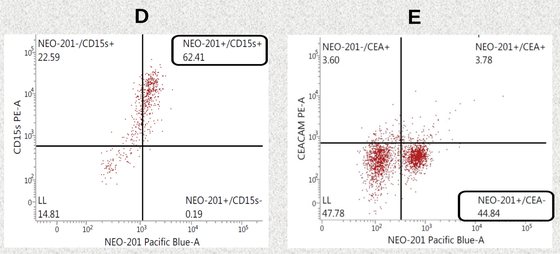
**(Healthy donor 1)**




**A**: **86.27 %** of **isolated cells** are $CD4^+$

**B**: **10.15%** of $CD4^+$ **cells** are $CD25^{high}CD127^-$

**C**: **97.30%** of $CD4^+/CD25^{high}/CD127^-$ **cells** are $FOXp3^+$



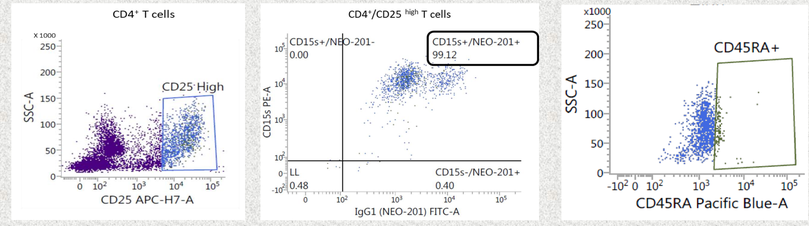

**D**: **62.41%** of **FOXp3+ cells** are **NEO-$201^+$/$CD15s^+$**

**E**: **NEO-$201^+$/$CD15s^+$ cells are CEA (CEACAM5) and CEACAM6 negative**

**E:** Cells were stained with **PE Mouse Anti-Human CD66 antibody** (Clone B1.1/CD66) which recognizes *CEACAM1* ***CEACAM6***, *CEACAM3* and ***CEACAM5***

**EasySep™ Human Biotin NEO-$201^+$ Selection Kit**

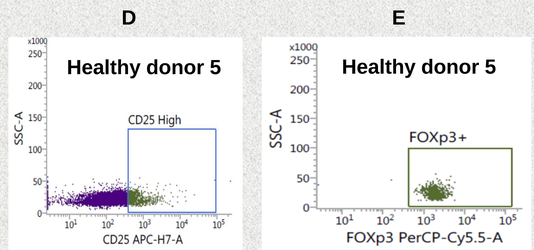
**(Healthy donor 4)**




**A**: **15.69%** of **cells are $CD25^{high}$**

**B**: **99.12%** of **$CD25^{high}$ cells** are **NEO $201^+$/ $CD15s^+$**

**C**: **NEO $201^+$/$CD15s^+$ cells are CD45RA negative**



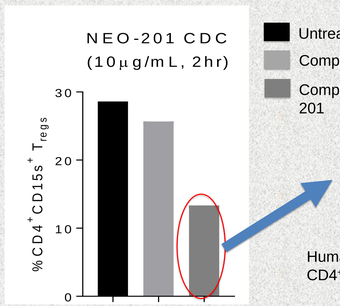

**D**: **10.32%** of **cells are $CD25^{high}$**

**E**: **99.75%** of $CD4^+/CD25^{high}$ **cells** are **$FOXp3^+$**

2 STEP human regulatory T cells isolation:

1) **$CD4^+$ T cells were isolated from whole PBMCs**
2) **$CD4^+$ T cells were bounded to biotinylated NEO-201 to isolate NEO-$201^+$ Tregs**

### 5. NEO-201 mediates CDC activity against isolated $CD4^+/CD15s^+$/NEO-$201^+$ Tregs.




Untreated
Complement
Complement + NEO-201

**50 % killing compared to untreated cells**

Human Tregs were isolated using the EasySep™ Human $CD4^+CD127^{low}CD25^+$ Regulatory T Cell Isolation kit

## Conclusions

This study demonstrates that NEO-201 can mediate immune killing through additional mechanisms including blocking the interaction between CEACAM5 on tumor cells and CEACAM1 on NK cells to reverse CEACAM1-dependent inhibition of NK cytotoxicity as well as to recognize and eliminate human Tregs through CDC. Ongoing studies are looking at leveraging this phenomenon by combining NEO-201 with checkpoint inhibitors.

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