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Cancer Res 2011;71:1263-1271. Published OnlineFirst February 8, 2011.

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Clinical Impact of Different Classes of Infiltrating T Cytotoxic and Helper Cells (Th1, Th2, Treg, Th17) in Patients with Colorectal Cancer

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Abstract

The tumor microenvironment includes a complex network of immune T-cell subpopulations. In this study, we systematically analyzed the balance between cytotoxic T cells and different subsets of helper T cells in human colorectal cancers and we correlated their impact on disease-free survival. A panel of immune related genes were analyzed in 125 frozen colorectal tumor specimens. Infiltrating cytotoxic T cells, Treg, Th1, and Th17 cells were also quantified in the center and the invasive margin of the tumors. By hierarchical clustering of a correlation matrix we identified functional clusters of genes associated with Th17 (RORC, IL17A), Th2 (IL4, IL5, IL13), Th1 (Tbet, IRF1, IL12Rb2, STAT4), and cytotoxicity (GNLY, GZMB, PRF1). Patients with high expression of the Th17 cluster had a poor prognosis, whereas patients with high expression of the Th1 cluster had prolonged disease-free survival. In contrast, none of the Th2 clusters were predictive of prognosis. Combined analysis of cytotoxic/Th1 and Th17 clusters improved the ability to discriminate relapse. *In situ* analysis of the density of IL17+ cells and CD8+ cells in tumor tissues confirmed the results. Our findings argue that functional Th1 and Th17 clusters yield opposite effects on patient survival in colorectal cancer, and they provide complementary information that may improve prognosis. *Cancer Res*; 71(4); 1263–71. ©2011 AACR.

Introduction

Cancer progression is a complex process involving host–tumor interactions through multiple molecular and cellular factors of the tumor microenvironment. In mice, the immune cells appear to prevent the development of tumors and inhibit tumor progression (1). However, through inflammation-dependent mechanisms, the innate immune system can promote tumor development (2, 3). In humans, lymphocytes have been shown to participate in antitumoral responses. Tumor-infiltrating T cells are associated with improved clinical outcome and survival in colorectal cancer patients (4). Similar results were found in breast (5, 6) and lung cancer (7–11).

We previously showed that a high intratumoral memory T cells density correlated with the decrease of early metastatic events and the prevention of relapse in colorectal cancer patients (12). Furthermore, we demonstrated that the functional orientation, density, and location of immune cells profoundly influence the clinical outcome of the patients, regardless of cancer stages (4). In opposition to patients with metastasis or low density of immune cells, a functional coordination of the immune response was observed in patients without metastasis and with high density of immune cells (13).

We previously analyzed adaptive immune response, immunosuppression, and inflammation. Adaptive immune response correlated with the absence of tumor recurrence, whereas inflammation and immunosuppression did not. Here, we analyzed the cytotoxic CD8 T cells markers in relation to T helper subpopulations (Th1, Th2, Th17, and Treg).

Unsupervised hierarchical clustering of a correlation matrix revealed functional clusters of genes associated with Th17, Treg, Th2, Th1, and cytotoxic. Patients with high expression of the Th17 cluster had a poor prognosis whereas patients with high expression of the Th1 cluster had a prolonged disease-free survival. In contrast, no prediction of the prognosis was associated with Th2 or Treg clusters. The combined analysis of cytotoxic and Th17 clusters gave a better discrimination for relapse. These results were confirmed by *in situ* analysis.

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-10-2907

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Materials and Methods

Patients and database

Patients with colorectal cancer who underwent a primary resection at the Laennec/HEGP (Hopital Européen George Pompidou) Hospital between 1986 and 2004 were randomly selected (cohort 1, $n = 125$ and cohort 2, $n = 106$; Supplementary Table S4). The validation cohort ($n = 415$) was previously described (4). Time to recurrence or disease-free time was defined as the time period from the date of surgery to the confirmed tumor relapse date for relapsed patients and from the date of surgery to the date of last follow-up for disease-free patients (Supplementary Table S5). A secure Web-based database, TME.db, integrated the clinical data and the data from high-throughput technologies (36).

Real-time PCR analysis

Tissue samples were snap-frozen. Total RNA was extracted by homogenization with RNeasy isolation kit (Qiagen). The integrity and the quality of the RNA ($n = 125$) were evaluated on Bioanalyzer-2100 (Agilent Technologies). Quantitative real-time PCR was performed using Low-Density-Arrays and the 7900 robotic real-time PCR-system (Applied-Biosystems) according to the manufacturer's instructions. 18S primers and probes were used as internal controls. Data were analyzed using the SDS Software v2.3 (Applied-Biosystems).

Tissue microarrays construction

Using a tissue microarray instrument (Beecher Instruments, Alphelys, Plaisir, France), we selected 2 different and representative areas of the tumor. The center of the tumor (CT) and the invasive margin (IM) were punched (0.6 mm and 1 mm-diameter, respectively) from paraffin-embedded tissue-blocks. Tissue microarrays were constructed and cut into 5- μ m sections for immunohistochemical staining.

Immunohistochemistry

After antigen retrieval and quenching of endogenous peroxidase activity, sections were incubated for 60 min at room temperature with antibodies against CD8 (4B11; Neomarkers), FoxP3 (ab20034; abcam), CCL5 (NBP1-19769), RORC (NLS5188; Novus Biological), T-bet (4B10), CCL24 (G-17), and IL17 (H-132; Santa Cruz Biotechnology). Envision+ system (enzyme-conjugated polymer backbone coupled to secondary antibodies) and DAB-chromogen were applied (Dako). Tissue sections were counterstained with Harris's hematoxylin. Slides were analyzed using an image analysis workstation (SpotBrowser). The density was recorded as the number of positive cells per tissue surface area (mm^2). For each duplicate, the mean density was used for further statistical analysis.

Statistical analysis

Genesis software was used to visualize and cluster the correlation matrix and the genes expression. Kaplan-Meier curves were used to assess the influence of immune parameters on disease-free survival. The significance of these parameters was calculated with the log-rank test. We applied 2 different methods to assess the cutoffs for the separation of

patients, and similar results were found. First, we used hierarchical clusters of gene expression data (LDA) to define the cutoffs. Second, we applied cutoffs based on the patients' disease-free survival using the median and "minimum P -value" approach to separate patients into a Hi and Lo group. For pairwise comparisons Wilcoxon rank-sum test was used. $P < 0.05$ was considered statistically significant. All analyzes were performed with the statistical software R and Statview.

Results

Gene expression in normal versus tumoral tissue

We first investigated the expression of 45 immune genes related to cytotoxic, Th1, Th2, Th17, Treg cells in 125 colorectal tumors and 3 adjacent normal mucosa from the same patient by real-time PCR. The expression levels of regulatory T cells (FoxP3, CTLA4, TGF β 1) and Th1/cytotoxic T cells (IFNG, TAP1, GZMB) genes was significantly higher in tumors than in normal tissue (Fig. 1 and data not shown). We observed similar increase for Th2 (IL4, IL5, IL13) and Th17 (IL17A) genes. Among genes investigated the chemokine CCL24 was significantly different between tumors compared to normal mucosa.

Gene expression associated with the Th17 patient groups

According to the expression of the Th17 genes (IL17A and RORC), the patients were classified in 3 groups: a Lo group with low expression, a Het group with heterogeneous expression and Hi group with high expression of those genes. The

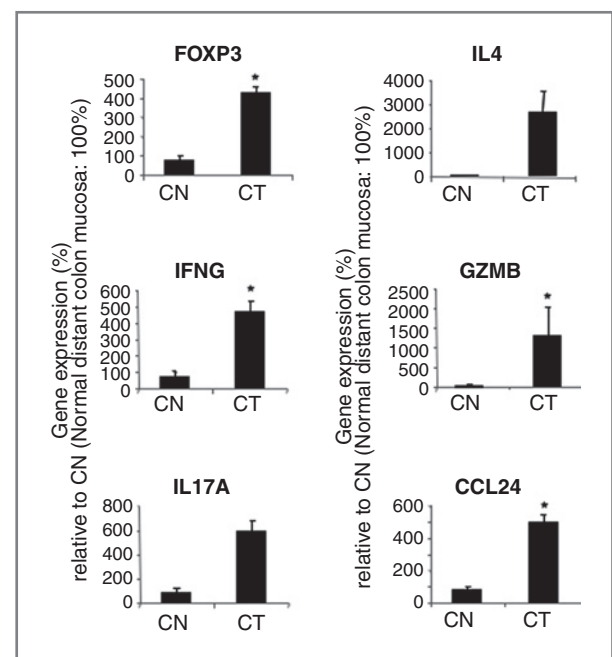
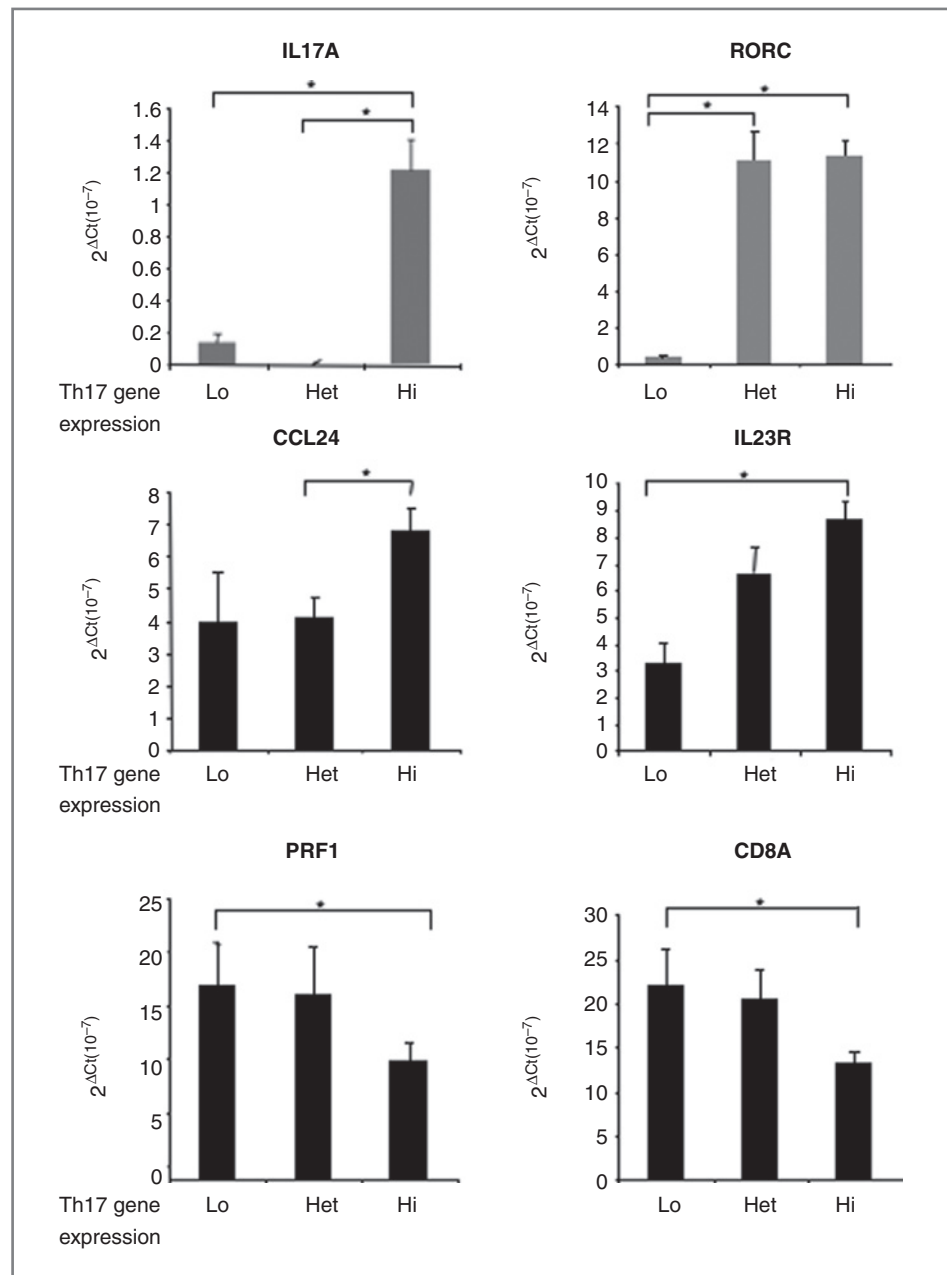


Figure 1. Immune gene expression level within primary colorectal tumors (CT; $n = 125$) compared to normal tissue (CN; $n = 3$). The percentage of the fold increase or decrease compared to CN is shown. *, $P < 0.05$ Wilcoxon test.

Figure 2. Immune gene expression in Th17-based patient groups. Patients ($n = 125$) were classified in 3 groups (Lo, Het, Hi) according to the Th17 gene expression (IL17A and RORC). Relative expression levels were adjusted for the level of 18S mRNA for each sample. The levels were represented as mean of $2^{-\Delta\Delta C_t}$ values. *, $P < 0.05$ Wilcoxon test.



expression of IL17A and RORC was used as control (Fig. 2). In Lo and Het groups, the expression level of IL17A was very low and high in the Th17 cluster Hi group. The expression level of RORC was low in the Th17 cluster Lo group and high in the other groups. The expression of CCL24 and IL23R was significantly increased in the Th17-Hi patient group, whereas the expression of Th1 and cytotoxic genes (PRF1 and CD8A) significantly decreased.

Correlation matrix of T-helper cells

The intratumoral immune coordination was assessed by analyzing the correlation between the selected 45 immune cell markers populations. Pairwise comparisons of gene expres-

sion levels were done by measuring Pearson correlation coefficients (r). The relationships implied by these correlations were visualized as an unsupervised hierarchical cluster of r values (Fig. 3A). We identified eight clusters of co-modulated genes. Two clusters were composed essentially by Th1 genes: Th1 cytotoxic cluster A.1 (IRF1, GZMB, IL27, GNLY, PRF1, CCL5, CD8A, STAT1) and Th1 cluster A.2 (IL12RB1, CD28, CCR5, HLA DMB, IL12RB2, CD38, CXCR6, TBX21). Three clusters had predominantly Th2 genes: Th2 cluster B.1 (IFNGR1, STAT3, IFNGR2, IL10RB, IL4R, STAT6), Th2 cluster B.2 (IL4, IL5, IL13), and Th2 cluster B.3 (CCR7, CD3E, CD40LG, CCL19, CCR4, GATA3). Two regulatory genes, IL10 and TGF β clustered together (C.1). A Treg Th2 cluster (C.2) including

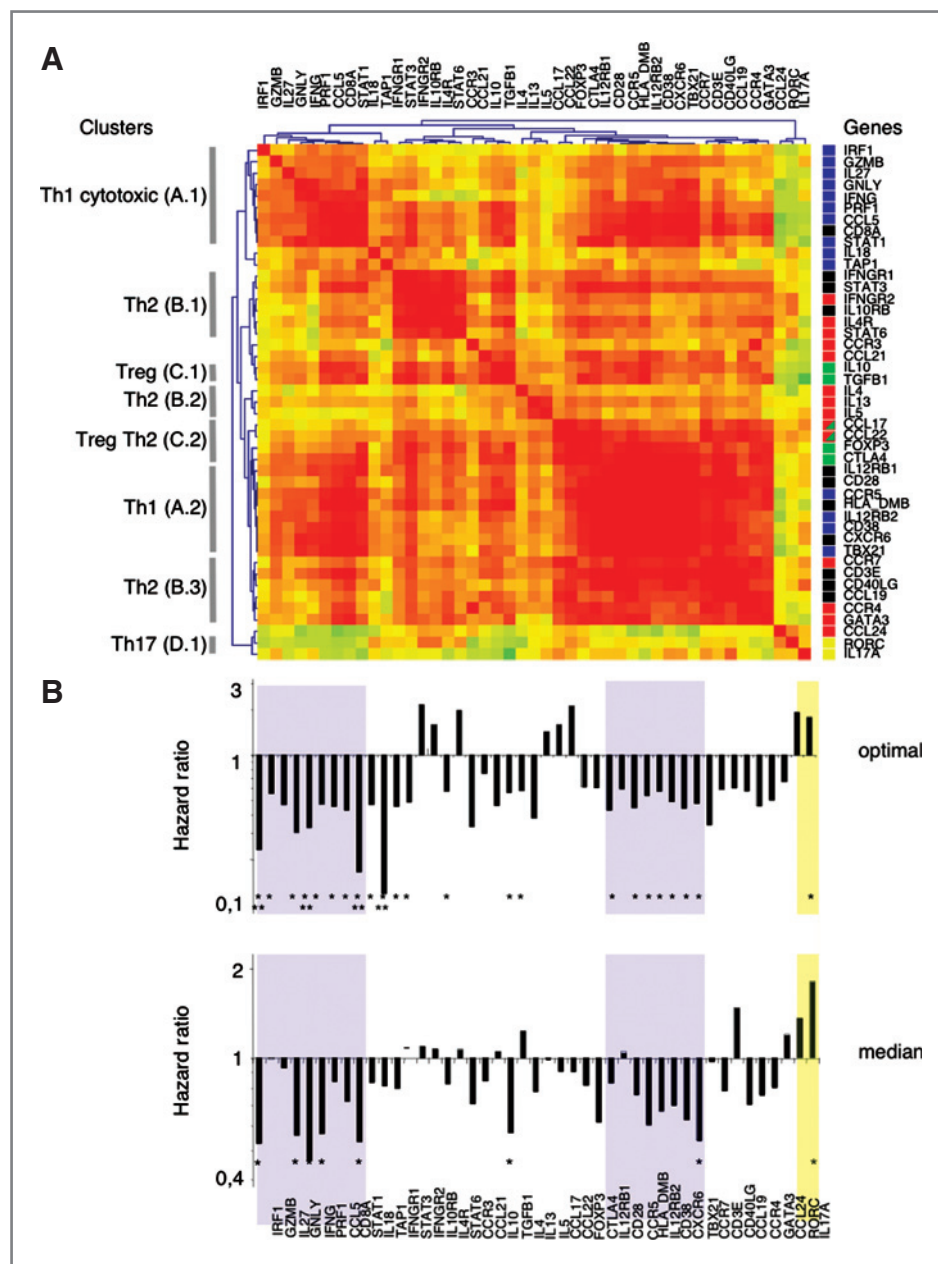


Figure 3. A, unsupervised hierarchical clustering of a gene-gene correlation from 125 frozen tumors. Pearson correlation coefficients (R) were calculated for combinations of the 45 immune cell markers. The negative correlation ($R = -0.8$) is shown in green, the positive correlation ($R = 0.8$) in red, and the absence of correlation ($R = 0$) in yellow. The marker type is shown on a color bar, on the right side of the figure. Th1 cells genes are represented in blue, Th2 cells genes in red, regulatory T cells genes in green, Th17 cells genes in yellow, and total T cells in black. Markers involved in 2 types of processes are represented with 2 colors. Functional clusters are marked on the left side of the figure. B, hazard ratio (HR) for DFS comparing patients with low against high gene expression. The order of the genes reflects the clustered correlation matrix. The cut point value for the expression of each gene was defined at the optimal (top) or the median (bottom) cutoff of the cohort. *, $P < 0.05$, **, P -corrected < 0.05 . Logrank test and Altman correction were applied. The clusters A.1 and A.2 are marked in blue and the D.1 in yellow.

FoxP3, CTLA4, and 2 chemokines (CCL17, CCL22) involved in both Treg and Th2 processes was found. The last cluster was composed with Th17 genes (RORC, IL17A) and the chemokine CCL24. Thus, the gene-gene correlation matrix revealed a functional coordination of T helper subsets at the tumor site. In order to validate the Th17 gene expression coordination, we performed *in situ* double staining with anti-CCL24 antibody and with anti-RORC or anti-IL17A antibodies by immunofluorescence. Interestingly, RORC+ or IL17A+ cells were found to be CCL24+ (Supplementary Fig. S1).

We performed disease-free survival analysis for up to 10 years after primary tumor resection (Fig. 3B and Supplementary Table S1). We determined the optimal and the median

cut-off values for each gene. Logrank P -value and hazard ratio associated with disease-free survival were then calculated. Most of genes from the Th1 cluster (A.1 and A.2) were logrank significant and had a hazard ratio lower than 1 at the optimal and median cut-off illustrating their beneficial effect on the patient survival. In contrast, the gene from the Th17 cluster had a hazard ratio higher than 1 and an opposite effect on the survival.

Disease-free survival associated with the functional clusters

To better characterize the mechanisms involved in anti-tumoral activity, we investigated the impact of the mRNA

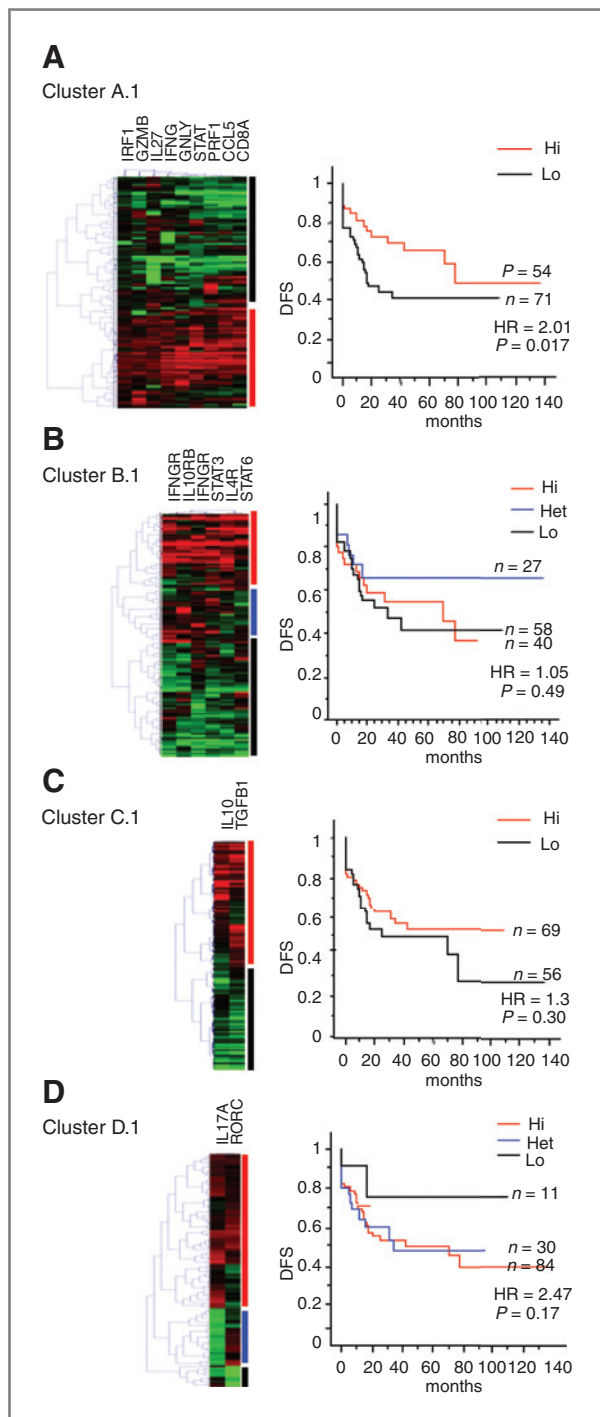


Figure 4. Hierarchical clustering of the Th1 (A), Th2 (B), Treg (C), and Th17 (D) genes in 125 patients. High expressed genes are shown with red and low expressed genes with green. Kaplan-Meier curves illustrated the duration of disease free survival according to the high (Hi, red line), heterogenous (Het, blue), or low (Lo, black line) gene expression. Hazard ratio (HR) and logrank P value for DFS comparing patients with low against high gene expression were calculated.

expression level on the clinical outcome. For each gene cluster defined in the correlation matrix, groups of patients were defined according to their gene expression (Fig. 4 and Supplementary Fig. S2) using unsupervised hierarchical clustering. High expression of Th1 cytotoxic genes (cluster A.1) was associated with significantly improved disease-free survival rates ($P = 0.01$; Fig. 4A). Patients with low expression of those genes had an early recurrence. The median time to relapse was 18 and 78 months for patients with low and high expression, respectively. In the same way, patients with high expression of Th1 genes from cluster A.2 had prolonged disease-free survival (Supplementary Fig. S2A). The expression level of Th2 and regulatory T clusters had no apparent effect on clinical outcome (Fig. 4B and C and Supplementary Fig. S2B and C). On the contrary, patients with low expression of Th17 genes (cluster D.1) seemed to have a prolonged disease-free survival (Fig. 4D). Thus, 80% of the patients with low expression did not experience relapse.

Disease-free survival associated with the Th1 and Th17 clusters

We assessed the complementary effect of Th1 and Th17 immune reaction. Kaplan-Meier curves illustrating the combination of hi and lo groups from both Th1 cytotoxic (A.1) and Th17 (D.1) clusters can be seen in Figure 5. In the Th1 cytotoxic Hi group, patients with low expression of the gene from the Th17 cluster had a better survival than patients with high expression of those genes. Similarly in the Th1 cytotoxic lo group, patients with low expression of the gene from the Th17 cluster had a better survival than patients with high expression of those genes. Thus the best combination associated with prolonged disease-free survival was Th1-Hi and Th17-Lo (DFS at 5yrs: 100%) whereas the worst combination was Th1-Lo and Th17-Hi (DFS at 5yrs: 40%, HR = 1.29, $P = 0.005$).

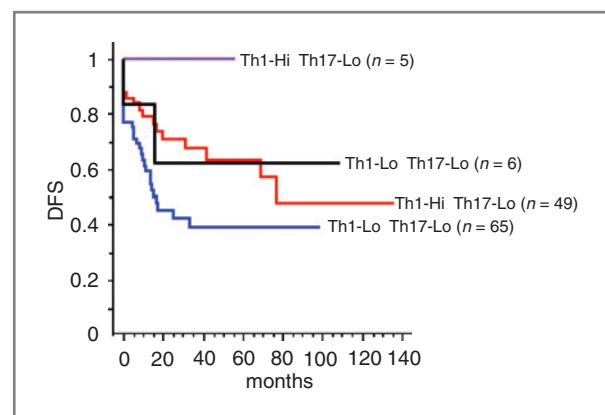


Figure 5. Disease free survival (DFS) of colorectal cancer patients according to the expression level of the gene from the Th1 cytotoxic gene cluster (A.1) in combination with the genes from Th17 genes cluster (D.1). The DFS of the patients with Th1-Hi Th17-Hi is shown in red, with Th1-Lo Th17-Lo in black, with Th1-Lo Th17-Hi in blue, and with Th1-Hi Th17-Lo in purple.

Validation study

We validated our mRNA results with *in situ* studies using tissue microarray from the center and the invasive margin of the tumor. Immunostaining for Th1 (CD8, T-bet), Th17 (IL17), and regulatory T cells (FOXP3) were illustrated (Fig. 6) and quantified with a dedicated image analysis workstation. A precise measurement of intratumoral immune cell density was performed by counting the immune cells and measuring the surface area of the tissue.

The percentage of the relapsing patients decreased with the CD8 density (Fig. 7A): 11.7%, 37.5%, and 63.3% in CD8 HiHi, Het, LoLo patients groups, respectively. Contrary to CD8, the frequency of relapsing patients increased with the IL17 density (63.3%, 0%, 0% in IL17 HiHi, Het, LoLo and CD8 HiHi patient groups, 60%, 37.5%, 0% in IL17 HiHi, Het, LoLo, and CD8 Het patient groups and 100%, 63.2%, 20% in IL17 HiHi, Het, LoLo, and CD8 LoLo patients groups, respectively). We evaluated the disease-free survival according to the Th1 and Th17 cell density. Kaplan–Meier curves illustrated the pejorative effect of IL17A on patient's survival. This confirmed the results at the gene expression level. Patients with low IL17 density in the 2 regions of the tumors had a better disease-free survival than patients with high IL17 density in both regions (Fig. 7B, HR = 3.08, $P = 0.0009$). Indeed, 70% of the patients in the LoLo group did not relapse. In contrast, 50% of the patients in the HiHi group had tumor recurrence after 1 year. On the contrary, patients with high density of CD8, T-Bet or FOXP3 had a better disease-free survival than patients with low density of those markers (Supplementary Fig. S3). The combination of the CD8 and IL17 markers defined groups of patients with very different outcome. Patients with CD8 LoLo had a dramatic outcome. Median disease-free survival was 9 months and all patients experienced tumor recurrence after 6 years. In contrast, patients with CD8 HiHi had a very good outcome

and patients with CD8-HiHi and a low density of IL17+ cells (IL17-LoLo) had the best outcome (Supplementary Fig. S4, HR = 1.48, $P = 0.0009$). Strikingly, in the group of patients with heterogeneous density of CD8, patients with low density of IL17 had a better disease-free survival whereas patients with high density of IL17 had a poor prognosis (Fig. 7C and Supplementary Fig. S4). 8% of patients relapsed in the group of patient with low density of IL17 cells whereas 40% and 70% of patients in the IL17-Het and IL17-HiHi groups relapsed, respectively. Longer disease-free survival was observed among patients with tumor containing a high density of CD8+ cells and a low density of IL17+ cells than patients among patients whose tumors had low densities of IL17+ cells and high densities of CD8+ cells (Supplementary Table S2). We validated the results by analyzing an independent cohort of 415 colorectal cancer patients by tissue microarrays. Similar results were found (Supplementary Fig. S5A and B). Longer disease-free survival was observed among patients with tumor containing a high density of CD8+ cells (CD8-HiHi), and patients with CD8-HiHi and a low density of IL17+ cells (IL17-LoLo) had the best outcome (Supplementary Fig. S5A and B). Patients with CD8-LoLo had a very bad outcome. Strikingly, the group of patients with CD8-LoLo and with high density of IL17 (IL17-HiHi) had the worst prognosis. Similar hazard-ratios and P -values were found in both cohorts (HR = 1.49 and HR = 1.37, $P = 0.0002$, respectively).

Finally, we performed multivariate analysis integrating the classical clinical parameters with the immune score. We applied stepwise Akaike information criterion (AIC)-based Cox multivariate analysis to reduce the number of parameters to the most informative ones. Supplementary Table S3 shows that the immune parameter (CD8/IL17) in the whole as well as in the reduced model remains significant.

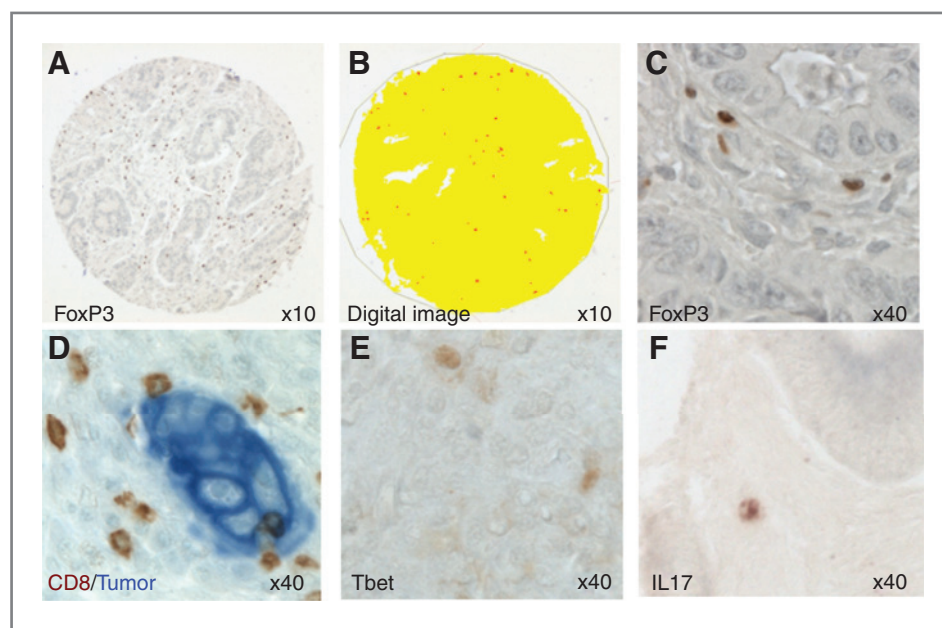


Figure 6. A representative example of FoxP3 (A, C), CD8 (D), Tbet (E), and IL17 (F) immunostaining of colorectal cancer tissue microarray. B, digital image analyzed with the image software SpotBrowser with tissue represented in yellow and the FoxP3 cells represented in red. The densities of these immune cells were recorded as the number of positive cells per unit of tissue surface area (mm^2).

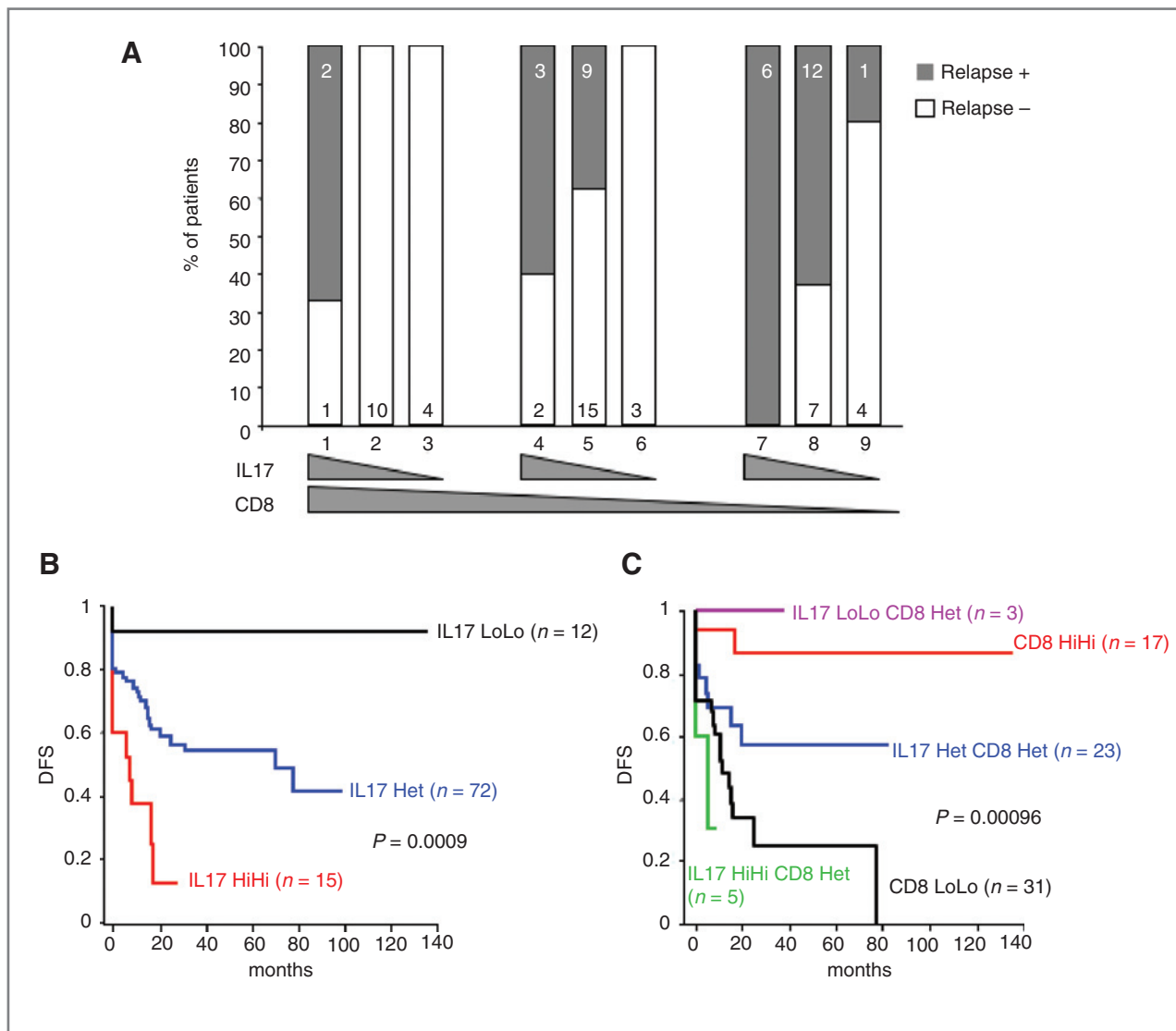


Figure 7. A, the frequency of patients with (black columns) or without (white columns) relapse classified according to the densities of immune cells within colorectal tumors. Patients ($n = 71$) were classified according to the CD8 and IL17 cell densities in 2 regions of the tumor, the center and the invasive margin of the tumor. High CD8 densities in the 2 regions of CD8 were represented in the histograms 1–3, heterogeneous densities in the histograms 4–6, and low densities in the histograms 7–9. High densities in the 2 regions of IL17 were represented in the histograms 1,4,7, heterogeneous densities in the histograms 2,5,8, and low densities in the histograms 3,6,9. Kaplan Meier curves illustrate the duration of disease free survival according to the IL17 cell density (B) in combination with CD8 cell density (C) in 2 region of the tumor, the center and the invasive margin of the tumor. Groups of patients with low densities in the 2 regions were named LoLo, those with high densities in the 2 regions HiHi, and the others with heterogeneous densities Het. The cutoff value for the density of CD8 and IL17 cells was defined at the optimal P -value of the cohort. Hazard ratio (HR) and logrank P corrected values for DFS comparing patients with low against high gene expression.

Discussion

We analyzed T helper subpopulations in colorectal cancer. We showed that the immune markers expression was different in adjacent mucosa and tumoral tissues suggesting that specific T helper subsets are recruited at the tumor site. Unsupervised hierarchical clustering of a correlation matrix revealed functional clusters of immune genes: Th17, Th1, Th2, Treg, and cytotoxic. Thus, immune coordination between T helper subsets was found within the tumors. Th1, cytotoxic

and Th17 clusters were associated with a particular clinical outcome. Patients with high expression of the Th17 cluster genes had a poor prognosis whereas patients with high expression of Th1 or cytotoxic cluster genes had a prolonged disease-free survival. The combined analysis of the cytotoxic and Th17 clusters gave a better discrimination for relapse. These results were confirmed by *in situ* analysis: patients with high density of IL17+ cells had a poor prognosis whereas patients with high density of CD8+ cells had a prolonged disease-free survival. The combination of these 2 markers gave

also a better discrimination of patients. In particular, patients with heterogeneous densities of CD8 cells between tumor regions having an intermediate outcome could be discriminated in good or bad survivor depending on the densities of the IL17+ cells.

We previously showed that Th1 pattern was associated with increased expression of CX3CL1 (14) and memory T-cell response associated with increased CXCL9 and CXCL10 expression (14). Here we show for the first time that Th17 produced CCL24, a chemokine known to affect the eosinophils (15–17).

We have shown that high Th1 and cytotoxic gene levels were associated with a good outcome. These results have been validated with the CD8 and T-bet protein studies that confirms previous findings (4, 13, 18–20).

In contrast to Th1, we have shown that patients with a low Th17 response had a better disease-free survival. However the Th17 responses decreased with the tumor progression in the tissue (T-stage). On the contrary, the Th17 response increased with advanced gastric cancer (21). Kryczek and colleagues (22) observed an opposite effect in ovarian cancer: tumor infiltrating Th17 are reduced in more advanced diseases and a low level of IL17 is associated with a bad outcome. It was found that IL-1 β , but not IL-1 α , IL-6, TGF- β , and IL-23, is crucial for Th17 cell induction. Consistent with this observation, the levels of IL-1 α and IL-23 were found negligible in ovarian cancer ascites (22). It suggests that IL-1 α and IL-23 play a minor role in Th17 cell development in human ovarian cancer, in contrast to other pathologic conditions such as colorectal cancer or psoriasis (23). However, in ovarian cancer, Th17 cells synergistically induced the production of CXCL9 and CXCL10, and in turn promoted effector T-cell migration toward tumor (22). Thus, Th17 cells may not mediate a direct tumor cytotoxic activity against tumor cells. In colorectal cancer, we found that high expression of Th1 and cytotoxic genes were associated with a good outcome, whereas IL17 was associated with bad outcome. However, the positive effect of Th1 on patient's survival seems to be superior to the negative effect of Th17 on patient's survival.

We found 3 different clusters associated with Th2 response, but none of these clusters was associated with a particular outcome. Previously, Th2 response was associated with the tumor immune evasion in mice studies (24, 25), or not associated with clinical outcome in human studies (26, 27). Our study does not support a major role of Th2 cells on patient's prognosis.

We found 2 different clusters related to regulatory genes, including cytokines, chemokines and transcription factors that were not associated with a particular outcome. At the

protein level, patients with high density of FoxP3 positive cells had a better disease-free survival. This result confirms the analysis of the FoxP3 mRNA expression: high level seems to be better for patient's survival. The role of regulatory T cells in cancer is disputed. In breast cancer, FoxP3 expression is associated with the worst overall survival (28, 29). Curiel and colleagues (30) reported that the presence of high numbers of CD3+CD4+CD25+, FoxP3+ cells in malignant ascites of ovarian carcinoma correlated with tumor staging and reduced survival. In colorectal cancer, Loddenkemper and colleagues (31) did not find any difference between patients with high or low Treg infiltration whereas Salama and colleagues (32) found an improved survival associated with a high density of tumor infiltrating FoxP3 suggesting no major immunosuppressive role of Treg cells in colorectal cancer. However, the facts that Treg can loose FoxP3 expression, and change phenotype (33), and normal T cells can acquire FoxP3 after multiple stimulation, without becoming regulatory (34), add complexity to these analysis (35). Finally, as Treg are positively correlated with effectors T-cells and Th1 cells, the positive impact of Treg cells could be the reflection of an indirect effect.

In summary, unsupervised hierarchical clustering of a correlation matrix revealed functional clusters of genes associated with Th17, Treg, Th2, Th1, and cytotoxic T cells. Patients with high expression of the Th17 cluster had a poor prognosis whereas patients with high expression of the Th1 cluster had a prolonged disease-free survival. Both gene expression experiments and *in situ* immunohistochemistry analysis revealed that the combined analysis of cytotoxic and Th17 clusters gave a better discrimination for relapse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by grants from the Association pour la Recherche sur le Cancer (ARC), the National Cancer Institute (INCA), the Canceropole Ile de France, Ville de Paris, INSERM, the Austrian Federal Ministry of Science and Research (GEN-AU project, Bioinformatics Integration Network), the Austrian Science Fund, and the European Commission (7FP, Geninca Consortium, grant number 202230).

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Received August 9, 2010; revised October 3, 2010; accepted November 4, 2010; published OnlineFirst February 8, 2011.

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Supplementary Online information for

Clinical impact of infiltrating cytotoxic- and helper-T cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer

Short title: Clinical impact of Th1 and Th17 in colorectal cancer

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Supplementary Figure 1

A representative example of CCL24/RORC (**A**), CCL24/IL17 (**B**), and IL17/CCL5 (**C**) immunostaining of colorectal cancer.

Supplementary Figure 2

Hierarchical tree structure classifying the 126 patients according to the mRNA level of genes from the Th1 (**A**) Th2 (**B**) Treg (**C**) clusters from maximal (red) to minimal (green) expression levels.

Kaplan-Meier curves illustrated the duration of disease free survival according to the high (Hi, red line) or low (Lo, black line) gene expression. Hazard ratio (HR) for DFS comparing patients with low against high gene expression. Logrank p value

Supplementary Figure 3

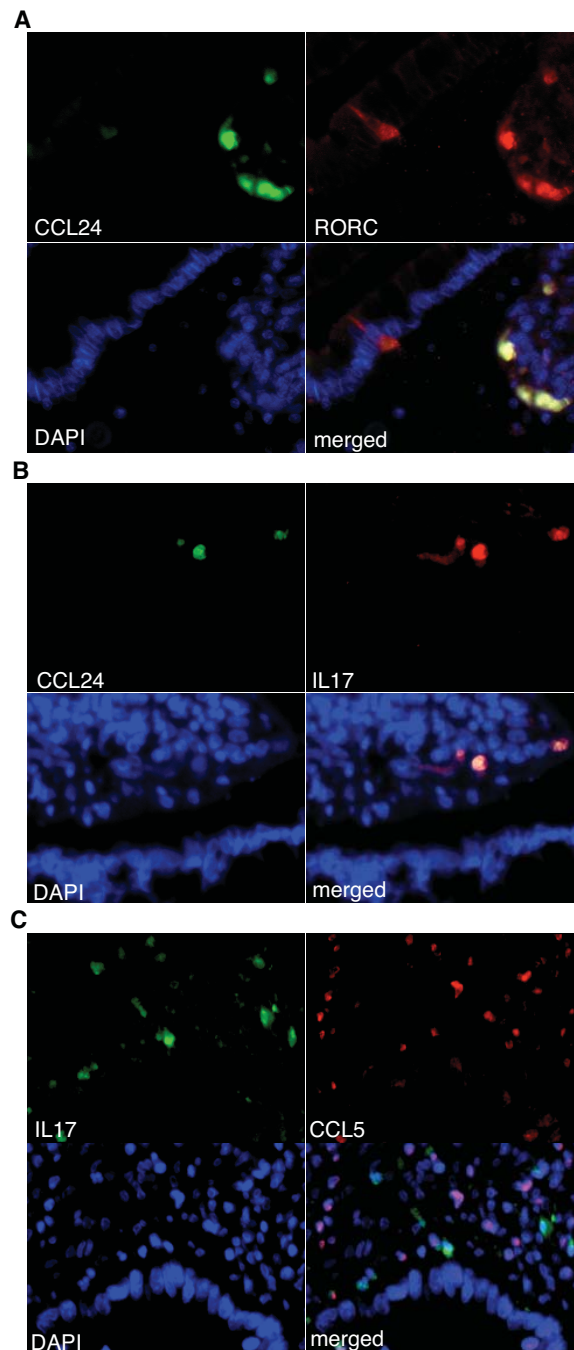
Kaplan Meier curves illustrate the duration of Disease free survival according to the T-Bet (A), FoxP3 (B) and CD8 (C) cells density in two region of the tumor, the center and the invasive margin of the tumor. The cutoff values were defined at the optimal p-value of the cohort.

Supplementary Figure 4

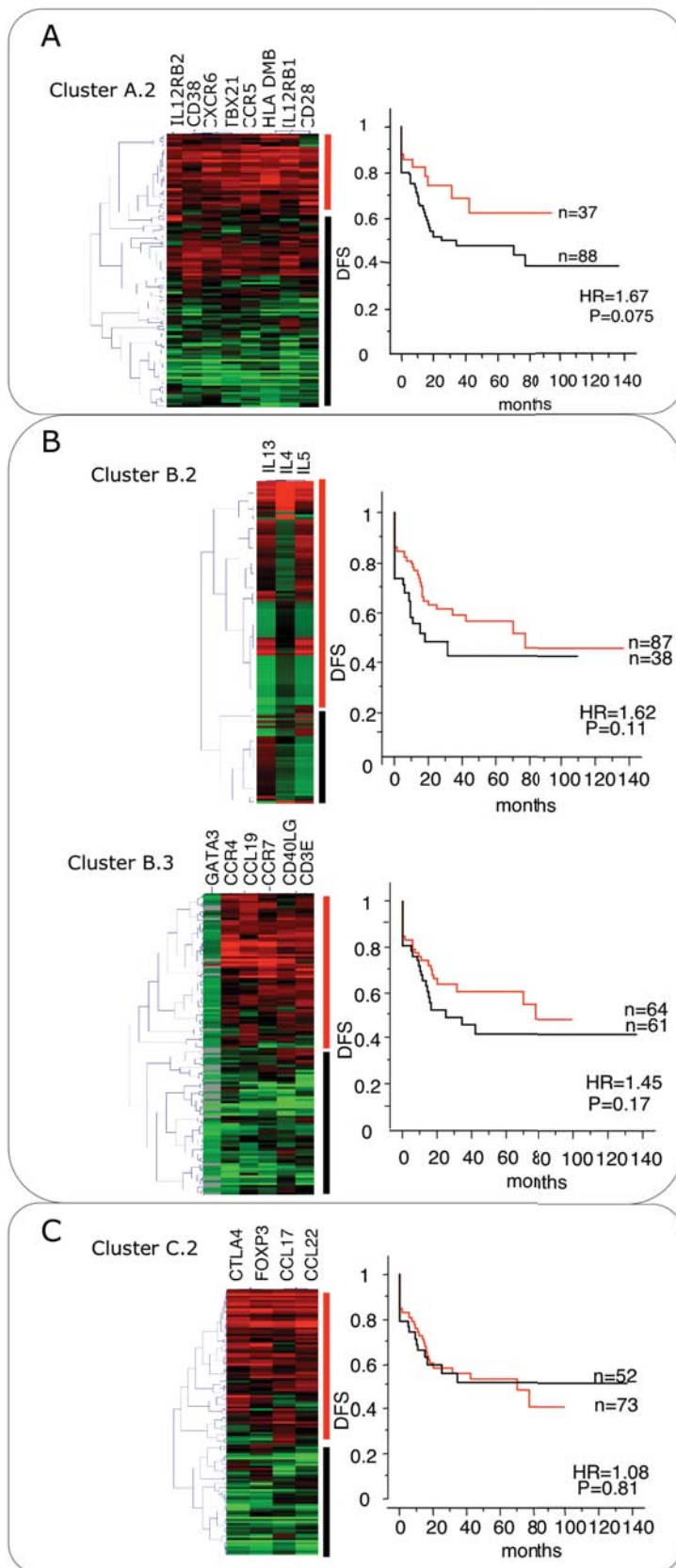
Kaplan Meier curves illustrate the duration of Disease free survival according to the IL17 cells density in combination with CD8 cells density in two region of the tumor, the center and the invasive margin of the tumor. The cutoff value for the density of IL17 and CD8 cells was defined at the optimal p-value of the cohort.

Supplementary Figure 5

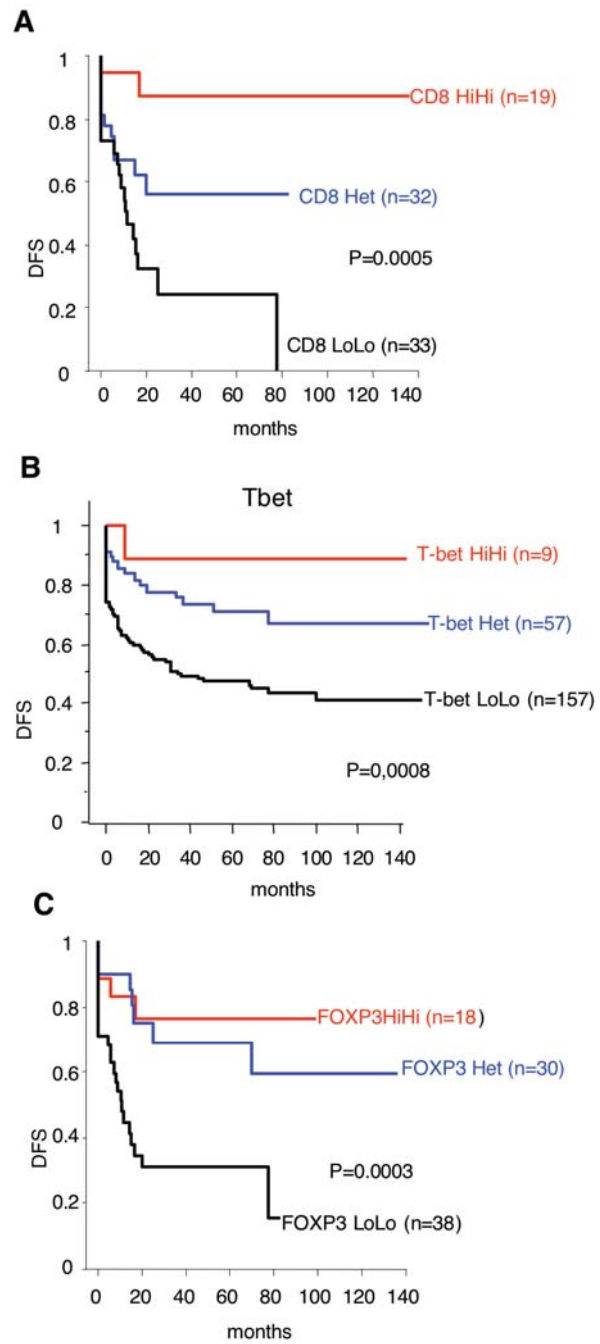
Kaplan Meier curves illustrate the duration of Disease free survival according to the IL17 cells density in combination with CD8 cells density in two region of the tumor, the center and the invasive margin of the tumor. Panel (**A**) illustrates cohort 2 (n=106 patients), and panel (**B**) illustrates the validation cohort (n=415).



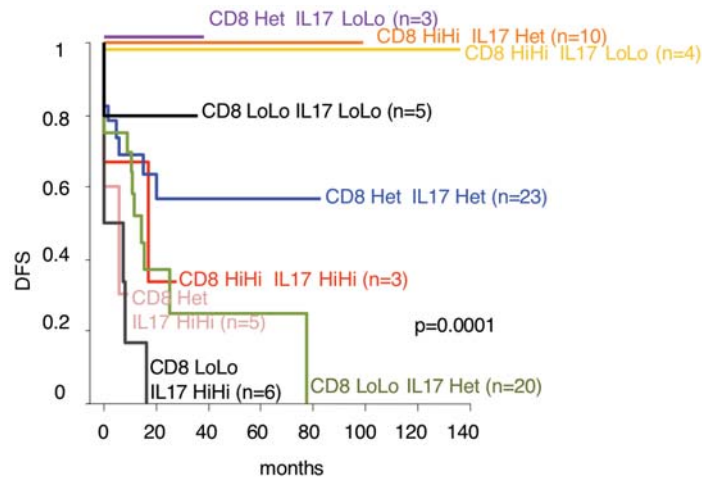
Supplementary Figure 1



Supplementary Figure 2

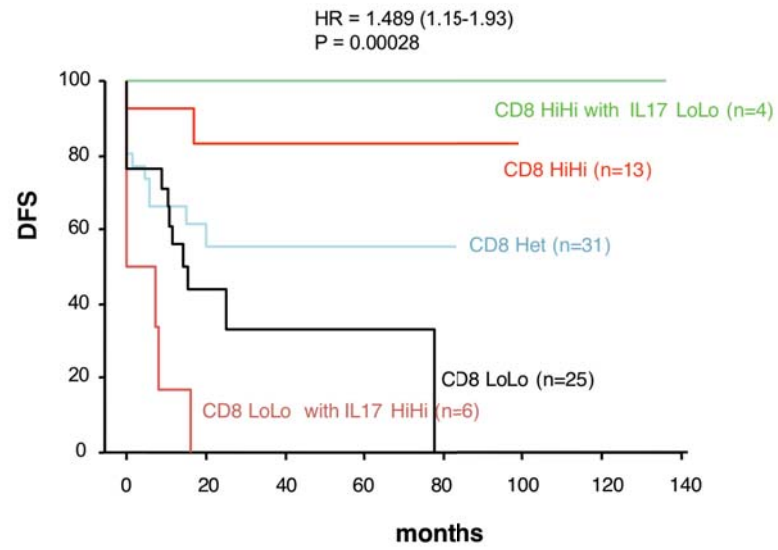


Supplementary Figure 3

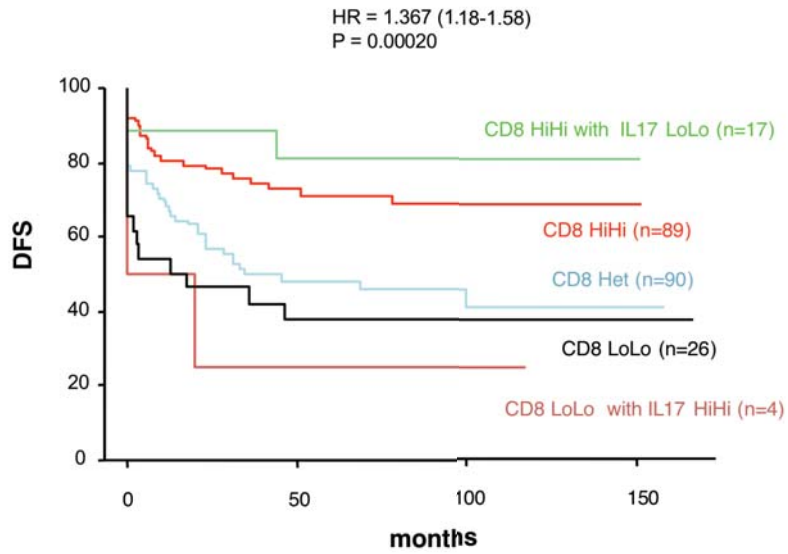


Supplementary Figure 4

A



B



Supplementary Figure 5

Gene	Hazard ratio (optimal)	P value* (optimal)	P value ** (optimal)	Hazard ratio (median)	P value* (median)
IRF1	0.23 (0.52-0.1)	<0.001 S	0.006 S	0.522 (0.93-0.29)	0.02 S
GZMB	0.55 (0.96-0.32)	0.03 S	0.66	0.985 (1.69-0.57)	0.954
IL27	0.46 (1.16-0.18)	0.104	0.104	0.926 (1.61-0.53)	0.797
GNLY	0.3 (0.65-0.14)	0.001 S	0.057	0.555 (0.98-0.32)	0.036 S
IFNG	0.33 (0.64-0.17)	0.001 S	0.036 S	0.46 (0.22-0.87)	0.007 S
PRF1	0.47 (0.81-0.27)	0.005 S	0.186	0.562 (0.99-0.32)	0.038 S
CCL5	0.56 (1.04-0.3)	0.027 S	0.617	0.834 (1.45-0.48)	0.5
CD8A	0.43 (0.83-0.22)	0.007 S	0.248	0.717 (1.25-0.41)	0.24
STAT1	0.17 (0.53-0.05)	<0.001 S	0.028 S	0.528 (0.93-0.3)	0.022 S
IL18	0.47 (0.85-0.25)	0.012 S	0.354	0.827 (1.43-0.48)	0.464
TAP1	0.12 (0.49-0.03)	<0.001 S	0.021 S	0.805 (0.72-2.16)	0.406
IFNGR1	0.45 (0.84-0.24)	0.01 S	0.311	0.791 (1.37-0.45)	0.4
STAT3	0.48 (1-0.23)	0.048 S	0.879	0.999 (1.72-0.57)	0.951
IFNGR2	0.47 (1.49-0.14)	0.18	0.18	1.088 (0.63-1.89)	0.801
IL10RB	0.63 (1.11-0.36)	0.121	0.121	1.072 (1.61-0.54)	0.842
IL4R	0.57 (0.99-0.33)	0.037 S	0.757	0.816 (1.43-0.47)	0.436
STAT6	1.98 (0.93-4.2)	0.072	0.072	1.064 (1.64-0.54)	0.841
CCR3	0.33 (1.06-0.1)	0.052	0.052	0.704 (1.22-0.4)	0.213
CCL21	0.75 (1.33-0.42)	0.319	0.319	0.837 (1.45-0.48)	0.509
IL10	0.46 (0.9-0.23)	0.015 S	0.424	1.047 (1.67-0.55)	0.909
TGFB1	0.56 (0.98-0.32)	0.037 S	0.751	0.567 (0.99-0.32)	0.041 S
IL4	0.58 (1.45-0.23)	0.237	0.237	1.221 (1.43-0.47)	0.531
IL13	0.38 (1.05-0.14)	0.051	0.051	0.773 (1.33-0.44)	0.368
IL5	1.42 (0.79-2.54)	0.209	0.209	0.983 (1.69-0.56)	0.991
CCL17	1.59 (0.9-2.81)	0.105	0.105	0.902 (0.64-1.92)	0.721
CCL22	2.12 (0.91-4.98)	0.072	0.072	0.898 (0.64-1.93)	0.751
FOXP3	0.61 (1.05-0.35)	0.07	0.07	0.81 (0.71-2.14)	0.45
CTLA4	0.6 (1.05-0.34)	0.074	0.074	0.615 (1.08-0.35)	0.087
IL12RB1	0.43 (0.84-0.22)	0.011 S	0.335	0.825 (1.43-0.48)	0.511
CD28	0.6 (1.1-0.32)	0.094	0.094	1.04 (1.67-0.56)	0.953
CCR5	0.44 (0.99-0.2)	0.042 S	0.809	0.757 (0.76-2.29)	0.328
HLA_DMB	0.53 (0.95-0.3)	0.032 S	0.691	0.6 (1.05-0.34)	0.068
IL12RB2	0.57 (1.01-0.33)	0.046 S	0.859	0.664 (1.16-0.38)	0.131
CD38	0.49 (0.9-0.26)	0.018 S	0.068	0.697 (0.82-2.51)	0.195
CXCR6	0.44 (0.83-0.23)	0.009 S	0.299	0.623 (1.09-0.36)	0.091
TBX21	0.47 (0.85-0.26)	0.01 S	0.317	0.531 (0.93-0.3)	0.024 S
CCR7	0.34 (1.1-0.11)	0.06	0.06	0.966 (1.67-0.56)	0.939
CD3E	0.59 (1.04-0.33)	0.062	0.062	0.778 (1.35-0.45)	0.367
CD40LG	0.6 (1.09-0.33)	0.087	0.087	1.454 (0.83-2.53)	0.195
CCL19	0.57 (1.08-0.3)	0.075	0.075	0.699 (1.22-0.4)	0.204
CCR4	0.46 (1.01-0.21)	0.052	0.052	0.752 (1.3-0.43)	0.302
GATA3	0.5 (1.03-0.24)	0.059	0.059	0.798 (0.69-2.27)	0.475
CCL24	0.66 (1.15-0.38)	0.137	0.137	1.184 (1.47-0.49)	0.553
RORC	1.92 (0.86-4.28)	0.101	0.101	1.349 (1.28-0.43)	0.283
IL17A	1.78 (1.01-3.11)	0.037 S	0.753	1.778 (0.99-0.32)	0.037 S

*Log-rank P value. **P value correction for optimal cutoff with Altman et al. Het: Hi/Lo and Lo/Hi. NR: Not Reached.

Supplementary Table 1

Multivariate analysis of Disease-free survival (DFS) among patients with AJCC/UICC-TNM Stage I/II/III/IV colorectal cancer cohort 2 (TMA 106pts) according to clinical parameters or immune parameters

	No. of patients (percentage)	Rate at 5 yr % (95% CI)	Hazard ratio (95% CI)	P value*	P value* **	Hazard ratio (95% CI)	P value*	P value**
CD8 CT								
Lo	55 (53.4)	32.4 (19.9-52.6)	3.78 (1.86-7.66)	0.0001	s	0.0047	s	
Hi	48 (46.6)	76.2 (63.9-90.9)	1.0 (reference)					0.0047 s
CD8 IM								
Lo	56 (65.9)	42 (29.5-59.9)	2.73 (1.19-6.27)	0.0138	s	0.3955		0.3955
Hi	29 (34.1)	68.5 (51-92)	1.0 (reference)				0.0138 s	
CD8 CT/IM								
LoLo	33 (39.3)	24.4 (11-54.4)	11.08 (2.54-48.43)	0.0001	s	0.0053	s	0.39 (0.23-0.65)
Het	32 (38.1)	55.9 (39-79.9)	4.5 (1-20.19)	0.0306	s	0.6726		0.0005 s
HiHi	19 (22.6)	87.4 (72.4-100)	1.0 (reference)					0.0276 s
IL17 CT								
Lo	14 (13.5)	92.9 (80.3-100)	0.14 (0.02-1.02)	0.0206	s	0.5200		7.11 (0.98-51.66)
Hi	90 (86.5)	48.4 (38.1-61.5)	1.0 (reference)				0.0206 s	0.5200
IL17 IM								
Lo	85 (84.2)	58 (47.1-71.5)	0.36 (0.18-0.72)	0.0031	s	0.1277		2.78 (1.38-5.6)
Hi	16 (15.8)	20.8 (6.6-65.7)	1.0 (reference)				0.0031 s	0.1277
IL17 CT/IM								
LoLo	12 (12.1)	91.7 (77.3-100)	0.08 (0.01-0.64)	0.0021	s	0.0935		3.08 (1.67-5.7)
Het	72 (72.7)	54.4 (42.9-68.9)	0.35 (0.17-0.71)	0.0024	s	0.1041		0.0009 s
HiHi	15 (15.2)	12.5 (2.2-71.1)	1.0 (reference)					0.0475 s
Immune Score CD8/IL17								
I0	31 (39.2)	25.1 (11.3-55.8)	10.07 (2.29-44.22)	0.0002	s	0.0119	s	0.68 (0.53-0.87)
I1 - IL17 LoLo	3 (3.8)	100 (100-100)	NaN (NaN-NaN)	NaN		NaN		0.0010 s
I1 - IL17 Het	23 (29.1)	56.7 (38.2-83.9)	3.91 (0.84-18.16)	0.0590		NaN		
I1 - IL17 HiHi	5 (6.3)	30 (6.3-100)	NaN (NaN-NaN)	NaN		NaN		
I2	17 (21.5)	86.3 (70.1-100)	1.0 (reference)					

† The stage was determined by pathological (p) examination. Tis denotes carcinoma in situ, T1 tumor invading submucosa, T2 tumor invading muscularis propria, T3 tumor penetrating muscularis propria and invading subserosa, and T4 tumor completely through the intestine's wall and invading serosa and nearby tissues. #: Bowel perforation presentation.

*Log-rank P value. **P value correction with Altman et al. Het: Hi/Lo and Lo/Hi. NR: Not Reached. s:significant.

CI denotes confidence interval. CT: center of the tumor and IM: Invasive margin. I0: CD8 LoLo, I1: CD8 Het and I2: CD8 HiHi.

Supplementary Table 2

Multivariate proportional hazard Cox analysis for DFS among patients with UICC-TNM Stage I/II/III/IV colorectal cancer					
Clinical parameters + Immune Score CD8/IL17					
Variable	PHA test ***	Hazard ration	(95% CI)	P-value	
Age (yrs) (<65=0, 65-75=1, 75-85=2, >85=3)	0.9538	1.01	(0.67-1.53)	0.9551	
Tumor (T) stage (TisT1 = 0, T2 = 1, T3 = 2, T4 = 3)	0.8819	2.64	(1.24-5.61)	0.0118	s
N stage (N0 = 0, N1 = 1, N2 = 2)	0.0035	s	(0.76-1.89)	0.4365	
Tumor Location (Left colon=0, Right colon=1, Transverse colon=2, Sigmoid colon=3, Rectum=4)	0.3126	0.89	(0.67-1.18)	0.4082	
Histological grade (Well differentiated=0, Moderately differentiated=1, Poor differentiated=2)	0.4494	0.97	(0.48-1.96)	0.9280	
Mucinous Colloide Type (No = 0, Yes = 1)	0.4801	1.08	(0.42-2.75)	0.8767	
Occlusion (No = 0, Yes = 1)	0.2639	0.81	(0.23-2.9)	0.7454	
Perforation (No = 0, Yes = 1)	0.8252	0.79	(0.09-7.35)	0.8370	
Immune Score CD8/IL17 (I0=0, I1-IL17LoLo=1, I1-IL17Het=2, I1-IL17HiHi=3, I2=4)	0.0347	s	(0.49-0.89)	0.0073	s
		0.69**	(0.52-0.9)		
** Leave-one-out method, correction using $C=1-(SE[coef]/coef)^2 = 0.91$					
Final Model for DFS after stepwise (stepAIC) selection					
Variable	PHA test ***	Hazard ration	(95% CI)	P-value	
Tumor (T) stage (TisT1 = 0, T2 = 1, T3 = 2, T4 = 3)	0.5720	2.64	(1.35-5.16)	0.0045	s
Immune Score CD8/IL17 (I0=0, I1-IL17LoLo=1, I1-IL17Het=2, I1-IL17HiHi=3, I2=4)	0.0510	0.67	(0.51-0.88)	0.0034	s
		0.68**	(0.53-0.88)		
** Leave-one-out method, correction using $C=1-(SE[coef]/coef)^2 = 0.95$					
* Immune Score: CD8CT/IMCD45ROCT/IM, minimum <i>P</i> -value cut-off with 4 groups: (Im4)-4Hi, (Im3)-3Hi, (Im2)-2Hi, (Im1)-1Hi, (Im0)-0Hi					
** heuristic shrinkage factor. Corrected by Hollaender et al. Similar results obtained using bootstrap. #: Bowel perforation presentation (Yes, No).					
*** PHA (proportional hazards assumption) test ($P<0.05$ violates the hazards assumption)					

Supplementary Table 3

Comparison of CRC cohorts according to clinical parameters

Cohorts	Percentage (total number) of patients		pv*
	LDA (125 pts) (cohort 1)	TMA (106 pts) (cohort 2)	
AJCC/UICC (TNM) stage			0.6519
I	23 (18.4)	13 (12.3)	
II	39 (31.2)	35 (33)	
III	34 (27.2)	31 (29.2)	
IV	29 (23.2)	27 (25.5)	
Tumor (T) stage †			0.6739
pTis-1	7 (5.6)	3 (2.8)	
pT2	19 (15.2)	13 (12.3)	
pT3	77 (61.6)	70 (66)	
pT4	22 (17.6)	20 (18.9)	
N stage			0.8490
N0	70 (56)	57 (53.8)	
N1	30 (24)	25 (23.6)	
N2	25 (20)	24 (22.6)	
M stage			0.7585
M0	96 (76.8)	79 (74.5)	
M+	29 (23.2)	27 (25.5)	
Age (yrs)			0.9927
< 65	34 (27.2)	28 (26.4)	
65-75	30 (24)	24 (22.6)	
75-85	42 (33.6)	38 (35.8)	
> 85	19 (15.2)	16 (15.1)	
Tumor Location			0.9283
Left colon	15 (12)	14 (13.2)	
Right colon	37 (29.6)	34 (32.1)	
Transverse colon	11 (8.8)	8 (7.5)	
Sigmoid colon	38 (30.4)	34 (32.1)	
Rectum	24 (19.2)	16 (15.1)	
Histological grade			0.8376
Well Differentiated	80 (64)	67 (63.2)	
Moderately Differentiated	41 (32.8)	37 (34.9)	
Poorly Differentiated	4 (3.2)	2 (1.9)	
Mucinous Colloid Type			0.8770
No	95 (76.6)	80 (77.7)	
Yes	29 (23.4)	23 (22.3)	
Occlusion			1.0000
No	108 (86.4)	91 (85.8)	
Yes	17 (13.6)	15 (14.2)	
Perforation#			1.0000
No	122 (97.6)	103 (97.2)	
Yes	3 (2.4)	3 (2.8)	

† The stage was determined by pathological (p) examination. Tis denotes carcinoma in situ, T1 tumor invading submucosa, T2 tumor invading muscularis propria, T3 tumor penetrating muscularis propria and invading subserosa, and T4 tumor completely through the intestine's wall and invading serosa and nearby tissues. #: Bowel perforation presentation. pv* Fisher's exact test P value.

Univariate analysis of Disease-free survival (DFS) among patients with AJCC/UICC-TNM Stage I/II/III/IV colorectal cancer cohort 1 /2 according to clinical parameters					
	LDA 125 patients (cohort 1)			TMA 106 patients (cohort 2)	
	Hazard ratio (95% CI)	P value*		Hazard ratio (95% CI)	P value*
According to clinical parameters					
AJCC/UICC (TNM) stage (I = 0, II = 1, III = 2, IV = 3)	2.78 (1.94-3.98)	<0.0001	s	2.62 (1.76-3.9)	<0.0001 s
Tumor (T) stage † (TisT1 = 0, T2 = 1, T3 = 2, T4 = 3)	1.76 (1.15-2.69)	0.0390	s	1.6 (0.98-2.64)	0.0754 .
N Stage (N0 = 0, N1 = 1, N2 = 2)	1.59 (1.14-2.22)	0.0188	s	1.36 (0.95-1.95)	0.0520 .
M Stage (M0 = 0, M+ = 1)	12.3 (6.74-22.43)	<0.0001	s	11.57 (6.12-21.88)	<0.0001 s
Age (yrs) (<65=0, 65-75=1, 75-85=2, >85=3)	0.94 (0.71-1.24)	0.3481		0.93 (0.68-1.27)	0.1921
Tumor Location (Left colon=0, Right colon=1, Transverse colon=2, Sigmoid colon=3, Rectum=4)	1.18 (0.98-1.41)	0.2143		1.26 (1.03-1.54)	0.0237 s
Histological grade (Well differentiated=0, Moderately differentiated=1, Poor differentiated=2)	0.78 (0.48-1.28)	0.1654		0.87 (0.5-1.5)	0.3828
Mucinous Colloide Type (No = 0, Yes = 1)	1.1 (0.56-2.15)	0.7933		1.01 (0.48-2.11)	0.9970
Occlusion (No = 0, Yes = 1)	1.33 (0.62-2.83)	0.4686		0.98 (0.41-2.32)	0.9563
Perforation# (No = 0, Yes = 1)	4.15 (0.97-17.65)	0.0337	s	3.63 (0.85-15.48)	0.0545 .
† The stage was determined by pathological (p) examination. Tis denotes carcinoma in situ, T1 tumor invading submucosa, T2 tumor invading muscularis propria, T3 tumor penetrating muscularis propria and invading subserosa, and T4 tumor completely through the intestine's wall and invading serosa and nearby tissues.					
#: Bowel perforation presentation. *: Log-rank P value. Het: Hi/Lo and Lo/Hi. NR: Not Reached. 's':significant p<0.05. '.': 0.1>p>=0.5. CI denotes confidence interval.					

Supplementary Table 5