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Putative contribution of CD56 positive cells in cetuximab treatment efficacy in first-line metastatic colorectal cancer patients

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Abstract

Background: Activity of cetuximab, a chimeric monoclonal antibody targeting the epidermal growth factor receptor, is largely attributed to its direct antiproliferative and proapoptotic effects. Antibody-dependent cell-mediated cytotoxicity (ADCC) could be another possible mechanism of cetuximab antitumor effects and its specific contribution on the clinical activity of cetuximab is unknown. **Methods:** We assessed immune cells infiltrate (CD56, CD68, CD3, CD4, CD8, Foxp3) in the primary tumor of metastatic colorectal cancer (mCRC) patients treated with a first-line cetuximab-based chemotherapy in the framework of prospective trials (treatment group) and in a matched group of mCRC patients who received the same chemotherapy regimen without cetuximab (control group). The relationship between intra-tumoral immune effector cells, the K-ras status and the efficacy of the treatment were investigated. We also evaluated *in vitro*, the ADCC activity in healthy donors and chemonaive mCRC patients and the specific contribution of CD56⁺ cells. **Results:** ADCC activity against DLD1 CRC cell line is maintained in cancer patients and significantly declined after CD56⁺ cells depletion. In multivariate analysis, K-ras wild-type (HR: 4.7 (95% CI 1.8-12.3), p=0.001) and tumor infiltrating CD56⁺ cells (HR: 2.6, (95%CI:1.14-6.0), p=0.019) were independent favourable prognostic factors for PFS and response only in the cetuximab treatment group. By contrast CD56⁺ cells failed to predict PFS and response in the control group. **Conclusions:** CD56⁺ cells, mainly NK cells, may be the major effector of ADCC related-cetuximab activity. Assessment of CD56⁺ cells infiltrate in primary colorectal adenocarcinoma may provide additional information to K-ras status in predicting response and PFS in mCRC patients treated with first-line cetuximab-based chemotherapy.

Background

Cetuximab is a chimeric immunoglobulin G 1 (IgG1) monoclonal antibody (mAb) which binds the epidermal growth factor receptor (EGFR) with high affinity and inhibits ligand binding [1]. Cetuximab is active in chemotherapy resistant metastatic colorectal cancer (mCRC) [2,3] and enhances response rate and progression-free survival (PFS) in first-line therapy in combination with Folfiri and Folfox [4,5]. Clinical studies of cetuximab therapy in mCRC have failed to show a significant correlation between EGFR-staining intensity and patients' response to cetuximab treatment [2,3]. Therefore, identifying molecular markers that can select patients who are likely to benefit from cetuximab is crucial to avoid chemotherapy toxicity and reduce treatment cost. Recently the absence of K-ras mutation appears to be a reliable marker in predicting cetuximab efficacy, both in first-line and in third-line of the anti-EGFR therapies [4-8]. Other factors such as, EGFR amplification [9-11], epiregulin and amphiregulin expression [12], nuclear factor-kB tumor expression [13], PTEN [14], BRAF [15] or PIK3CA [16] were also suggested to predict response to cetuximab but these additional biomarkers require further validation before incorporation into clinical practice.

The activity of cetuximab has largely been attributed to the direct antiproliferative and proapoptotic effects of the antibody. However, another possible mechanism of its antitumor effects is mediated through antibody-dependent cell-mediated cytotoxicity (ADCC). The ADCC mediated through Fc receptors (Fc γ R) carried by NK cells, macrophages and polymorphonuclear leukocytes, is a well-recognized immune effector mechanism in the antitumor effect of IgG1 [17]. Of these cells, NK cells represent the principal ADCC effector cells [18,19]. Recently, some polymorphisms on genes encoding for activating receptors Fc γ RIIa and Fc γ RIIIa were found to affect

the clinical efficacy of cetuximab [20,21]. The recruitment of Foxp3-positive regulatory T cells (Treg) into tumor likely represents one of the mechanisms by which malignant cells evade host immune response. The intratumoral density of foxp3 has been reported to be associated with overall survival [22]. Once activated, Tregs can inhibit the function of dendritic cells, NK cells, B cells and other immune cells [23-25] and consequently alter ADCC activity.

Based on the potential value of ADCC in cetuximab activity, we assessed in mCRC patients, the role of peripheral blood mononuclear cells (PBMC) and their CD56+ subpopulation in ADCC activity and we evaluated the relationship between the intratumoral immune cells and the efficacy of first-line cetuximab-based chemotherapy.

Methods

Patients and tissue samples: a retrospective study review was conducted from data in our institution's prospectively collected gastrointestinal cancer database. Chemonaive patients with mCRC who underwent surgical resection of their primary tumor and diagnosed with *synchronous* metastases were included in the analysis. We analyzed the primary tumor of 33 chemonaïve mCRC patients treated with first-line cetuximab (Erbix®), Merck, Darmstadt, Germany) containing chemotherapy regimen in the framework of phase II and III studies. Patients received mostly standard FOLFIRI regimen or FOLFOX accordingly to study recommendations [4, 26, 27]. Thirty-five chemonaïve mCRC patients with *synchronous* metastasis, who underwent resection of the primary tumor before starting a similar chemotherapy regimen that did not contain cetuximab were used as control group. The treatment and the control group were case-matched for the following parameters: sex, age,

primary tumor location, tumor stage, performance status, metastatic sites, type of chemotherapy administered and treatment duration.

Formalin-fixed and paraffin-embedded (FFPE) samples of the primary tumor were obtained for immunohistochemical and PCR analysis.

The study was approved by the ethic committee of the Erasme University Hospital and all patients provided written informed consent.

Clinical evaluation and tumor response criteria:

We considered PFS to assess the cetuximab-based chemotherapy efficacy in first line and not OS which is influenced by second and third-lines chemotherapy and liver mets surgery. Tumor response was evaluated by computerized tomodensitometry according to the Response Evaluation Criteria in Solid Tumors (RECIST) [28] and classified in complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). The overall best response (OR) was defined as the best response recorded from the start of the treatment until disease progression, recurrence or start of the new therapy. Response was centrally confirmed in the setting of the referenced trials. For the analysis, CR and PR patients were grouped in responders; patients with SD and PD were grouped in non-responder patients.

Immunohistochemical assessment: Hematoxylin & eosine sections of the tumors were examined by a pathologist for confirmation of the histologic diagnosis and the optimal block donors selected. The FFPE tissues were deparaffinized in xylene, and rehydrated in graded alcohols and water. Colorectal tumor sections were incubated with monoclonal antibodies against CD3, CD4, CD8, CD56, CD68, Foxp3 and EGFR and DAB-chromogen were applied (Dako, Copenhagen, Denmark) (Additional file 1: supplemental Table S1). Appropriate negative and positive controls were used.

Evaluation of Immunohistochemical variables: The slides were examined independently by two observers (RM, NN) blinded to both clinical and pathologic data. Twenty representative fields of the tumour invasive margin (IM) were chosen from each slide, and the stained cells were counted by means of a 10x10 ocular grid at x 200 magnification (observed area 0.25mm²) using confocal microscopy. For each case, the total number of CD3, CD8, CD4, ,FOXP3, CD56, CD68 positive cells (representing lymphocytes T (LT), LTCD8, LTCD4, Treg, natural killers (NK) and macrophages, respectively) per square millimeter was calculated. Variations in the enumeration within a range of 5% were re-evaluated and a consensus decision was made.

Expression of EGFR was quantified using a visual grading system based on the extent of staining (percentage of positive tumor cells graded on scale of 0 to 3: 0, none; 1, 1-30%, 2, 31-60%, 3, > 60%) and the intensity of staining (graded on a scale of 0 to 3: 0, none; 1, weak staining; 2, moderate staining; 3, strong staining). Membranous and cytoplasmic staining were evaluated.

DNA extraction and mutation analysis: Presence of tumor cells (>75%) in each tumor block was firstly histologically controlled by H&E coloration. Thereafter, DNA was extracted from FFPE samples after macrodissection. The presence of K-ras was determined by allelic discrimination assay on a 7500HT Real Time PCR System. K-ras mutations located within the codon 12 (n=6) and 13 (n=1) were screened for. All mutations were confirmed by direct sequencing (8).

Cell Lines and cell culture: The EGFR overexpressing DLD1 colorectal glandular carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). DLD1 has been previously found to carry mutated K-ras. To confirm that DLD1 indeed harbour mutated K-ras alleles, we extracted the

corresponding genomic DNA and sequence the K-ras locus. We confirmed that DLD1 display Gly13Asp K-ras mutation (data not shown). The DLD1 cells were maintained in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibico BRL, Grant Island, NY, USA), sodium pyruvate 1%, 100 units/ml penicillin and 100µg/ml streptomycin. The expression of EGFR by the DLD1 cell line was confirmed by immunocytochemistry (data not shown)).

Preparation of human peripheral blood mononuclear cells (PBMC) : PBMC from 5 healthy medication-free donors (3 male, 2 female; mean age: 33.8 years, range:27-48 years) and 5 chemo-naïve mCRC patients (2 female, 3 male, mean age 55.4 years, range 49-61 years) were isolated from heparinised peripheral blood on a Ficoll gradient.

Flow cytometry analysis:

Immunophenotyping of PBMCs before and after depletion of CD56⁺ cells was performed by incubation with appropriate combination of fluorochrome-labeled monoclonal antibodies. Major lymphocyte populations CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD3⁻CD16⁺56⁺ (NK), CD3⁺CD16⁺56⁺ (NKT), CD19⁺ (B) were determined by two cocktails MoAb: anti-CD45 PerCP, anti-CD3 FITC, anti-CD8 PE, anti-CD4 APC and anti-CD45 PerCP, anti-CD3 FITC, anti-CD16+56 PE and CD19 APC. Data acquisition was performed with a FACS Canto flow cytometer and data analysed using BD FACS Diva software (BD Biosciences, Mountain View, CA).

Purification of effector cells and interleukine-2(IL-2) treatment: Highly depleted PBMC from CD56⁺ cells (CD56 depleted PBMC), were obtained by magnetic activated cells sorting (MACS) using the system from Miltenyi Biotec according to the manufacturer's instructions. The CD56 depleted PBMC and non depleted PBMC

were used separately in ADCC assays. Between 92% to 94% of the CD56⁺ cells were sorted after MACS depletion. As IL-2 is known to activate PBMC, we tested the effect of human recombinant IL-2 (R&D Systems) on cetuximab-mediated ADCC in CD56 depleted and non depleted PBMC . The two populations were cultured in medium alone or enriched with rhIL-2 (10 ng/ml) for 18h prior to use in ADCC assays.

ADCC assay : 5x10⁴target cells/well were plated in a 96 flat bottom wells plate in 200µl of medium, 24 hours before adding effectors cells. Human PBMC (effector) or IL-2 activated PBMC, were added at different E:T ratios ranging from 20:1 to 2:1 and incubated for 24h. Cetuximab or polyclonal human IgG (Sigma, St Louis, MO, USA) were added to the individual wells at different concentrations ranging from 0 to 100 µg/ml.

Accordingly to *Heo et al.*, cytotoxicity was evaluated using a 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [29,30] (additional file 2). Experiments conducted in a preliminary phase to select optimal conditions for the ADCC effect showed that (a) target cells were not killed after exposure to cetuximab in the absence of PBMC (b) 10µg/ml was the optimal cetuximab concentration for saturating ADCC assay by PBMC.

Statistical Analysis: The PFS was estimated by the Kaplan-Meier method and the two groups were compared with by the log-rank test. For all immunohistochemical markers, the cut off for definition of subgroups was the median value. The non-parametric χ^2 test and the Fisher's exact tests were carried out as appropriate to compare categorical variables. The Mann-Witney U test was used for the comparison of the treatment and the control groups regarding the tumor infiltrating immune cells and ADCC activity. Correlations were analyzed by Spearman's correlation coefficient

test. Multivariate analyses used a step-down procedure based on the likelihood ratio test. A p-value ≤ 0.1 in univariate analysis was required to consider the variable for multivariate analysis. The Two-tailed $p < 0.05$ was judged to be significant. All analysis were performed with SPSS 10.0 software (SPSS, Chicago, IL).

Results

Patients characteristics:

The baseline and treatment characteristics of patients from treatment and control group are summarized in table 1. The two groups were similar regarding all variables matched including the proportion of patients treated with the FOLFIRI ***Immune cells***

infiltrate in primary CRC and treatment efficacy:

Positive CD4, CD3, CD8, CD68 and Foxp3 cells were detected in 33/33 (100%) tumor samples (figure 1) and their mean number were similar between the two patient groups (table 2). The number of intratumoral macrophages (CD68⁺ cells) and Lymphocytes (CD3⁺, CD4⁺, CD8⁺, Foxp3⁺ cells) were similar between responders and non responders and we found no association with the PFS both in the treatment and the control group (table 3).

For CD56⁺ cells, two distinct immunologic pattern were clearly observed consisting in, either tumors with strongly positive CD56 staining (CD56 positive tumors) or tumors with undetectable CD56 staining (CD56 negative tumors) (figure 1). These two pattern were found both in the treatment and the control groups (table 2) and were compared for OR and PFS and correlated with the K-ras status. In the control group, no difference has been detected in OR and PFS between patients with CD56 negative tumor cells and those with CD56 positive tumor (table 3, figure 1). By contrast, in the treatment group, CD56 positive tumors were more frequent in responder than in non-responder patients (10/16 (63%) *versus* 3/19 (18%), $p=0.011$)

and patients with CD56 positive tumor had a longer PFS than those with CD56 negative one (8.8 months (95% CI: 3.3-13.5) versus 3.9 months (95% CI: 3.1-4.7)) ($p=0.005$) (table 3). Neither the CD56 tumor status ($r=-0.267$, $p=0.134$) and the number of CD56⁺ cells ($r=-0.295$, $p=0.101$) were correlated with the K-ras status of the tumor.

EGFR expression: as previously observed, no significant correlation was found between EGFR expression, OR rate and PFS.

K-ras mutation Status and treatment efficacy:

K-ras mutations were detected in the tumor of 13/33 (39%) patients from the cetuximab treatment group. Wild type (WT) status was found in 13/16 (81%) responders and in 7/17 (41%) non responders ($p=0.008$). WT group had a prolonged PFS (9.2 months (95% CI: 4.9 to 16.4)) as compared to the mutated group (4.5 months (95% CI: 2.6-6.2; $p=0.0007$)) (table 3).

Multivariate analysis:

CD56⁺ cells infiltrate and WT K-ras status were independent predictors of OR. Both CD56 negative tumors (Hazard ratio: 2.6 (95% CI: 1.14-6.00); $p=0.019$) and K-ras mutations (Hazard ratio: 4.74 (95% CI: 1.8-12.3); $p=0.001$) contribute as significant independent negative prognostic factors for the PFS (table 3). Interestingly, in the group of WT K-ras tumors ($n=20$), there is a trend for a higher OR and a prolonged PFS in patients with CD56 positive tumor as compared with CD56 negative tumor patients and the whole group (CD56⁺ and CD56⁻) (additional file 3, Supplemental Table S2).

Cetuximab-mediated ADCC in vitro activity is maintained in mCRC patients

CD56⁺ cells represented a minor fraction (range between 5 and 14%) of the whole PBMC population.

Adjunction of cetuximab enhanced the cytotoxicity of PBMC (ADCC) as compared to the PBMC activity (PBMC alone). At the higher E:T ratios of 10:1 and 20:1, cetuximab enhanced tumoral cell lysis, as compared to the baseline activity of PBMC; both in healthy volunteers ($p= 0.008$ at an E:T ratio of 10:1, from 26% to 60%; and $p= 0.009$ at an E:T ratio of 20:1, from 30% to 74%) and in mCRC patients ($p= 0.001$ at an E:T ratio of 10:1, from 19% to 56%; and $p= 0.002$ at an E:T ratio of 20:1 from 25% to 71%) (figure 2 and 3B). PBMC activity was not increased by the control antibody. Interestingly, PBMC and ADCC activity was similar between mCRC patients and healthy volunteers.

CD56⁺ cells are responsible for cetuximab-mediated ADCC in vitro activity and its majoration by IL-2

While CD56⁺ cells represent a minor fraction of the PBMC, depletion of CD56⁺ cells significantly reduced the ADCC activity at the different E:T ratio ($p < 0.05$ at all E:T ratios) even after IL-2 stimulation (figure 3C, 3D) underlying the major role of this immune cells in ADCC activity.

Discussion

One of the most important mechanisms of cetuximab and panitumumab, the monoclonal antibodies that target the EGFR, is through the inhibition of the EGF receptor/ligand interaction. The other pathway through which cetuximab only, as an IgG1, may exert its antitumor effect is ADCC [12,14]. Several *in vitro* and *in vivo* studies have shown that this chimeric IgG1 mAb binds to the antigen, the EGFR, on the surface of tumor cells while its Fc portion binds to the immune effector cells through FC γ R. Consequently, this binding activates the immune cells leading to tumor cells killing. ADCC activity of mAbs has been well described for trastuzumab, a

human IgG1 anti human EGFR 2 (Her-2) antibody and for rituximab, a chimeric IgG1 mAb for B-cell differentiation antigen CD20 [31-33].

The array of cellular effectors potentially exerting ADCC includes the CD3⁻ CD56⁺ NK lymphocytes [34] but also CD3⁺CD16⁺ T-cell subset [35], CD16⁺CD33⁺ macrophages [36] and CD16⁺ granulocytes [37]. Among the mononuclear cells (MNC), the NK cells (CD3⁻ CD56⁺) play a predominant role in the ADCC through low affinity type II (Fcγ RIIc) and type IIIA (Fcγ RIIIa) Fc receptors present on their surface [38,39]. The polymorphonuclear cells are also able to induce ADCC by the engagement of their CD32 receptor but needs higher concentration levels of antibody compare with the MNC.

Since only less than 50% of WT-Kras mCRC patients seem to benefit from cetuximab-based treatment, it may be highly relevant to identify other biomarkers than K-ras status to predict cetuximab efficacy. To this end, the evaluation of the ADCC actors as putative surrogate markers of cetuximab-based therapy seems to be attractive. As there is still debate whether ADCC plays a role in metastatic cancer patients who mostly have suppressed immune function, we choose to evaluate cetuximab-mediated ADCC activity in front-line chemo-naïve mCRC patients. We only used an EGFR⁺ cell line and no control EGFR⁻ cell since the activation of ADCC activity by cetuximab requires the expression of its target, the EGFR [40]. Our functional assay provided evidences that CD56⁺ cells are the main effectors of cetuximab-mediated ADCC in mCRC patients. Furthermore, the PBMC of mCRC were capable to initiate ADCC comparable to healthy donors suggesting that their function is maintained. Furthermore, we observed a significant *in vitro* ADCC activity despite the mutated K-ras status of the DLD1 cell line. Our results are thus in accordance with a recent report showing that cetuximab-related ADCC is irrespective

of the K-ras status [40]. This property could be exploited particularly in patients with K-ras-mutated tumors, who otherwise have a low probability of responding to cetuximab [4,6-7]. In this way, approaches to enhance ADCC activity, such as immunostimulation and new generation of EGFR-directed mAbs, may be promising in the management of this subset of patients.

Whether ADCC contributes to the clinical efficacy of cetuximab, remains to be determined. Indeed, cetuximab and panitumumab displayed similar clinical activity despite the fact that panitumumab is a fully human IgG2 monoclonal antibody with low ability to recruit immune cells compared to IgG1. The adding value of cetuximab-mediated ADCC might deserve direct comparison between cetuximab and panitumumab. On the other hand, two studies have demonstrated that FC γ R gene polymorphisms (Fc γ R3A-V158F and Fc γ R2a-H131R) are associated with clinical outcome (response rate and PFS) in mCRC patients treated with cetuximab administered in combination with irinotecan or as a single-agent [20,21]. These findings may support the contribution of ADCC in cetuximab efficacy. In this line, it would be helpful to identify which surrogate markers may help us to predict this contribution to efficacy.

Complementary to our in vitro assay, we observed that the OR and PFS were significantly higher in patients with CD56 positive tumors as compared with CD56 negative tumors. This was not observed in a matched control group. Nevertheless, only evaluation in prospective randomized trials may confirm that CD56 tumor status is specifically predictive for ADCC contribution and not only prognostic.

When adjusted for K-ras status in multivariate model, the CD56 tumor status remains an independent marker of response to treatment (OR) and of better PFS and could

be therefore of additional interest in predicting enhanced cetuximab activity in the WT population.

Our data raise additional questions regarding the role of NK cells. The phenotype and properties of tumor-infiltrating NK cells seems to be different from that of peripheral NK cells. Peripheral NK cells are CD3⁻ CD56^{low} CD16⁺ while intratumoral NK cells are usually CD3⁻ CD56^{bright} CD16⁻ and unable to do ADCC [41,42]. The number of intratumoral CD56⁺ cells was found to be low (mean number: 6.4/mm² and 5.6/mm² in the treatment and the control group respectively) and it may be thus questionable how this small number can truly impact on CTX efficacy. However, while the circulating CD56⁺ cells represent a minor fraction of PBMC, we observed *in vitro* that their low number does not preclude for ADCC intensity. Additionally, some studies have evidenced significant immune cells recruitment into the tumors after trastuzumab or rituximab based therapy [38,43,44] even when trastuzumab was associated with immunosuppressive chemotherapy [38]. This recruitment was more significant in tumors exhibiting higher *in situ* infiltration lymphocytes infiltrate at the baseline [44]. All together, these data suggest that intratumoral CD56⁺ cells might be a surrogate marker of subsequent recruitment.

Conclusion

In summary, the presence of tumor-infiltrating CD56⁺ cells is an independent predictor for PFS and OR in mCRC patients treated with first line cetuximab based-chemotherapy. This suggests that ADCC, mainly through its central effector, the NK cells, could influence response to cetuximab-based chemotherapy. The exact relationship between TIL of the primary cancer and peripheral NK cells activity should be further explored, notably in assessing the role of ADCC in the adjuvant setting in patients treated with cetuximab.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RM designed the study, performed IHC and manual analysis, *in vitro* experiments, statistical analysis and drafted the manuscript, JDS performed PCR analysis, NN performed IHC and manual evaluation, PD performed IHC and manual evaluation, AL designed the study and performed *in vitro* experiments, JD, designed the study and drafted the manuscript, IS designed the study and drafted the manuscript, ST designed the study and drafted the manuscript, JLVL designed the study and drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends:

Figure 1: Sections of colorectal adenocarcinoma with results of the immunostaining: CD3, CD4, CD8, CD56, Foxp3 (magnification x 100).

The CD56- section (magnification X40) reveals no CD56 positive immune cells (considered as NK negative tumor) but staining of Meisner plexus used as interne positive control.

Figure 2: PFS according to tumor CD56 status in the treatment group (A) and the control group (B).

Figure 3: Cytotoxicity against colorectal cancer cell line mediated by cetuximab.

A-B: PBMCs from healthy volunteers (n=5, mean age 31 years) and from 5 mCRC patients (n= 5) mean age 52 years) were tested for cytotoxicity against DLD1, using four E:T ratio in the absence (PBMCs activity) or presence of cetuximab (10µg/ml).

Points mean of a triplicate experiment; bars: SD. **C-D:** PBMCs from 5 healthy donors and 5 mCRC were tested for the cytotoxicity in the presence or the absence of cetuximab, for the effect of IL-2 stimulation and CD56⁺ cells depletion. Columns, mean; bars, SD.

Tables

Table 1. Patients characteristics

Variables	Treatment group (n=33)	Control group (n=35)	p-value
Age, median (range)	59 (43-75)	62 (41-78)	0.74
Sex, n			
Male	19	20	
Female	14	15	0.97
ECOG PS, median (range)	0 (0-1)	0 (0-2)	0.37
Primary tumour location			
Right	11	11	
Transverse	1	2	
Left	18	19	
rectum	3	3	0.91
Metastatic sites			
Liver	24	26	
Lymph nodes	7	8	
Lung	2	1	0.98
Chemotherapy regimen			
Folfiri + cetuximab	32	0	
Folfiri	0	33	
Folfox + cetuximab	1	0	0.94
Folfox	0	2	
Overall Best Response			
CR	2	1	
PR	14	15	
SD	6	9	
PD	11	10	0.36
Treatment duration months, median	4.9	5.3	0.51
PFS Months, median	5.7	5.4	0.52

Abbreviations: NS: non significant, ECOG PS: Eastern Cooperative Oncology Group Performance Status.

Table 2. Mean numbers of tumor-infiltrating immune cells in treatment and control groups.

Variables	Treatment Group		Control Group		Treatment vs Control group
	Mean cells/mm ² (SD)	Median cells/mm ² (range)	Mean cells/mm ² (SD)	Median cells/mm ² (range)	p-value
CD3+	139 (108)	99 (0-340)	106 (89)	144 (0-410)	0.82
CD4+	33 (27)	42 (0-114)	24 (19)	28 (0-82)	0.21
CD8+	111 (106)	65 (29-331)	126 (101)	68 (28-361)	0.71
CD68+	171 (80)	88 (31-465)	139 (127)	151 (22-385)	0.27
Foxp3+	43 (36)	40 (14-150)	114 (101)	76 (12-228)	0.54
CD56+	6.4	0 (0-18)	5.6	0 (0-15)	0.32

Abbreviations: SD: Standard Deviation

Table 3. Univariate and multivariate analysis of factors associated with OR and PFS

UNIVARIATE ANALYSIS				
Variables	OR		PFS	
	HR (95% CI)	p-value	HR (95%CI)	p-value
Treatment Group				
CD3+	0.80 (0.20-3.25)	0.70	0.94 (0.45-1.94)	0.91
CD4+	1.31 (0.32-1.49)	0.36	0.74 (0.35-1.58)	0.41
CD8+	0.86 (0.22-3.58)	0.74	0.84 (0.45-1.72)	0.62
CD56+	7.75 (1.13-42.6)	0.012	2.70 (1.25-5.88)	0.005
CD68+	1.25 (0.79-7.72)	0.55	1.16 (0.55-2.46)	0.69
Foxp3+	0.54 (0.20-3.50)	0.98	0.89 (0.43-1.83)	0.81
K-ras status				
WT vs MT	12.81 (2.15-7.7)	0.008	4.36 (1.77-10.7)	0.0007
Control Group				
CD3+	0.91 (0.41-4.11)	0.63	0.84 (0.38-3.98)	0.74
CD4+	1.51 (0.61-2.74)	0.54	0.94 (0.52-2.98)	0.52
CD8+	1.32 (0.59-2.42)	0.31	1.20 (0.59-2.31)	0.76
CD56+	1.21 (0.74-2.07)	0.29	1.11 (0.81-1.56)	0.14
CD68+	0.84 (0.58-2.11)	0.36	0.87 (0.44-3.52)	0.27
Foxp3+	0.66 (0.34-1.92)	0.68	0.71 (0.88-1.56)	0.13
MULTIVARIATE ANALYSIS				
Variables	OR		PFS	
	HR (95% CI)	p-value	HR (95%CI)	p-value
CD56				
Negative	1		1	
Positive	7.05 (1.49-45.76)	0.04	2.62 (1.14-6.00)	0.0019
K-ras				
Mutated	1		1	
Wild Type	11.7 (1.77-8.33)	0.013	4.74 (1.8-12.3)	0.001

Abbreviations: CI: Confident interval, MT: mutated, WT: wild type, OR: overall response rate, PFS: Progression-free survival

Additional files

Additional file 1:

Title: Supplemental Table S1

Description: Summary of the immunohistochemistry.

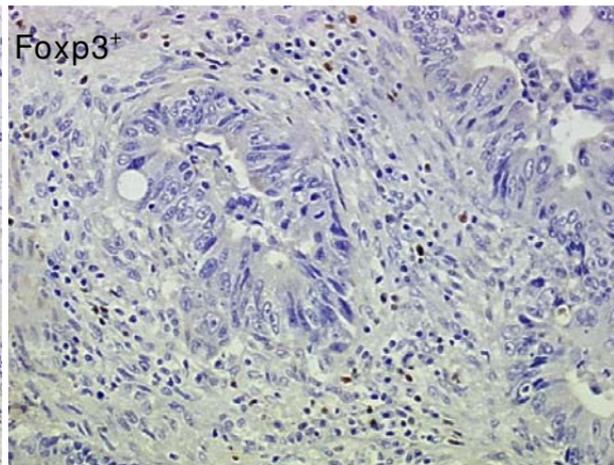
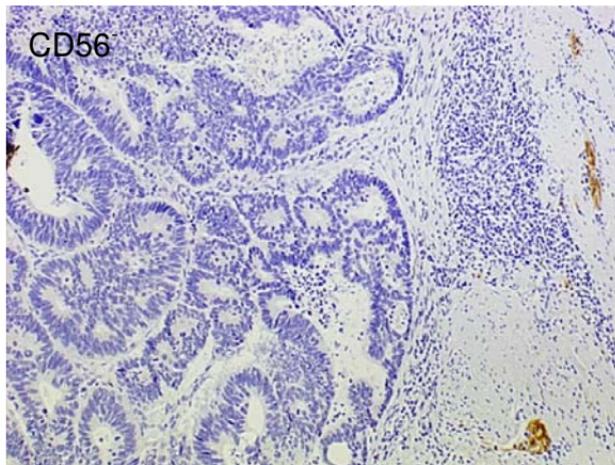
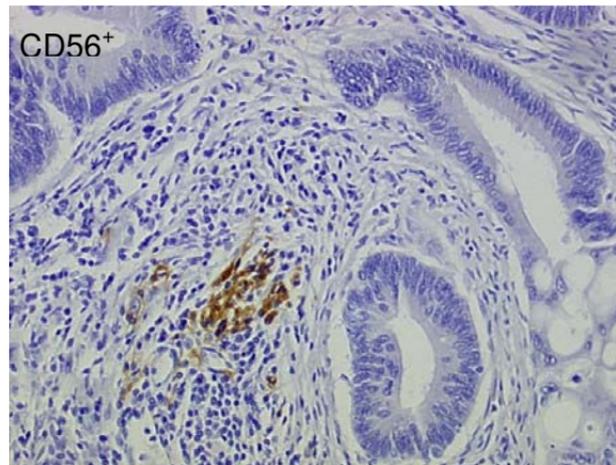
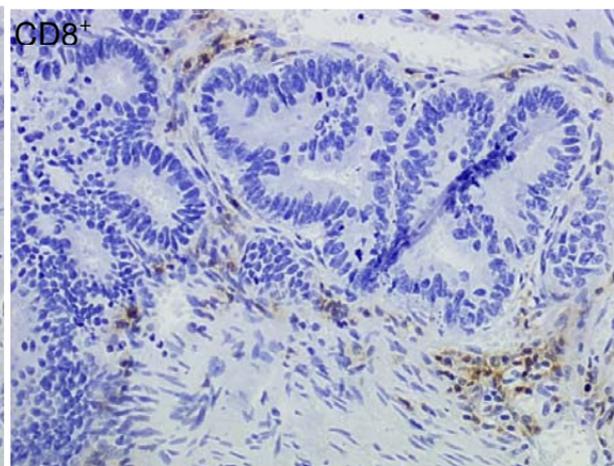
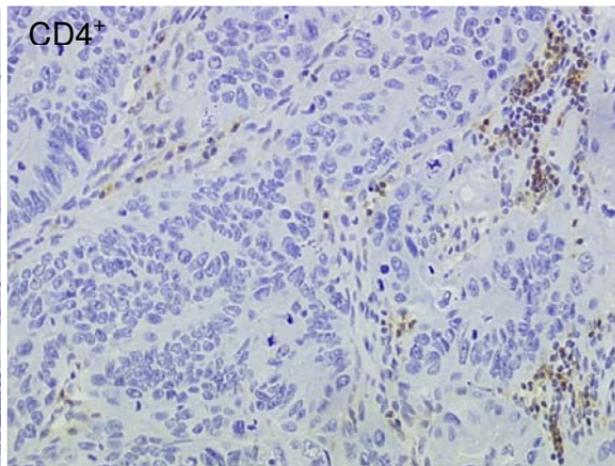
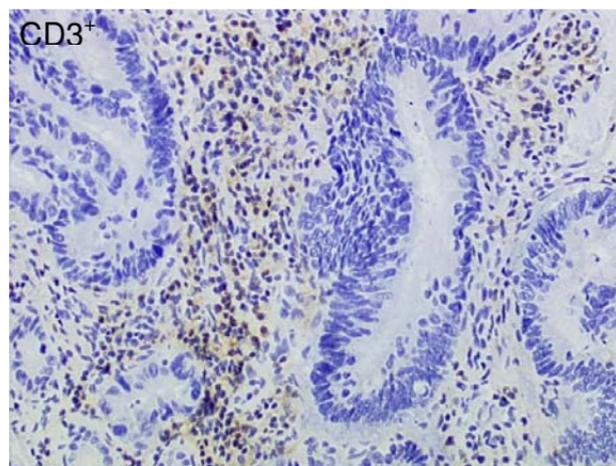
Additional file 2:

Title: Description of the MTT assay

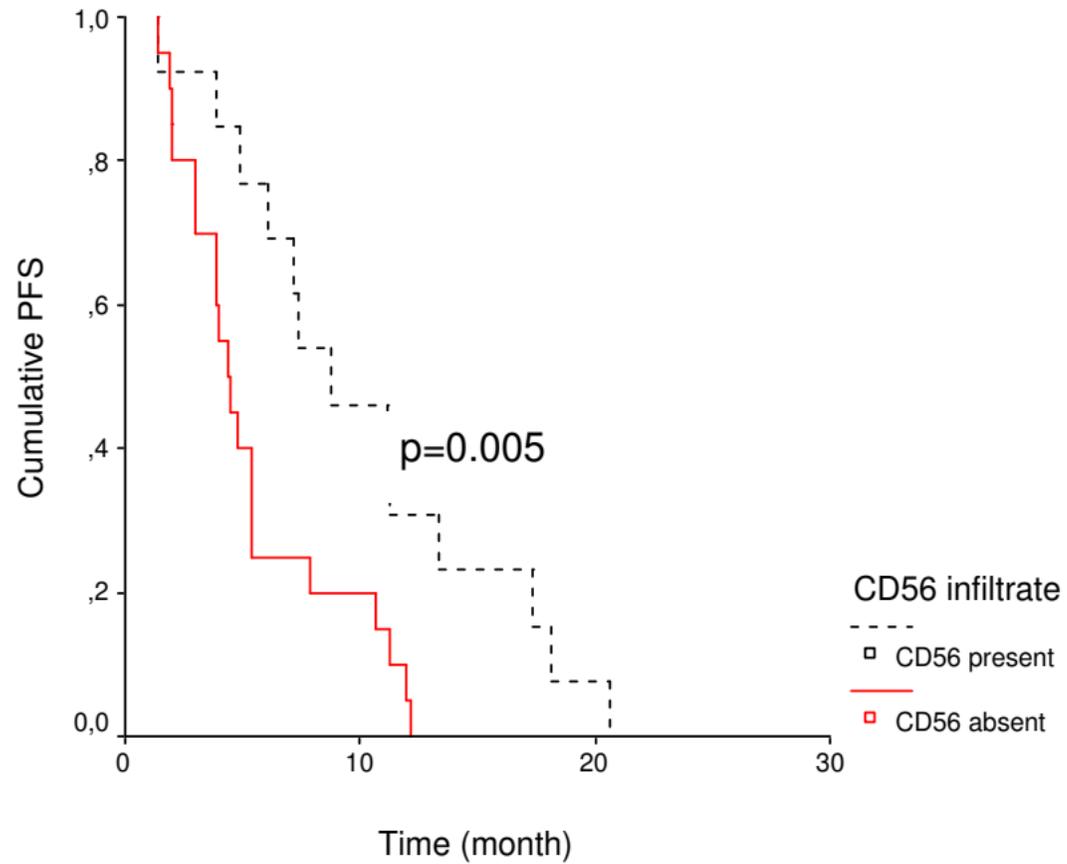
Additional file 3:

Title: Supplemental Table S2

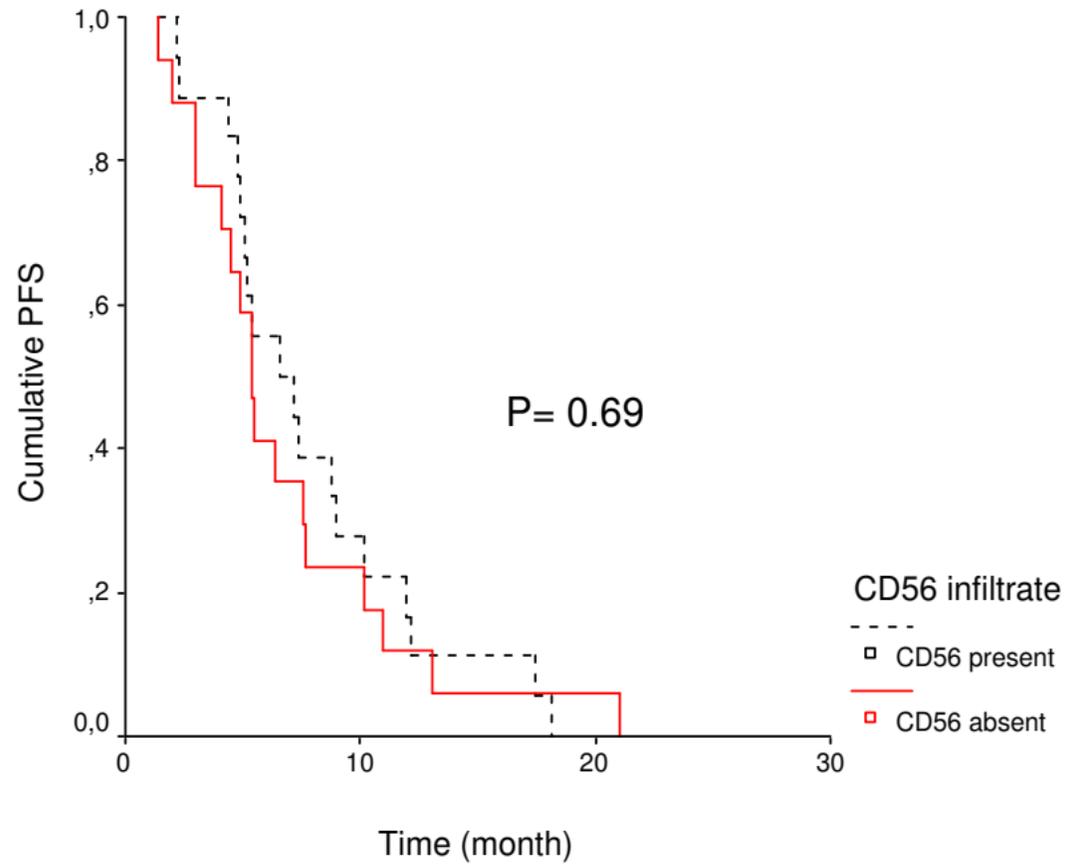
Description: Cetuximab-based chemotherapy efficacy in WT tumors based on the presence or absence of tumor infiltrating CD56+ cells.



A.

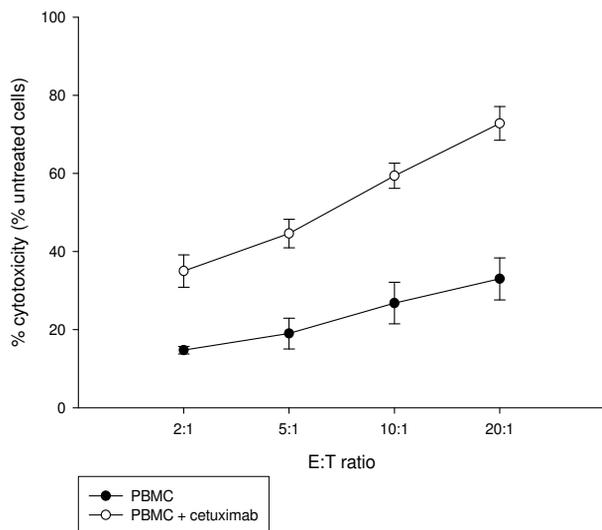


B.



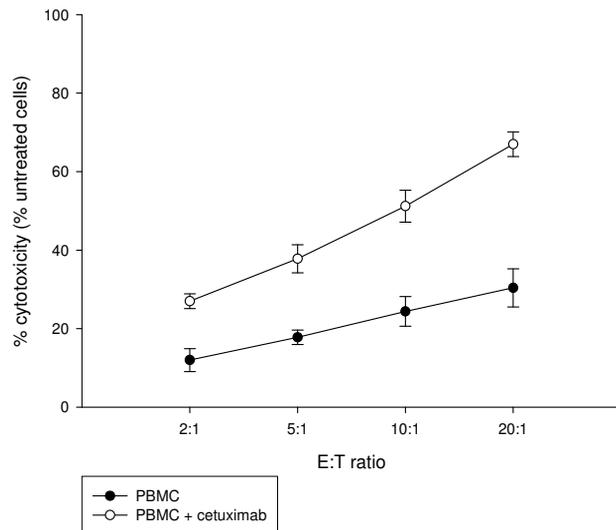
A.

Healthy volunteers



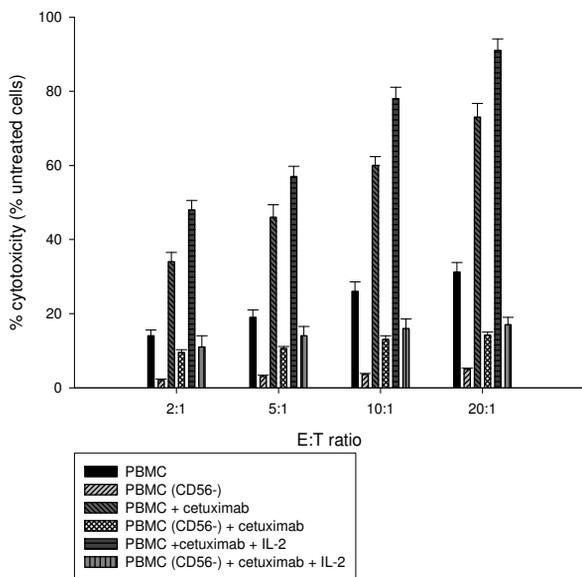
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Colorectal cancer patients



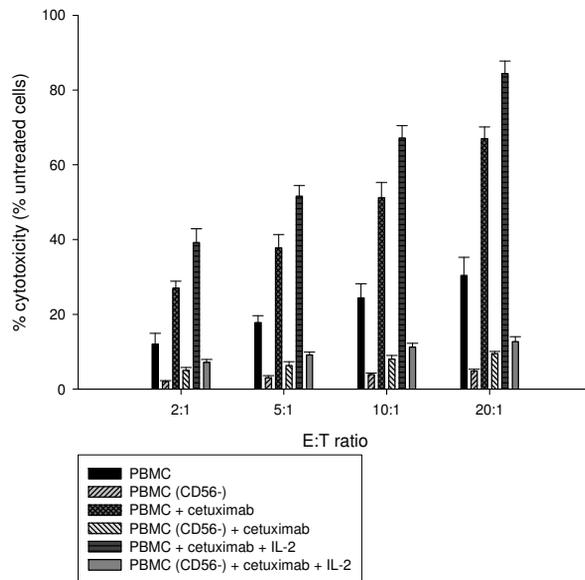
C.

Healthy volunteers



D.

Colorectal cancer patients



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Additional file 1: Additionalfile1.doc, 29K

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Additional file 3: Additionalfile3.doc, 27K

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