Infiltration of Lynch Colorectal Cancers by Activated Immune Cells Associates with Early Staging of the Primary Tumor and Absence of Lymph Node Metastases

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Abstract

Purpose: Lynch syndrome colorectal cancers often lose human leukocyte antigen (HLA) class I expression. The outgrowth of clones with immune evasive phenotypes is thought to be positively selected by the action of cytotoxic T cells that target HLA class I–positive cancer cells. To investigate this hypothesis, we related the type and density of tumor lymphocytic infiltrate in Lynch colorectal cancers with their HLA class I phenotype and clinicopathologic stage.

Experimental Design: HLA class I expression was assessed by means of immunohistochemistry. Characterization of tumor-infiltrating lymphocytes was carried out by using a triple immunofluorescence procedure that allowed the simultaneous detection of CD3, CD8, and granzyme B (GZMB)–positive cells. Additional markers were also used for further characterization of an elusive CD3+/CD8-/GZMB+ cell population.

Results: We discovered that high tumor infiltration by activated CD8+ T cells correlated with aberrant HLA class I expression and associated with early tumor stages (P < 0.05). CD8+ T cells were most abundant in HLA class I heterogeneous tumors (P = 0.02) and frequent in HLA class I–negative cases (P = 0.04) when compared with HLA class I–positive carcinomas. An elusive immune cell population (CD45+/CD8-/CD56+/GZMB+) was characteristic for HLA class I–negative tumors lacking lymph node metastases (P < 0.01).

Conclusions: The immune system assumes an important role in counteracting the progression of Lynch colorectal cancers and in selecting abnormal HLA class I phenotypes. Our findings support the development of clinical strategies that explore the natural antitumor immune responses occurring in Lynch syndrome carriers.
**Translational Relevance**

Lynch syndrome–associated colorectal cancers are known to evoke a strong reaction from the immune system of patients. We discovered that early staged tumors were more densely infiltrated by activated CD8⁺ T cells than tumors diagnosed at advanced stages. Moreover, we observed an immune cell population that was specifically associated with nonmetastasized Lynch syndrome colorectal cancers. Those cells lacked most common lymphocytic surface antigens. Altogether, our findings support that the immune system plays a major role in countering colorectal cancer progression in patients with Lynch syndrome. We propose the development of novel clinical strategies, inspired by the natural antitumor immune response occurring in patients with Lynch syndrome. Considering that Lynch colorectal cancers rapidly acquire immune evasive phenotypes, special relevance should be given to prophylactic approaches.

Accordingly, those reports might carry a possible bias: Because both pronounced lymphocytic infiltration and HLA class I loss are hallmarks of mismatch repair–deficient tumors it is difficult to establish a causal relation between the presence of lymphocytes and HLA class I abnormalities on consecutive series of colorectal cancers (8, 25, 26). Therefore, the current study was conducted on a homogeneous cohort of genetically proven Lynch colorectal carcinomas. We sought to characterize and quantify the lymphocytic infiltration present in Lynch colorectal carcinomas and relate it with their HLA class I expression status and clinicopathologic stage.

**Materials and Methods**

**Patient material**

A cohort of 90 colorectal carcinomas, derived from 86 patients with Lynch syndrome, was compiled. Corresponding formalin-fixed, paraffin-embedded tissues were collected throughout The Netherlands. All patients were carriers of genetically proven, pathogenic, germline mutations in *MLH1* (*n* = 31), *MSH2* (*n* = 25), *MSH6* (*n* = 24), or *PMS2* (*n* = 6) as determined by the Leiden Diagnostic Genome Centre of the Leiden University Medical Centre (LUMC), Leiden, The Netherlands. In addition, all except one patient, for whom extended clinical information was not available, fulfilled the revised Bethesda criteria for Lynch syndrome (27). Pathologic tumor– (lymph) node–metastasis (TNM) staging was retrieved from 79 tumors of which 5 tumors were staged as T1, whereas 19, 43, and 12 cases were staged as T2, T3, and T4, respectively. Twenty-four cases presented with lymph node metastases. Metastases in distant organs were only observed in 2 cases. The study was approved by the Medical Ethical Committee of the LUMC (protocol P01-019). Patient samples were handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

**Immunohistochemistry**

Expression of HLA class I and β2M were assessed by means of a 2-step indirect immunohistochemistry procedure on 4-μm tissue sections. Following deparaffinization and rehydration, the tissue sections underwent heat-mediated antigen retrieval in a 10 mmol/L citrate buffer solution (pH 6). After cooling, endogenous peroxidase activity blockage was carried out with a 0.3% hydrogen peroxide/methanol solution. Thereafter, the sections were incubated overnight with one of the following primary antibodies: the monoclonal antibody (mAb) HCA2, which recognizes β2M-free HLA-A (except -A24), -B7301, and -G heavy chains (28, 29); the mAb HC10, which recognizes a determinant expressed on all β2M-free HLA-B and -C heavy chains and on β2M-free HLA-A10, -A28, -A29, -A30, -A31, -A32, and -A33 heavy chains (refs. 29, 30; supernatants kindly provided by Prof. J. Neefjes, Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands, and Prof. H.L. Ploegh, MIT, Boston, MA); and the rabbit anti-β2M heavy chain.
polyclonal antibody A0072 (Dako). The following day, primary antibody binding was detected with the Bright-Vision Poly-HRP (horseradish peroxidase) Detection System (Immunologic). Scoring of HLA class I and β2M expression in tumor cells was always carried out against an internal positive control, provided by the staining of stromal cells. Negative controls were generated by replacing the primary antibodies by a 1% bovine serum albumin (BSA)/PBS solution during the procedure.

A double-staining immunohistochemistry procedure was carried out to study the morphology of CD3+/CD8−/GZMB+ cell population prompted us to screen additional tissue sections with primary antibodies directed against various hematopoietic markers. Anti-CD2, -CD16, -CD45, -CD56, -CD57, -CD68, -CD117, -NKp46, and anti-TCR-γ antibodies were used in combination with anti-CD8 and anti-GZMB antibodies (antigens and dilutions provided in Supplementary Table S1). Immunofluorescence was detected with a LSM700 confocal laser microscope (Carl Zeiss), equipped with a ZEISS LCI Plan-NEOFLUAR ×25/0, 8 DIC Imm Korr objective. Approximately 4 mm² of tumor tissue was scanned. Density of lymphocytic infiltration was determined by dividing the number of intraepithelial (or immediately adjacent to the epithelium) lymphocytes by the tumor area. Both lymphocyte counting and measurement of the tumor area were conducted with the ZEN2009 LE software (Carl Zeiss). Negative controls were generated by replacing primary antibodies by a 1% BSA/PBS solution.

**Statistical analyses**

All statistical tests and graph construction were carried out with GraphPad PRISM (version 5.04). The Mann–Whitney U test was used when assessing the differences in the amount of lymphocytic infiltrate between HLA class I phenotypes and lymph node–negative and –positive tumors. ANOVA was conducted for comparing lymphocyte density among the different T tumor stages (TNM classification).

## Results

**Lymphocytic infiltration of Lynch carcinomas associates with the HLA class I phenotype**

Expression of HLA class I and β2M was assessed in 90 Lynch colorectal carcinomas. Altogether, 83% of tumors presented HLA class I defects (Table 1). Membranous HLA class I expression was completely lost in 58 tumors, whereas a heterogeneous pattern of HLA class I staining was observed in 17 carcinomas (Supplementary Fig. S1). The latter presented fields where tumor cells conserved membranous expression of HLA class I together with additional tumor areas where HLA class I expression was completely lost (Supplementary Fig. S1C and S1D). HLA class I

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### Table 1. HLA class I phenotypes and density of lymphocytic infiltration in Lynch colorectal cancers

<table>
<thead>
<tr>
<th>HLA class I status</th>
<th>Tumors, n (%)</th>
<th>% β2M loss</th>
<th>CD3+/CD8− T cells (CD3+/CD8−)</th>
<th>Activated CD8+ T cells (CD3+/CD8+/GZMB+)</th>
<th>CD3+/CD8− / GZMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>15 (17)</td>
<td>—</td>
<td>6.13</td>
<td>20.78</td>
<td>49.16</td>
</tr>
<tr>
<td>Heterogeneous</td>
<td>17 (19)</td>
<td>26</td>
<td>8.73</td>
<td>19.56</td>
<td>72.88</td>
</tr>
<tr>
<td>Negative</td>
<td>58 (64)</td>
<td>59</td>
<td>11.98</td>
<td>23.51</td>
<td>60.58</td>
</tr>
</tbody>
</table>

*Medians differ significantly from the HLA class I-positive group.

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alterations were accompanied by aberrant β2M expression in 51% of cases (Table 1). There was no difference in the distribution of HLA class I phenotypes between Lynch colorectal carcinomas with mutations in different mismatch repair genes (data not shown).

Qualitative and quantitative characterization of intraepithelial lymphocytic infiltration by means of triple immunofluorescence was possible for 83 tumors. In a few cases (n = 7), the staining procedure was not successful due to poor fixation and/or age of the tissue. The combination of CD3, CD8, and GZMB markers allowed the discrimination between CD3+/CD8− cells (presumably CD4+ and γδ T cells), CD3+/CD8+ cells (CD8+ T cells), and CD3−/CD8+/GZMB+ (activated CD8+ T cells; Fig. 1A–D). No significant difference was detected between distinct HLA class I phenotypes for total CD8+ T-cell density (Fig. 2A). However, HLA class I-negative and heterogeneous tumors were more densely infiltrated by activated CD8+ T cells than HLA class I-positive tumors (P = 0.02 and P = 0.04, respectively; Fig. 2B). CD3+/CD8− cells (CD4+ and γδ T cells) were more frequent in HLA class I-negative tumors when compared with HLA class I-positive (P = 0.01; Fig. 2C). Among HLA class I-negative tumors, only the density of CD3+/CD8− cells (CD4+ and γδ T cells) was related to the β2M expression status. β2M-negative cases were more densely infiltrated by these type of cells (P = 0.01; data not shown).

A population of cells with a CD3+/CD8−/GZMB− phenotype was detected in 82% of cases at various frequencies (Fig. 1C and D). These cells were often localized in the stromal compartment, immediately adjacent to the tumor fields, but less frequently within the epithelium. CD3−/CD8−/GZMB− cells were mostly restricted to HLA class I-negative tumors (P = 0.004; Fig. 2D).

The amount of CD8- and GZMB-positive cells relates to tumor stage of Lynch carcinomas

As the interaction between tumor and immune cells might have implications for tumor progression and...
dissemination, we compared the density of lymphocytic infiltration between different pathologic stages (TNM classification).

There was a clear correlation between the T staging of the primary tumor and the presence of CD8⁺ T cells, independently of their activation status (provided by GZMB staining). The total amount of CD8⁺ T cells was gradually smaller with increasing tumor stage (P < 0.05; ANOVA, Fig. 3A). This difference was most striking when the analysis was restricted to HLA class I-negative tumors (P = 0.002; Fig. 3B). The same trend was observed for activated CD8⁺ T cells in all tumors (P = 0.04) and HLA class I-negative cases (P = 0.004; Fig. 3C and D). No other lymphocyte population correlated with T staging.

Remarkably, abundance of the elusive CD3⁺/CD8⁻/GZMB⁺ cell population not only correlated with HLA-negative tumors but was also characteristic for lymph node–negative carcinomas (P < 0.01; Fig. 3E and F). All Lynch carcinomas with more than 10 CD3⁻/CD8⁻/GZMB⁺ cells per mm² of tumor area did not present lymph node metastases. Furthermore, the only 2 cases presenting metastases at distant organs also carried lower numbers of CD3⁻/CD8⁻/GZMB⁺ cells (0 and 7 cells/mm²). None of the CD3⁺ lymphocyte populations related to the lymph node status of tumors.

Characterization of the CD3⁻/CD8⁻/GZMB⁺ cell population

The potential clinical significance of the CD3⁻/CD8⁻/GZMB⁺ cell population in the progression of Lynch carcinomas compelled us to further characterize these cells. Their clear association to HLA class I loss and lymph node-negative tumors supported the investigation of additional markers with a focus on natural killer (NK) cells. CD2, CD16, CD45, CD56, CD57, CD68, CD117, NKp46, and TCR-γ expression was assessed simultaneously with CD8 and GZMB markers (antibody description in Supplementary Table S1).

The only marker that clearly associated with the CD3⁻/CD8⁻/GZMB⁺ cell population was CD45 (Fig. 1E), thus confirming the hematopoietic nucleated lineage of these cells. CD56⁺ cells were rare and located nearby blood vessels (Supplementary Fig. S2A). CD16 cells were abundant but did not colocalize with GZMB positivity (Supplementary Fig. S2B). CD57⁺/CD8⁻ cells were often found in the stromal compartment of tumors, and some displayed positivity for GZMB but CD57 failed to colocalize with the majority of CD8⁻/GZMB⁺ cells (Supplementary Fig. S2C). All other investigated markers did not colocalize with these cells, despite the presence of internal positive controls for the majority (data not shown). No NKp46⁺ cells were found in tumor tissues. We detected NKp46 expression in tonsil tissues to rule out a failure of the staining procedure.

As GZMB expression could be derived from granulocytes, we conducted a bright-field, double staining of CD8 and GZMB together with hematoxylin counterstaining to discern the nuclear morphology of CD8⁻/GZMB⁺ cells. The nuclei from CD8⁻/GZMB⁺ cells were nonlobated and easily discriminated from polymorphonuclear granulocytes (Fig. 1F and Supplementary Fig. S2D).

This CD3⁻/CD8⁻/GZMB⁺ cell population presented consistently throughout the staining procedures a
higher amount of GZMB$^+$ granules when compared with activated CD8$^+$ T cells. Morphologically, these cells also appeared to be considerably larger than T lymphocytes (Fig. 1F and Supplementary Fig. S2D).

Discussion

We characterized the quality and density of lymphocytic infiltrate, in a cohort of Lynch colorectal cancers, in the context of the HLA class I expression status of tumors and their clinicopathologic stage. We aimed to establish a relation between the makeup of the antitumor immune response and the HLA class I phenotype of tumors to support the notion of immune selection.

Both CD3$^+$/CD8$^-$ cells (presumably CD4$^+$ and γδ T cells) and activated CD8$^+$ T cells were more frequent in HLA class I-negative tumors than HLA class I-positive cases. Moreover, tumors presenting heterogeneous patterns of HLA class I expression were infiltrated by remarkably high numbers of activated CD8$^+$ T cells. It is tempting to speculate that these tumors were undergoing an active immune selection process, revealed by the presence of a heterogeneous HLA class I phenotype and the high affinity of activated CD8$^+$ T cells. It is less evident why tumors that lost HLA class I expression remained infiltrated by activated CD8$^+$ T cells as their effector function is dependent on the expression of HLA class I (31). Their persistent activation status might be supported by inflammatory signals derived from a past immune response against HLA class I-negative tumor cells. The elevated number of CD3$^+$/CD8$^-$ cells (CD4$^+$ and γδ T cells) in HLA class I-negative tumors than HLA class I-positive cases.

Figure 3. The density of lymphocytic infiltration was related to tumor stage both for the primary tumor (T) and lymph node status (N). Total CD8$^+$ T cells and activated CD8$^+$ T cells were more frequent in earlier stages of the primary tumor (A–D). The elusive CD3$^+$/CD8$^+$/GZMB$^+$ cell population was almost exclusively present in lymph node-negative (N0) carcinomas (E and F).
I–negative tumors alludes to the presence of T cells with helper and regulatory functions, capable of sustaining CD8+ T-cell activation (32, 33). On the other hand, the activation of CD8+ T cells might derive from the presence of antigen-presenting cells which conserve the capability of presenting tumor antigens through an HLA class II route (34). Colorectal tumor cells were also shown to express HLA class II and might thereby promote the migration and activation of CD8+ T cells (35). Michel and colleagues described that approximately one third of microsatellite instability-high (MSI-H) colorectal tumors presented membranous expression of HLA class II (36). We found a similar distribution for HLA-DR expression, the most abundantly expressed HLA class II molecule, in a subset of the current cohort. Its expression was associated with a higher density of infiltration by CD8+ T cells and CD3+/CD8+/GZMB+ cells (data not shown).

An elusive CD3+/CD8+/GZMB+ cell population was highly specific for HLA class I–negative tumors. Those cells were infrequent in tumors with heterogeneous expression of HLA class I and nearly absent in HLA class I–positive tumors. Strikingly, the presence of CD3+/CD8+/GZMB+ cells, in primary tumors, was highly predictive for the absence of lymph node metastases. Altogether, these observations compelled us to further characterize this population with focus on NK cell markers.

NK cells are CD3-negative cells possessing cytolytic activity, which can be triggered by the absence of HLA class I expression in target cells (37). They, thus, constitute an important component of the innate immune system, responsible for dealing with cells lacking important host markers–missing self hypothesis (38). Accordingly, NK cells have been regarded as promising vectors for the treatment of HLA class I–negative cancers (39). It is thought that tumor cells compensate for HLA class I loss by favoring the expression of additional NK-inactivating ligands and by losing antagonist ligands with NK-activating properties (40). Expression of HLA-G, an NK cell inhibitory ligand, was previously associated with a worse prognosis in colorectal cancer (41). We investigated its expression in a fraction of tumors composing the current cohort. HLA-G expression was never membranous and only detected in 16% of tumors (data not shown).

Several molecules have been proposed to be characteristic for NK cells or, at least, expressed by certain NK cell subsets (40). Our investigation included CD56, NKp46, and CD16, among others. All these markers were absent in the CD3+/CD8+/GZMB+ cell population, thus not supporting their classification as NK cells. Several other markers were investigated, which in turn excluded that these cells were mast cells or other granulocytes, macrophages, or γδ T cells. The only marker that clearly associated with the CD3+/CD8+/GZMB+ cell population was CD45, which confirmed their hematopoietic-nucleated lineage.

Considering that tumor staging is still the most important prognostic factor in colorectal cancer, the findings presented in this work might be of great relevance. We found a clear association between the type and density of immune cells, present in primary tumors, and their clinicopathologic stage. Furthermore, different immune cell populations related to the local invasive behavior of tumors or with their disseminating capacity. Our results support a role for CD8+ T cells in counteracting a local invasion of the mucosa by cancer cells but not in preventing the migration of tumor cells to adjacent lymph nodes. Conversely, the elusive CD45+/CD8+/CD56+/GZMB+ cell population was characteristic of tumors without lymph node metastases but did not relate to the extent of invasion of the primary tumor. Such a combination of immunologic responses might contribute to the improved survival of Lynch syndrome carriers when compared with patients with sporadic colorectal cancer (42, 43). The makeup and magnitude of the immune responses observed in patients were previously associated with the clinical prognosis and staging of colorectal cancer (44). Moreover, Galon and colleagues proposed that the type, density, and location of immune cells were better predictors of patient outcome than staging (45). However, the majority of studies did not discriminate between mismatch repair–deficient and mismatch repair–proficient colorectal cancers. Hence, the current study is the first to focus specifically on Lynch colorectal carcinomas.

While immune cells are important drivers of tumor selection, they could also hold the key for novel therapeutic or preventive interventions. The prophylactic vaccination of patients with Lynch syndrome with recurrent tumor antigens could elicit an early and robust CD8+ T-cell immune response (17, 18). Such a reaction could lead to tumor eradication or impede tumor progression from early stages. Of note, the presence of CD8+ T cells did not associate with the lymph node status of tumors, which highlights the role of the CD45+/CD8+/CD56+/GZMB+ cell population. The understanding of their role in counteracting tumor metastases could prove essential for the development of novel immunotherapeutic approaches targeting advanced tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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