Extent and location of tumor infiltrating lymphocytes in micro-satellite stable colorectal cancer predicts outcome to adjuvant Active Specific Immunotherapy

A.W. Turksma
M.C. Shamier
K.L.H. Lam
V.M.H. Coupé
V.A. de Weger
J.A.M. Belien
A.J. van den Eertwegh
G.A. Meijer
C.J.L.M. Meijer
E. Hooijberg

Clinical Cancer Research, under review
Abstract

Purpose: We investigated the prognostic and predictive value of tumor infiltrating lymphocytes (TIL) in colorectal cancer in a cohort of patients who previously took part in a trial on adjuvant Active Specific Immunotherapy (ASI).

Experimental Design: We determined the numbers of CD3+ and CD8+ cells in archival tumor samples of 110 colorectal cancer patients as total infiltrating TIL, intraepithelial TIL and stromal TIL. Survival curves were used to determine the prognostic and predictive value of TIL.

Results: Patients with microsatellite instable (MSI) tumors had higher numbers of intraepithelial CD3+ and CD8+ TIL compared to patients with microsatellite stable (MSS) tumors. Patients with MSI tumors had a good prognosis. Patients with MSS cancers with high intraepithelial or stromal TIL numbers had a better prognosis compared to those with low TIL numbers. Significant survival benefits were found for the disease specific survival (DSS) and recurrence free interval (RFI) for patients with high infiltrates of stromal CD3+ or intraepithelial CD8+ cells. Additionally high numbers of total CD8+ and high stromal CD8+ cells gave a significant difference in RFI compared to low TIL numbers. The numbers of TIL found in MSS tumors also have predictive value with respect to response to adjuvant ASI treatment. Compared to controls significance was reached in ASI treated patients with high total CD3, high stromal CD3 or high stromal CD8 numbers in DSS as well as RFI.

Conclusion: ASI therapy is effective in patients with MSS colorectal tumors harboring high numbers of preexisting stromal CD3 or CD8 positive TIL.

Statement of translational relevance

The presence or absence of intraepithelial or stromal tumor infiltrating lymphocytes (TIL) has prognostic value for patients with colorectal cancer and may also have predictive value with respect to response to therapy. Information about the presence of TIL in relation to microsatellite status of the colorectal cancer and the response to adjuvant Active Specific Immunotherapy (ASI) was lacking. Here, we investigated this retrospectively and concluded that patients with MSI cancers did well irrespective of extent or location of TIL. Patients with MSS cancers with high intraepithelial or stromal TIL numbers had a better prognosis compared to those with low TIL numbers. Furthermore, adjuvant ASI therapy was shown to be effective in patients with MSS colorectal tumors harboring high numbers of preexisting CD3 or CD8 positive T cells in the stroma. These data provide support for a differentiated approach for future development and clinical testing of adjuvant treatment of patients with colorectal cancer.
Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide for both males and females, with a 5-year survival rate of approximately 50%, depending on tumor stage. Mutations in oncogenes and tumor suppressor genes such as TP53, KRAS, BRAF, APC, B-Catenin, SMAD4 and AXIN contribute to the carcinogenesis in CRC [1].

Mutations can also arise in the DNA mismatch repair system in the tumor cells, which can lead to microsatellite instability. In CRC 15% to 20% is microsatellite instable (MSI) and 80% to 85% is microsatellite stable (MSS) largely overlapping with chromosomal instability [2, 3]. Frame shift mutations in protein coding sequences may lead to the formation of frame shift peptides in MSI tumors. Frame shift peptide derived T cell epitopes can be recognized as foreign by the immune system, and may therefore give rise to an increase of tumor specific T cells [4].

Currently the prognosis for CRC patients is determined by using the UICC TNM classification system [5]. The 5-year survival for CRC patients ranges from almost 100% for patients with stage I disease to less than 5% for patients with stage IV disease [1]. Besides the TNM criteria, tumor intrinsic factors can play a role in determining the prognosis for CRC patients. For example, KRAS mutations and specific TP53 mutations are correlated with a relatively poor prognosis [6, 7]. Low expression of p21 and cyclin D1 and high expression of p53 and AURKA cell-cycle proteins have been correlated with disease recurrences [8]. Conversely patients with MSI tumors have a relatively good prognosis [9].

TNM classification, genetic factors and microsatellite status of the tumor are important determinants of the prognosis for CRC patients. In recent years it has become more clear that the tumor microenvironment is also of importance for the prognosis for these patients [10, 11]. The presence of intraepithelial or stromal tumor infiltrating lymphocytes (TIL), has been shown to play an important role in the survival of patients with colorectal cancer [11-13]. Recently Galon and co-workers have proposed an alternative classification system to determine the prognosis; the Immunoscore [14, 15]. The Immunoscore is aimed at quantifying various types of TIL. Numbers of cells, positive for CD3 (expressed on all T cells), CD8 (expressed on cytotoxic T cells), granzyme B (cytotoxic protein expressed in CTL) or CD45RO (marker for memory T cells), were investigated for their correlation to prognosis [16, 17]. The markers CD3 and CD8 emerged as most informative and these are currently under investigation for implementation of the Immunoscore [18].

The first line of treatment for CRC patients is surgery, either or not followed by adjuvant chemotherapy for patients with advanced stage disease [19, 20]. Immunotherapeutic treatment options in the adjuvant setting have been explored as well [21]. Vermorken and coworkers conducted a multicenter clinical trial on adjuvant Active Specific Immunotherapy (ASI) for colon cancer patients [22]. A vaccine consisting of irradiated autologous tumor cells admixed with the adjuvant Bacillus Calmette-Guérin bacteria has been evaluated. In that study in colorectal cancer patients, a comparison was made between surgery alone and surgery followed by adjuvant ASI treatment. The recurrence free interval for stage II patients was significantly extended for patients treated with surgery plus ASI compared to surgery alone [22]. In a retrospective follow up study, tumor samples from these patients were analyzed for their microsatellite status; 17% of the samples were MSI and 83% were MSS tumors [23]. The patients with MSI tumors had an increased survival
rate independent of ASI therapy. Whereas MSS patients with Dukes B (stage II) tumors showed a significantly increased recurrence-free survival when they received ASI [23].

The Immunoscore does not make an a priory distinction between patients with microsatellite-stable or -instable tumors [18], even though these tumors display a very different course of disease progression and patient prognosis. In the current study we used archival tumor material derived from patients who participated in the large randomized trial testing the effects of Active Specific Immunotherapy previously [22]. Disease specific survival (DSS) and recurrence free interval (RFI) were evaluated at the 15 year post-treatment time point. First, we investigated the prognostic value of stromal and intraepithelial T cell infiltrates in patients with MSI tumors or MSS tumors. Second, we investigated the predictive value of stromal and intraepithelial T cell infiltrates on the clinical outcome after surgery alone or surgery followed by adjuvant ASI treatment of patients with MSS tumors.
Materials and methods

Patients

The patient population, inclusion criteria, and vaccination protocol have been described in detail previously [22]. In summary, eligible patients with Dukes A, B, or C (corresponding to the current stage I, II or III) resectable adenocarcinoma of the colon and a good performance status were randomly assigned postoperative ASI or no adjuvant treatment. From 254 patients included in the original ASI trial [22], and the follow up study on the effects of MSI/MSS [23], 110 tumor samples were available, and of sufficient quality, to be used in the current study. An overview of the patient characteristics is shown in Table 1.

Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) tumor samples of 3μm were deparaffinized in xylene and dehydrated in ethanol. Endogenous peroxidase was blocked in 0,3% H2O2/methanol. Antigen retrieval was performed by boiling the slides in either TRIS/EDTA (pH 9) or citrate (pH 6) in a microwave for 15 minutes. Antibodies were applied against CD3 (clone C8/144B, 1 : 200 dilution; Dako Cytomation, Glostrup, Denmark) and CD8 (clone C8/144B, 1 : 100 dilution; Dako Cytomation), and left for incubation for an hour at room temperature. All stainings were performed using the Powervision Plus (Dako Cytomation) method. Bound peroxidase was visualized with diaminobenzidine (DAB) and nuclei were counterstained with haematoxylin.

Table 1 Clinicopathological characteristics of colorectal patients

Tumor samples of a 110 patients out of 254 patients from a multicenter trial testing ASI therapy on CRC patients were used to quantify immune infiltrates (CD3+ and CD8+ cells). CD3 quantification was performed on 102 patients and CD8 quantification was performed on 108 patients.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>Total number</td>
<td>110</td>
</tr>
<tr>
<td>Age</td>
<td>Average</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>64</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>53</td>
</tr>
<tr>
<td>Stage</td>
<td>I</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>34</td>
</tr>
<tr>
<td>Treatment</td>
<td>Control</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>ASI</td>
<td>67</td>
</tr>
<tr>
<td>MS-status</td>
<td>MSS</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>MSI</td>
<td>27</td>
</tr>
<tr>
<td>Tumor Location</td>
<td>Right colon</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Transverse colon</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Left colon</td>
<td>61</td>
</tr>
<tr>
<td>IHC Analysis</td>
<td>CD3</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>108</td>
</tr>
</tbody>
</table>
**Quantification**

The stained tumor sections were digitized by the Mirax slide Scanner system (3DHISTECH, Budapest, Hungary) equipped with a 20x objective with a numerical aperture of 0.75 and a Sony DFW-X710 Fire Wire 1/3” type progressive SCAN IT CCD (pixel size 4.65 x 4.65 μm). The actual scan resolution (effective pixel size in the same sample plane) at 20x is 0.23 μm. Panoramic Viewer version 1.14.50 (3DHISTECH) software was used to view the virtual slides. Per slide 10 fields (20 times magnification, 1082x786 pixels, 670x486 μm) were randomly selected and explored in the TIFF image-format to be quantified after excluding areas of insufficient quality (i.e. tissue folds or scanning errors) and areas not containing tumor fields. Various areas in the selected fields were demarcated to distinguish tumor fields from tumor stroma and necrosis or artifacts. Lumen was automatically recognized as white regions and excluded from further analysis.

The images were analyzed using an in-house developed macro for ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) to quantify the number of positively staining cells, as previously described [24]. The densities were calculated by dividing the cell count by the corresponding surface area for every of the 10 fields per slide and then taking the average value. Every slide received three scores: total cell density (in tumor nests and stroma together), cell density in stroma and cell density in tumor nests, all in cells/mm².

**Table 2 T cell infiltrate cut off value analyses for DSS and RFI**

A cross validated method was used to calculate cut off values for the whole group of patients. Presented are the distribution of selected cut offs in the training sets, and the average, cross-validated HRR in the validation sets together with 95% credibility intervals.

<table>
<thead>
<tr>
<th></th>
<th>Total CD3</th>
<th>2.5th</th>
<th>97.5th percentile</th>
<th>HRR median</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DSS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD3</td>
<td>508</td>
<td>404 ; 603</td>
<td>0.58</td>
<td>0.23 to 1.53</td>
<td></td>
</tr>
<tr>
<td>Tumor nest CD3</td>
<td>239</td>
<td>122 ; 353</td>
<td>0.57</td>
<td>0.15 to 2.03</td>
<td></td>
</tr>
<tr>
<td>Stromal CD3</td>
<td>817</td>
<td>684 ; 898</td>
<td>0.41</td>
<td>0.14 to 0.98</td>
<td></td>
</tr>
<tr>
<td>Total CD8</td>
<td>244</td>
<td>181 ; 298</td>
<td>0.50</td>
<td>0.11 to 1.44</td>
<td></td>
</tr>
<tr>
<td>Tumor nest CD8</td>
<td>61</td>
<td>31 ; 156</td>
<td>0.32</td>
<td>0.12 to 0.86</td>
<td></td>
</tr>
<tr>
<td>Stromal CD8</td>
<td>386</td>
<td>287 ; 475</td>
<td>0.62</td>
<td>0.13 to 1.78</td>
<td></td>
</tr>
<tr>
<td><strong>RFI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD3</td>
<td>508</td>
<td>384 ; 658</td>
<td>0.58</td>
<td>0.28 to 1.39</td>
<td></td>
</tr>
<tr>
<td>Tumor nest CD3</td>
<td>332</td>
<td>216 ; 425</td>
<td>0.55</td>
<td>0.13 to 1.21</td>
<td></td>
</tr>
<tr>
<td>Stromal CD3</td>
<td>867</td>
<td>690 ; 920</td>
<td>0.50</td>
<td>0.21 to 1.24</td>
<td></td>
</tr>
<tr>
<td>Total CD8</td>
<td>244</td>
<td>181 ; 298</td>
<td>0.46</td>
<td>0.07 to 1.27</td>
<td></td>
</tr>
<tr>
<td>Tumor nest CD8</td>
<td>63</td>
<td>34 ; 156</td>
<td>0.29</td>
<td>0.12 to 0.67</td>
<td></td>
</tr>
<tr>
<td>Stromal CD8</td>
<td>425</td>
<td>345 ; 475</td>
<td>0.49</td>
<td>0.13 to 1.16</td>
<td></td>
</tr>
</tbody>
</table>
Statistics

Disease specific survival (DSS) was defined as the time in years after surgery until disease specific death in a follow-up period of 15 years. Recurrence free interval (RFI) was defined as the time in years after surgery until a recurrence of the disease was diagnosed in a follow-up period of 15 years. The relation between CD3 numbers and survival, and CD8 numbers and survival, was visualized using Kaplan Meier curves and tested using Gehan-Breslow-Wilcoxon statistics. Furthermore, the prognostic value of CD3 and CD8 expression was tested in a cross-validation procedure, repeated 500 times. In each cross-validation round, the study population was randomly subdivided in a training set (50%) and validation set (50%). The training set was used to determine the optimal cut off for dichotomizing intensity scores into low and high CD3 and CD8 numbers. This was done using Receiver Operating Characteristic (ROC) curve analysis for survival data, with 15-year DSS and RFI as the outcome of interest [25, 26]. The optimal cut off was defined as the point on the ROC curve giving the smallest distance to the point (1-specificity, sensitivity) = (0,1) (Table 2) [27]. Using these cut off values, CD3 and CD8 scores in the validation set were dichotomized and the crude hazard rate ratios (HRR) describing the relation between DSS/RFI with CD3 and CD8 expression, respectively, were calculated in a Cox regression analysis with DSS/RFI as outcome. We present the distribution of selected cut off s in the training sets, and the average, cross-validated HRR in the validation sets together with a 95% credibility interval, that is, the interval within which 95% of the cross-validated HRRs fall. Using the most frequently selected cut off for CD3 and CD8 expression in the training sets as the optimal cut off, we studied the relation between DSS / RFI and CD3 / CD8 expression in a number of subgroups. This was done by means of Kaplan-Meier analysis and Gehan-Breslow-Wilcoxon testing. The cross-validation procedure as well as statistical tests were executed using R Statistics 14.0 software (RStudio Inc., Boston, USA), IBM SPSS Statistics 20.0 software (SPSS Inc., Illinois, USA) or GraphPad Prism (Version 5 (2007), GraphPad Software, Inc., California, USA). P values <0.05 were considered significant.
**Results**

**Patient characteristics**

From 110 out of the 254 patients originally included in the ASI trial [22], tumor samples were still available and of sufficient quality to perform immuno-staining and quantification. Of these, 102 tumor samples were available for CD3 staining and 108 for CD8 staining. Intraepithelial T cells and T cells in the tumor surrounding, stroma were quantified for both CD3 and CD8. In the subsequent analysis we made three categories; 1) Intraepithelial plus stromal T cells referred to as “total T cell infiltrate”; 2) Intraepithelial T cells referred to as “tumor nest infiltrate”; and 3) T cells found in the stroma as “stromal infiltrate”. Patient characteristics are shown in Table 1. The currently investigated group contained most (27 out of 34) of the patients with MSI tumors and a random selection of half of the patients with MSS tumors (83 out of 162), identified in our previous study on the effects of micro satellite stability and adjuvant ASI treatment [23]. The distribution of patient characteristics between the various subgroups, shown in Table 1, were similar to that in the original study. Except for the percentage of patients in the ASI group, where we had 50% in the original study versus 61% in our current study.

**T cell numbers versus tumor stage or location**

The numbers of total CD3+ and CD8+ cells, tumor nest CD3+ and CD8+ cells and stromal CD3+ and CD8+ cells for each tumor stage are shown in Supplementary Figure 1. In this analysis we did not make a distinction between patients with either MSS or MSI tumors. Although only a few data points were available for stage I patients, we found they had significantly higher tumor nest CD3+ infiltrates than patients with stage II tumors (p < 0.05). For CD8, the total T cell infiltrate and the tumor nest infiltrates were significantly higher in stage I tumors compared to stage II and III tumors (p < 0.05). No significant differences in the numbers of CD3+ and CD8+

---

**Figure 1 Survival analysis comparing patients with low and high immune infiltrates**

The total patient group was divided in high or low infiltrates of CD3+ or CD8+ cells, either the total numbers, the infiltrates in the tumor nests or in the tumor stroma. A) Examples of typically high (upper picture) or low (bottom picture) CD3 infiltrates stained immunohistochemically. Brown spots were counted automatically, making the distinction between tumor nests and the surrounding stroma. B) Disease specific survival curves depicting high (green line) and low (red line) infiltrates of total CD3+ cells (upper graph), CD3+ cells in the tumor nests (middle graph) and CD3+ cells in the tumor stroma (bottom graph). C) Recurrence free survival curves depicting high (green line) and low (red line) infiltrates of total CD3+ cells (upper graph), CD3+ cells in the tumor nests (middle graph) and CD3+ cells in the tumor stroma (bottom graph). D) Examples of typically high (upper picture) or low (bottom picture) CD8 infiltrates stained immunohistochemically. Brown spots were counted automatically, making the distinction between tumor nests and the surrounding stroma. E) Disease specific survival curves depicting high (green line) and low (red line) infiltrates of total CD8+ cells (upper graph), CD8+ cells in the tumor nests (middle graph) and CD8+ cells in the tumor stroma (bottom graph). F) Recurrence free survival curves depicting high (green line) and low (red line) infiltrates of total CD8+ cells (upper graph), CD8+ cells in the tumor nests (middle graph) and CD8+ cells in the tumor stroma (bottom graph).
Predictive value of immune infiltrates in colorectal cancer

A. CD3

High

Low

B. DSS

Total CD3

C. RFI

Total CD3

D. CD8

High

Low

E. CD8

Total CD8

F. CD8

Total CD8

Tumor nest CD3

Tumor nest CD3

Stromal CD3

Stromal CD3

Tumor nest CD8

Tumor nest CD8

Stromal CD8

Stromal CD8

P = 0.07

P = 0.06

P = 0.03

P = 0.06

P = 0.03

P = 0.0007

P = 0.18

P = 0.09

P = 0.03

P = 0.03

P = 0.0002

P = 0.04
cells were observed between the patients with either stage II or stage III tumors.

It has been shown that the location of the tumor can be of influence in the prognosis of CRC patients [28]. We analyzed whether we could correlate the numbers of CD3+ and CD8+ cells with tumor location (data shown in Supplementary Figure 2). In this analysis we did not make a distinction between patients with either MSS or MSI tumors. Tumors from the right hand side of the colon had significantly more CD3+ and CD8+ cells in the tumor nests compared to tumors originating from the left hand side of the colon (p < 0.05), but this was not found for total T cells nor for stromal T cells.

**T cell infiltration versus microsatellite status of the tumor**

Next we compared the absolute numbers of TIL found in MSI tumors to those in MSS tumors (Supplementary Figure 3). No significant differences were found between MSI and MSS tumors for stromal CD3 nor for stromal CD8 infiltrates (Supplemental Figure 3A and B; right hand panels). In contrast to this the numbers of CD3 and CD8 cells found in the tumor nests were significantly higher in MSI tumors compared to MSS tumors (Supplemental Figure 3A and B; middle panels (p-values of <0.001)). Inherently, since no significant differences were found in the stroma values, the numbers of total CD3 and total CD8 followed the numbers found in the tumor nests.

**Determining cut off values of low versus high T cell infiltrates**

In Figure 1 examples of immunohistochemistry staining representative of high and low immune infiltrates are depicted (Figure 1A: CD3 staining; Figure 1D: CD8 staining). We next used the data points of all patients available, irrespective of both microsatellite status and treatment, to determine the cross-validated cut off values for low and high numbers of infiltrating T cells. Numbers of CD3+ and CD8+ cells were quantified in the tumor nest and the tumor surrounding stroma. These numbers were also used to determine total immune infiltrates. In Table 2 the median cut off values per marker are shown for the 15 years disease specific survival (DSS) and recurrence free interval (RFI) from the trainings set with 2.5th to 97.5th percentile. The right hand side of Table 2 shows the corresponding validation set data; the hazard rate ratios (HRR) with 95% credible interval (CI) for the different subgroups.

**Prognostic value of intra-epithelial and stromal T cell infiltrates in patients with CRC**

The relation between the CD3 markers indicated as “total CD3”, “tumor nest CD3” and “stromal CD3” and the 15 year DSS and RFI are shown in Figure 1B and 1C. Similarly, the data for the CD8 marker are shown in Figure 1E and 1F. The cut off values were taken from Table 2; low is defined as below, and high as above, these cut off values. The data were subsequently analyzed irrespective of microsatellite status and treatment. Overall, patients with high infiltrates showed a trend for an improved DSS and RFI compared to patients with low infiltrates (Figure 1B, C, E and F). Significant differences were found in DSS, where patients with high stromal CD3 or high tumor nest CD8 infiltrates fared better compared to patients with low infiltrates (p-values of 0.03 and 0.0007 respectively). Significant differences were also found for RFI, where patients with high stromal CD3, high total CD8, high tumor nest CD8 or high stromal CD8 infiltrates fared better compared to patients with low infiltrates (p-values of 0.03, 0.03, 0.0002 and 0.04 respectively). Stromal
CD3 (Figure 1B and C; p=0.03 for both) had its bearing on DSS as well as RFI as did tumor nest CD8 (Figure 1E and F; p=0.0007 and p=0.0002) and clearly have prognostic value for patients with CRC.

**Prognostic value of intra-epithelial and stromal T cell infiltrates in patients with MSS tumors**

Bearing the differences in the numbers of infiltrating CD3+ and CD8+ cells between MSI and MSS tumors in mind, we next analyzed the effects of high and low infiltrates for patients with MSS tumors only. A trend of improved DSS and RFI for patients with high infiltrates compared to those with low infiltrates was observed in this dataset. Statistical significance was found in DSS for stromal CD3 and tumor nest CD8 and for RFI for tumor nest CD8 (p-values of 0.015, 0.002 and 0.0007 respectively). High level infiltrates of stromal CD3 and tumor nest CD8 cells seem to have the highest prognostic value for the whole group of patients (MSS plus MSI, shown in Figure 1) as well as for the sub-group of patient with MSS tumors (shown in Figure 2).
CHAPTER 10

**Predictive value of T cell infiltration on response to adjuvant ASI treatment of patients with MSS tumors**

Previously we have shown that patients with MSI tumors did not benefit from adjuvant ASI treatment. The beneficial effects of adjuvant ASI was restricted to patients with MSS tumors [23]. We were curious to investigate whether the beneficial effects of ASI seen in the group of patients with MSS tumors correlated with the extent and location of the T cell infiltrates of the tumor and/or the stroma. For statistical reasons, detailed in the discussion, we have analyzed the data on all available patients with stage II or stage III CRC grouped together. In Figure 3 and Figure 4 we show Kaplan-Meier plots for the 15 year disease specific survival and recurrence free interval for all patients with MSS tumors. The parameters included the number and location of CD3+ and CD8+ cells combined with treatment arm. The control group has received surgery alone, whereas the ASI group has received surgery and adjuvant ASI treatment. Survival curves for patients in the control (C) or ASI group (A) combined with either high (H) or low (L) total CD3, tumor nest CD3 or stromal CD3 counts are shown for DSS in Figure 3A and for RFI in Figure 4A. Similar to this, the survival curves for the numbers of CD8+ cells are shown for DSS in Figure 3B and for RFI in Figure 4B. To determine whether the level of immune infiltrate can be used as a predictor for response to therapy we compared DSS and RFI in the ASI group to those in the control group, stratified according to level of the immune infiltration. The hazard rate ratios are given for the ASI patients compared to the control group.

**Figure 2 Survival analysis comparing MSS patients with low and high immune infiltrates.**
The MSS patient group was divided in high or low infiltrates of CD3+ or CD8+ cells, either the total numbers, the infiltrates in the tumor nests or in the tumor stroma. A) Disease specific survival curves depicting high (green line) and low (red line) infiltrates of total CD3+ cells (upper graph), CD3+ cells in the tumor nests (middle graph) and CD3+ cells in the tumor stroma (bottom graph). B) Recurrence free survival curves depicting high (green line) and low (red line) infiltrates of total CD3+ cells (upper graph), CD3+ cells in the tumor nests (middle graph) and CD3+ cells in the tumor stroma (bottom graph). C) Disease specific survival curves depicting high (green line) and low (red line) infiltrates of total CD8+ cells (upper graph), CD8+ cells in the tumor nests (middle graph) and CD8+ cells in the tumor stroma (bottom graph). D) Recurrence free survival curves depicting high (green line) and low (red line) infiltrates of total CD8+ cells (upper graph), CD8+ cells in the tumor nests (middle graph) and CD8+ cells in the tumor stroma (bottom graph).
Predictive value of immune infiltrates in colorectal cancer

A) DSS

B) RFI

Total CD3

Tumor nest CD3

Stromal CD3

Total CD8

Tumor nest CD8

Stromal CD8

Percent survival

Years

P = 0.15 High Low

# censored subjects: 33 26

# deaths/events: 8 10

# total: 41 36

P = 0.17 High Low

# censored subjects: 33 26

# deaths/events: 7 11

# total: 40 37

P = 0.015 * High Low

# censored subjects: 40 19

# deaths/events: 7 11

# total: 47 30

P = 0.09 High Low

# censored subjects: 31 30

# deaths/events: 6 14

# total: 37 44

P = 0.002 ** High Low

# censored subjects: 44 17

# deaths/events: 7 13

# total: 51 30

P = 0.32 High Low

# censored subjects: 33 28

# deaths/events: 8 12

# total: 41 40

P = 0.23 High Low

# censored subjects: 31 24

# deaths/events: 10 12

# total: 41 36

P = 0.28 High Low

# censored subjects: 19 36

# deaths/events: 5 17

# total: 24 53

P = 0.07 High Low

# censored subjects: 34 23

# deaths/events: 9 13

# total: 43 36

P = 0.07 High Low

# censored subjects: 30 27

# deaths/events: 7 17

# total: 37 44

P = 0.0007 *** High Low

# censored subjects: 42 15

# deaths/events: 8 16

# total: 50 31

P = 0.09 High Low

# censored subjects: 29 28

# deaths/events: 7 17

# total: 36 45
A trend for an improved DSS and RFI is noted for patients with high CD3 or high CD8 infiltrates if combined with adjuvant ASI treatment (Figures 3 and 4 panels A, B). Significance was reached for both DSS and RFI in ASI treated patients versus controls with high total CD3, high stromal CD3 or high stromal CD8 infiltration (Figures 3 and 4, panels C; p=0.01, 0.004 and 0.02 respectively). Additionally, this also holds true for RFI in ASI treated patients versus controls with high total CD8 infiltration (Figure 4, panel C: p=0.001, 0.02 and 0.001 respectively)). The HRR with 95% CI have been visualized in Figure 3 and 4 D.

Overall, patients with MSS tumors with low T cell infiltrates did less well compared to patients with high T cell infiltrates (Figure 2). In all cases, except for the tumor nest CD8 counts, the control group with high infiltrates clustered together with the low controls and the low ASI group (Figure 3 and 4 panels A, B). In patients with low infiltrates, adjuvant ASI did not result in improved DSS or RFI (Figure 3 and 4, panels C and D).

Based on the data presented here, stromal CD3 counts (for DSS) and tumor nest CD8 numbers (for DSS and RFI) appear to have high prognostic value for CRC patients with MSS tumors (Figure 2). Furthermore, based on the data presented in Figures 3 and 4, stromal CD3 and stromal CD8 numbers appear to have high predictive value with respect to response to adjuvant ASI therapy in CRC patients with MSS tumors.

Figure 3 Disease specific survival of MSS patients with high and low infiltrates and response to therapy
MSS patients were grouped according to number of infiltrating lymphocytes and type of therapy they received. A) Disease specific survival of MSS patients with high (H) and low (L) numbers of CD3+ cells with (A) or without (C) ASI treatment. The green line represents the survival of patients with high numbers of CD3+ cells who received ASI therapy (H / A). The blue line represents the survival of patients with high numbers of CD3+ cells who did not receive ASI therapy (H / C). The red line represents the survival of patients with low numbers of CD3+ cells who received ASI therapy (L / A). The orange line represents the survival of patients with low numbers of CD3+ cells who did not receive ASI therapy (L / C). B) Disease specific survival of MSS patients with high (H) and low (L) numbers of CD8+ cells with (A) or without (C) ASI treatment. The green line represents the survival of patients with high numbers of CD8+ cells who received ASI therapy (H / A). The blue line represents the survival of patients with high numbers of CD8+ cells who did not receive ASI therapy (H / C). The red line represents the survival of patients with low numbers of CD8+ cells who received ASI therapy (L / A). The orange line represents the survival of patients with low numbers of CD8+ cells who did not receive ASI therapy (L / C). C) Statistical analyses performed on the data shown in the graphs from 3A and 3B. Type of treatment (control or ASI) are compared within the high and low infiltrate subgroups. The number of patients and events are given per group. The hazard ratios with 95% confidence interval, the corresponding P value and significance are given. D) A forest plot depicting the HR ratio’s with error bars illustrating the 95% confidence interval of the data shown in Figure 3C. Closed symbols are given to the HR ratios of high infiltrates and open symbols are given to the HR ratios of high infiltrates.
Predictive value of immune infiltrates in colorectal cancer

**A**

- **Total CD3**
  - H/A
  - L/C
  - L/A
  - H/C

- **Tumor nest CD3**
  - H/A
  - L/C
  - L/A
  - H/C

- **Stromal CD3**
  - H/A
  - L/C
  - L/A
  - H/C

**B**

- **Total CD8**
  - H/A
  - L/C
  - L/A
  - H/C

- **Tumor nest CD8**
  - H/A
  - L/C
  - L/A
  - H/C

- **Stromal CD8**
  - H/A
  - L/C
  - L/A
  - H/C

**C**

<table>
<thead>
<tr>
<th>DSS</th>
<th>Control</th>
<th>ASI</th>
<th>HR (95% CI)</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD3</td>
<td>Low</td>
<td>3 (12)</td>
<td>7 (24)</td>
<td>1.15 (0.31 to 4.30)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>6 (16)</td>
<td>2 (25)</td>
<td>0.18 (0.04 to 0.77)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Tumor nest CD3</td>
<td>Low</td>
<td>5 (14)</td>
<td>6 (23)</td>
<td>0.70 (0.21 to 2.39)</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>4 (14)</td>
<td>3 (26)</td>
<td>0.30 (0.06 to 1.50)</td>
<td>0.10</td>
</tr>
<tr>
<td>Stromal CD3</td>
<td>Low</td>
<td>3 (10)</td>
<td>8 (20)</td>
<td>1.38 (0.40 to 4.80)</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>6 (18)</td>
<td>1 (29)</td>
<td>0.11 (0.02 to 0.51)</td>
<td>0.004**</td>
</tr>
<tr>
<td>Total CD8</td>
<td>Low</td>
<td>5 (13)</td>
<td>9 (31)</td>
<td>0.76 (0.24 to 2.38)</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>4 (14)</td>
<td>2 (23)</td>
<td>0.23 (0.04 to 1.26)</td>
<td>0.06</td>
</tr>
<tr>
<td>Tumor nest CD8</td>
<td>Low</td>
<td>5 (8)</td>
<td>8 (22)</td>
<td>0.44 (0.12 to 1.60)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>4 (19)</td>
<td>3 (32)</td>
<td>0.37 (0.08 to 1.77)</td>
<td>0.14</td>
</tr>
<tr>
<td>Stromal CD8</td>
<td>Low</td>
<td>4 (13)</td>
<td>8 (27)</td>
<td>1.06 (0.32 to 3.48)</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>5 (14)</td>
<td>3 (27)</td>
<td>0.20 (0.04 to 0.92)</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

**D**

- **High infiltrates**
  - Total CD3
  - Tumor nest CD3
  - Stromal CD3
  - Total CD8
  - Tumor nest CD8
  - Stromal CD8

- **Low infiltrates**
  - Total CD3
  - Tumor nest CD3
  - Stromal CD3
  - Total CD8
  - Tumor nest CD8
  - Stromal CD8

HR ratios with 95% CI
MSS patients were grouped according to number of infiltrating lymphocytes and type of therapy they received. A) Recurrence free interval of MSS patients with high (H) and low (L) numbers of CD3+ cells with (A) or without (C) ASI treatment. The green line represents the recurrence free survival of patients with high numbers of CD3+ cells who received ASI therapy (H / A). The blue line represents the recurrence free survival of patients with high numbers of CD3+ cells who did not receive ASI therapy (H / C). The red line represents the recurrence free survival of patients with low numbers of CD3+ cells who received ASI therapy (L / A). The orange line represents the recurrence free survival of patients with low numbers of CD3+ cells who did not receive ASI therapy (L / C). B) recurrence free interval of MSS patients with high (H) and low (L) numbers of CD8+ cells with (A) or without (C) ASI treatment. The green line represents recurrence free the survival of patients with high numbers of CD8+ cells who received ASI therapy (H / A). The blue line represents the recurrence free survival of patients with high numbers of CD8+ cells who did not receive ASI therapy (H / C). The red line represents the recurrence free survival of patients with low numbers of CD8+ cells who received ASI therapy (L / A). The orange line represents the recurrence free survival of patients with low numbers of CD8+ cells who did not receive ASI therapy (L / C). C) Statistical analyses performed on the data shown in the graphs from 4A and 4B. Type of treatment (control or ASI) are compared within the high and low infiltrate subgroups. The number of patients and events are given per group. The hazard ratios with 95% confidence interval, the corresponding P value and significance are given. D) A forest plot depicting the HR ratio’s with error bars illustrating the 95% confidence interval of the data shown in Figure 4C. Closed symbols are given to the HR ratios of high infiltrates and open symbols are given to the HR ratios of high infiltrates.
Predictive value of immune infiltrates in colorectal cancer

A

Total CD3

Tumor nest CD3

Stromal CD3

B

Total CD8

Tumor nest CD8

Stromal CD8

C

<table>
<thead>
<tr>
<th>RFI</th>
<th>Control</th>
<th>ASI</th>
<th></th>
<th>HR(95% CI)</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. events(total)</td>
<td>No. events(total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD3</td>
<td>Low</td>
<td>3(12)</td>
<td>9(24)</td>
<td>1.52 (0.46 to 5.01)</td>
<td>0.60</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>8(16)</td>
<td>2(25)</td>
<td>0.12 (0.03 to 0.44)</td>
<td><strong>0.001</strong></td>
<td>ns</td>
</tr>
<tr>
<td>Tumor nest CD3</td>
<td>Low</td>
<td>8(20)</td>
<td>9(33)</td>
<td>0.64 (0.24 to 1.71)</td>
<td>0.36</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3(8)</td>
<td>2(16)</td>
<td>0.21 (0.03 to 1.46)</td>
<td>0.08</td>
<td>ns</td>
</tr>
<tr>
<td>Stromal CD3</td>
<td>Low</td>
<td>4(11)</td>
<td>9(25)</td>
<td>0.92 (0.28 to 3.05)</td>
<td>0.79</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>7(19)</td>
<td>2(24)</td>
<td>0.21 (0.05 to 0.79)</td>
<td><strong>0.02</strong></td>
<td>ns</td>
</tr>
<tr>
<td>Total CD8</td>
<td>Low</td>
<td>6(13)</td>
<td>11(31)</td>
<td>0.70 (0.24 to 2.03)</td>
<td>0.50</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>5(14)</td>
<td>2(23)</td>
<td>0.17 (0.03 to 0.82)</td>
<td><strong>0.02</strong></td>
<td>ns</td>
</tr>
<tr>
<td>Tumor nest CD8</td>
<td>Low</td>
<td>6(9)</td>
<td>10(22)</td>
<td>0.51 (0.16 to 1.56)</td>
<td>0.24</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>5(18)</td>
<td>3(32)</td>
<td>0.26 (0.06 to 1.14)</td>
<td>0.06</td>
<td>ns</td>
</tr>
<tr>
<td>Stromal CD8</td>
<td>Low</td>
<td>6(14)</td>
<td>11(31)</td>
<td>0.80 (0.28 to 2.24)</td>
<td>0.66</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>5(13)</td>
<td>2(23)</td>
<td>0.14 (0.03 to 0.72)</td>
<td><strong>0.01</strong></td>
<td>ns</td>
</tr>
</tbody>
</table>

D

RFI MSS (ASI versus Control)

High infiltrates

Total CD3

Tumor nest CD3

Stromal CD3

Total CD8

Tumor nest CD8

Stromal CD8

Low infiltrates

Total CD3

Tumor nest CD3

Stromal CD3

Total CD8

Tumor nest CD8

Stromal CD8

HR ratios with 95% CI
Discussion

The presence of tumor infiltrating lymphocytes in colorectal cancer has been documented in a number of studies [29-37]. In recent years Galon and co-workers have introduced the concept of the so-called Immunoscore, which is aimed at providing additional prognostic value to the universally implemented TNM classification for patients with CRC, based on the presence of infiltrating T cells [14, 16, 18, 38]. With a few exceptions [39, 40], most of the studies on infiltrating T cells have not taken the microsatellite status of CRC into account as one of the important parameters influencing clinical outcome. The microsatellite status of CRC tumors has a strong prognostic value [3, 9, 41]. Adjuvant ASI treatment has beneficial effects in patients with stage II CRC [22]. Recently we have documented that these beneficial effects of adjuvant ASI treatment were restricted to patients with MSS tumors, since patients with MSI tumors did well irrespective of treatment [23]. Here we report on the presence of infiltrating T cells in the tumor nests and the stroma of tumors obtained from patients with MSI (n=27) or MSS (n=83) tumors, and correlate the presence of these T cells with prognostic value for the patients and predictive value with respect to adjuvant ASI treatment. Larger numbers could not be included because of a lack of sufficient tumor material since parts had already been used previously [23], or because of poor quality and staining since the material was obtained in the 1990s [22].

In order to calculate the optimal cut off point to distinguish between high and low infiltrates we made use of a training set and a validation set. Cut off values were calculated by using a ROC based method including ‘time’ as an additional dimension to specificity and sensitivity. We were able to extend the follow up from the standard 5 year evaluation point, to 15 years post treatment. We calculated cut off values for DSS and RFI separately and found that for most parameters indicated in Table 2, the cut off values were near identical. For tumor nest CD3 the cut off values differed more than 10% between DSS and RFI (Table 2). However, these differences had no bearing on DSS and RFI (Figure 1B and 1C, p=0.06 and 0.09 respectively).

After applying these cut off values to investigate tumor infiltrates for all patients irrespective of MS-status, we found significant differences in DSS for stromal CD3 and tumor nest CD8. Similar findings were reported by Deschoolmeester and co-workers. In that data set a significant difference was also found for tumor nest CD3 in overall survival [39]. We additionally found significant differences in RFI for total CD8 and stromal CD8 infiltrates (Figure 1). Differences in the number of patient samples and in quantitation methods may explain these subtle differences, but in general high T cell infiltrates are indicative for a more favorable prognosis.

The data, presented in Supplemental Figure 3, on T cell infiltrates in MSI and MSS tumors, showed significant differences in total CD3+ and total CD8+ cells. In the patient group with MSI tumors the T cell numbers are significantly higher than in MSS tumors. This is in agreement with data reported by other investigators using immunohistochemistry and mRNA expression levels [42]. Furthermore, we found significant differences between MSI and MSS tumors and tumor nest infiltrating CD3+ and tumor nest CD8+ cells, but not in stromal CD3+ and stromal CD8+ cells. This is in good agreement with what has been reported by others employing immunohistochemistry [4, 43].

Bearing the differences in the number of infiltrating CD3+ and CD8+ cells between MSI
and MSS tumors in mind, we next analyzed the effects of high and low infiltrates for patients with MSS tumors only. We found clear differences between patients with MSS tumors with either low or high T cell infiltrates. A favorable trend was seen for all parameters in the group of patients with high infiltrates, and significance was reached in DSS for stromal CD3, tumor nest CD8 and in RFI for tumor nest CD8 high infiltrates (Figure 2). These parameters are of high prognostic value for the patient group with MSS tumors.

The prognostic value of TIL in CRC has been documented by a number of groups [4, 13, 16, 39, 44, 45], but although an association between TIL and response to chemotherapy has been found in several studies [36, 46], the predictive value of TIL is still under investigation and under debate [47-50]. We therefore asked ourselves the question whether the presence and location of CD3+ or CD8+ TIL in MSS CRC have predictive value with respect to clinical outcome after adjuvant ASI treatment.

A breakdown of the data on T cell infiltration combined with treatment arm is shown in Figure 3 (DSS) and Figure 4 (RFI). Adjuvant ASI treatment of patients with MSS tumors with low T cell infiltrates does not improve on DSS nor on RFI. Surprisingly not all patients with high T cell infiltrates did better than those with low infiltrates; in this case tumor nest CD8 seems to do make a difference. On the other hand adjuvant ASI treatment of the patient group with high CD3 or CD8 infiltration of the stromal compartment, performed best. Where tumor nest located CD8 infiltration had prognostic value, it seems that high level stromal CD3 and stromal CD8 infiltration is a good predictor for response to therapy.

Previously Vermorken et al. have shown that the beneficial effects of ASI treatment was restricted to patients with Dukes B (stage II) CRC [22]. Additionally we have shown that the patients with MSI CRC did well irrespective of treatment, and that the beneficial effects of adjuvant ASI was restricted to those patients with stage II (Dukes B) MSS cancers [23]. In the current study we speculated that patients with MSS tumors with high T cell infiltration would benefit most from adjuvant ASI treatment. It should be noted that tumor material was available from 83 patients with MSS CRC, which were separated according to the extent of T cell infiltration (high versus low) and treatment arm (control versus adjuvant ASI). A further separation on the basis of tumor stage (stage II versus stage III) would result in a lack of power in the subsequent statistical analysis. Therefore we have grouped the tumor material of patients with stage II and stage III CRC together in our analysis.

Based on the current data on T cell infiltration and clinical outcome, we can state the following with respect to the prognostic and predictive value of TIL; 1) patients with MSS tumors with low T cell infiltrates compared to those with high T cell infiltrates do less well in both DSS and RFI; 2) patients with MSS tumors with low T cell infiltrates do not benefit from adjuvant ASI; and 3) patients with MSS tumors with high T cell infiltrates benefit significantly from adjuvant ASI treatment. Stromal CD3 numbers (for DSS) and intraepithelial CD8 numbers (for DSS and RFI) appear to have prognostic value for CRC patients with MSS tumors. Whereas stromal CD3 and stromal CD8 numbers appear to have predictive value with respect to response to adjuvant ASI therapy in CRC patients with MSS tumors. Based on the data presented here and published previously by us [23], we suggest that the value of the Immunoscore could be further improved by distinguishing patients with microsatellite instable tumors from those with microsatellite stable tumors.
Acknowledgements

The authors thank the following pathologists from the participating hospitals in retrieving the FFPE material: Drs. M. Flens, Zaans Medisch Centrum, Zaandam; M. Brinkhuis, Stichting Laboratorium Pathologie Oost, Enschede; H. Doornewaard, Gelre Ziekenhuis, Apeldoorn; A. Uyterlinde, Medisch Centrum Alkmaar, Alkmaar; and E. Barbe, St Lucas, Amsterdam. This work was supported by grant VU2007-3814 from the Dutch Cancer Society.
Predictive value of immune infiltrates in colorectal cancer

References


Supplementary Figures

Infiltrating T cells and tumor stage

Supplementary Figure 1) Infiltrating T cells and tumor stage.
The numbers of infiltrating CD3+ and CD8+ cells are shown per tumor stage, ranging from stage I to III. The top row of graphs shows the CD3+ cells; total numbers of CD3+ cells in the graph on the left hand site, CD3+ cells in the tumor nests in the middle graph and stromal CD3+ cells in the graph on the right hand site. The bottom row of graphs shows the CD8+ cells; total numbers of CD8+ cells in the graph on the left hand site, CD8+ cells in the tumor nests in the middle graph and stromal CD8+ cells in the graph on the right hand site. Statistical significant differences in T cell infiltrates between stage I, II and II tumors are shown in the graph with an asterisk (p < 0.05).
Infiltrating T cells and tumor location

Supplementary Figure 2) Infiltrating T cells and tumor location.
The numbers of infiltrating CD3 and CD8 are shown per tumor location, right, transverse or left colon. The top row of graphs shows the CD3+ cells; total numbers of CD3+ cells in the graph on the left hand site, CD3+ cells in the tumor nests in the middle graph and stromal CD3+ cells in the graph on the right hand site. The bottom row of graphs shows the CD8+ cells; total numbers of CD8+ cells in the graph on the left hand site, CD8+ cells in the tumor nests in the middle graph and stromal CD8+ cells in the graph on the right hand site. Statistical significant differences in T cell infiltrates between tumors located on the right, transverse or left colon are shown in the graph with an asterisk (p < 0.05).
**Supplementary Figure 3**) Differences between MSS and MSI patients.
Numbers of CD3 and CD8 infiltrates in MSI tumors compared to MSS tumors. **A)** MSI tumors have a higher number of total CD3+ cells (left hand graph, \( p < 0.001 \)) and a higher number of CD3+ cells in the tumor nests (middle graph, \( p < 0.001 \)). No differences were found between MSI and MSS tumors when we compare the number of CD3+ cells in the tumor stroma. **B)** MSI tumors have a higher number of total CD8+ cells (left hand graph, \( p < 0.01 \)) and a higher number of CD8+ cells in the tumor nests (middle graph, \( p < 0.001 \)). No differences were found between MSI and MSS tumors when we compare the number of CD8+ cells in the tumor stroma.