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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Running title: Anti-tumor immune response in Lynch colorectal cancers

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**Translational relevance:**

Lynch syndrome-associated colorectal cancers are known to evoke a strong reaction from the host’s immune system. 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 non-metastasized 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 counteracting colorectal cancer progression in Lynch syndrome patients. We propose the development of novel clinical strategies, inspired by the natural anti-tumor immune response occurring in Lynch syndrome patients. Considering that Lynch colorectal cancers rapidly acquire immune evasive phenotypes, special relevance should be given to prophylactic approaches.
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. In order 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 clinicopathological stage.

Experimental design: HLA class I expression was assessed by means of immunohistochemistry. Characterization of tumor-infiltrating lymphocytes was carried out by employing a triple immunofluorescence procedure that allowed the simultaneous detection of CD3, CD8 and granzyme B (GZMB) positive cells. Additional markers were also employed 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 < .05). CD8+ T-cells were most abundant in HLA class I heterogeneous tumors (P = .02) and frequent in HLA class I negative cases (P = .04), when compared to 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 < .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 host’s natural anti-tumor immune responses.
Introduction:

Expression of Human Leukocyte Antigen (HLA) class I/antigen complexes, in human cells, is essential for a competent immune surveillance (1). CD8+ T-cells are capable of recognizing and eliminating target cells that present non-self antigens in an HLA class I context (2). Accordingly, HLA class I loss is interpreted as a mechanism, adopted by tumors, to escape immune surveillance and thereby avoid tumor cell recognition and destruction (3). We, and others, previously reported that the majority of DNA mismatch and base excision repair deficient colorectal cancers lose HLA class I expression (4-7). Those include sporadic mismatch repair deficient as well as Lynch syndrome colorectal cancers and MUTYH-associated polyposis colorectal cancers, respectively. The frequency of HLA class I deficiencies in these tumors was found to be considerably higher than the one observed for DNA repair proficient colorectal cancers (5, 7). Both mismatch and base excision repair deficient colorectal cancers are thought to be particularly prone to evoke anti-tumor immune responses due to their pronounced mutator phenotypes (8). Such immune reaction will act as a vector of selective pressure that favors the outgrowth of tumor clones that acquired immune evasive phenotypes. Multiple mechanisms have been shown to underlie defects in HLA class I expression by tumor cells; they include mutations in the individual HLA class I genes, HLA-A, -B and –C, located on chromosome 6p21.3 (9); loss of heterozygosity at 6p21.3 (10); mutations in \( \beta 2 \)-microglobulin (\( \beta 2 M \)) (11), the molecular chaperone required for the cell surface expression of HLA class I antigens; and defects in components of the HLA class I-associated antigen-processing machinery (12, 13). For unknown reasons \( \beta 2 M \) defects were preferentially associated with HLA class I loss in hereditary colorectal cancers (Lynch syndrome and MAP), while sporadic mismatch repair deficient tumors were frequently affected by antigen-processing machinery defects (5, 14). The loss of HLA class I expression, in mismatch and base excision repair deficient colorectal cancers, constitutes a strong handicap for the employment of T-cell based immunotherapeutical approaches (15, 16). On the other hand, evidence for T-cell mediated anti-tumor immune responses could support the development of prophylactic vaccination strategies based on peptides that are
frequently mutated in colorectal cancers (17, 18). This approach is of particular importance for individuals carrying an increased risk for developing colorectal cancer at an early age. Lynch syndrome is an autosomal, dominant disease caused by the germline inactivation of one copy of either MLH1, MSH2, MSH6 or PMS2 mismatch repair genes (19). Lynch syndrome patients have an increased lifetime risk of developing colorectal cancer, as well as other cancer types, when compared to the general population (20). Currently, endoscopic surveillance constitutes the most effective approach to increase life expectancy of affected individuals (21).

The development of T-cell based prophylactic vaccination strategies, for Lynch syndrome patients, requires additional evidence that T-cells are the drivers of immune selection in Lynch colorectal carcinomas. It has been previously reported that colorectal cancers carrying HLA class I defects are more densely infiltrated by T-cells (22-24). However, these observations were not reproduced specifically in cohorts of mismatch repair deficient tumors. Accordingly, those reports might carry a possible bias: since 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 performed 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 clinicopathological stage.
Material & Methods:

Patient Material:

A cohort of 90 colorectal carcinomas, derived from 86 Lynch syndrome patients, was compiled. Corresponding formalin-fixed, paraffin-embedded tissues were collected throughout The Netherlands. All patients were carriers of genetically proven, pathogenic, germline mutations in either 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. Additionally, all except one patient, for which extended clinical information was not available, fulfilled the revised Bethesda Criteria for Lynch syndrome (27). Pathologic tumor TNM staging was retrieved from 79 tumors: five tumors were staged as T1 while 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 two 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 two-step indirect immunohistochemistry procedure on 4 μm tissue sections. Following deparaffinization and rehydration, the tissue sections underwent heat-mediated antigen retrieval in a 10 mM citrate buffer solution (pH 6). After cooling, endogenous peroxidase activity blockage was performed with a 0.3% hydrogen peroxide/methanol solution. Thereafter, the sections were incubated over-night 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 (29, 30) (supernatants kindly provided by Prof. J. Neefjes, NKI, Amsterdam, The Netherlands and Prof. H. L. Ploegh, MIT, Boston, MA, USA); and the
rabbit anti-β2M polyclonal antibody A0072 (Dako). The following day, primary antibody binding was detected with the BrightVision Poly-HRP detection system (Immunologic). Scoring of HLA class I and β2M expression in tumor cells was always performed against an internal positive control, provided by the staining of stromal cells. Negative controls were generated by replacing the primary antibodies by a 1% BSA/PBS solution during the procedure. A double-staining immunohistochemistry procedure was carried-out to study the morphology of CD3-/CD8-/GZMB+ cells. Tissue sections were treated as described for the HLA class I and β2M staining but heat-mediated antigen retrieval was done in a 1 mM EDTA solution (pH 9) instead. Tissue sections were incubated over-night with a mixture of anti-CD8 (4B11, IgG2b) and anti-granzyme B (GZMB) (GrB-7, IgG2a) antibodies. Further detail on the primary antibodies is provided in Supplementary Table 1. The following day, tissue sections were incubated with an anti-mouse IgG2a-HRP and goat anti-mouse IgG2b-AP solution (1:200, Southern Biotech). Staining development was performed with diaminobenzidine (Dako) and Fast Red (Roche Applied Science) according to manufacturer’s instructions.

Immunofluorescence:
Characterization of lymphocytic infiltration in Lynch carcinomas was performed with triple immunofluorescence. After deparaffinization and rehydration of the 4 μm tissue sections, heat-mediated antigen retrieval with a 1mM EDTA solution (pH 9) and blockage of non-specific antibody binding with 10% normal goat serum (DAKO) were performed. A solution containing three primary antibodies was applied to the tissue sections over-night. The next day, a mixture of fluorescently labeled secondary antibodies was employed to detect primary antibody binding. A detailed list of primary and secondary antibodies utilized is provided in Supplementary Table 1. Initial characterization of the lymphocytic infiltrate was done with a combination of anti-CD3, anti-CD8 and anti-GZMB antibodies. The CD3+/CD8- cell population was considered to be composed of CD4+ and γδ T-cells. The discovery of an elusive 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, -CD68, -CD117, -NKp46 and anti-TCR-γ antibodies were employed in combination with anti-CD8 and anti-GZMB antibodies (Antigens and dilutions provided in Supplementary Table 1). Immunofluorescence was detected with a LSM700 confocal laser microscope (Carl Zeiss), equipped with a ZEISS LCI Plan-NEOFUAR 25x/0,8 DIC Imm Korr objective. Approximately 4 mm² of tumor tissue were 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 performed 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 performed with Graphpad PRISM (version 5.04). The Mann-Whitney U test was employed when assessing the differences in the amount of lymphocytic infiltrate between HLA class I phenotypes and lymph node negative and positive tumors. Analysis of variance (ANOVA) was performed for comparing lymphocyte density amongst 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 while a heterogeneous pattern of HLA class I staining was observed in 17 carcinomas (Supplementary Figure 1). 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 Figure 1 C, D). HLA class I 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 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) (Figure 1 A - D). No significant difference was detected between distinct HLA class I phenotypes for total CD8+ T-cell density (Figure 2 A). However, HLA class I negative and heterogeneous tumors were more densely infiltrated by activated CD8+ T-cells than HLA class I positive tumors (P = .02 and P = .04, respectively) (Figure 2 B). CD3+/CD8- cells (CD4+ and γδ T-cells) were more frequent in HLA class I negative tumors when compared to HLA class I positive (P = .01) (Figure 2 C). Amongst 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 = .01) (data not shown).

A population of cells with a CD3-/CD8-/GZMB+ phenotype was detected in 82% of cases at various frequencies (Figure 1 C, 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 \( (P = .004) \) (Figure 2 D).

**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 \( (P < .05; \text{ANOVA, Figure 3 A}) \). This difference was most striking when the analysis was restricted to HLA class I negative tumors \( (P = .002, \text{Figure 3 B}) \). The same trend was observed for activated CD8+ T-cells in all tumors \( (P = .04) \) and HLA class I negative cases \( (P = .004) \) (Figure 3 C, 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 \( (P < .01) \) (Figure 3 E, 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 two 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 tumor’s lymph node status.

**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 1).

The only marker that clearly associated with the CD3-/CD8-/GZMB+ cell population was CD45 (Figure 1 E), thus confirming the hematopoietic nucleated lineage of these cells. CD56+ cells were rare and located nearby blood vessels (Supplementary Figure 2 A). CD16 cells were abundant but did not co-localize with GZMB positivity (Supplementary Figure 2 B). CD57+/CD8- cells were often found in the tumor stroma and some displayed positivity for GZMB but CD57 failed to co-localize with the majority of CD8-/GZMB+ cells (Supplementary Figure 2 C). All other investigated markers did not co-localize with these cells, despite the presence of internal positive controls for the majority (data not shown). No NKp46+ cells were found in the tumor tissues. We detected NKp46 expression in tonsil tissues in order to rule out a failure of the staining procedure.

As GZMB expression could be derived from granulocytes, we performed a bright-field, double staining of CD8 and GZMB together with hematoxylin counter-staining in order to discern the nuclear morphology of CD8-/GZMB+ cells. The nuclei from CD8-/GZMB+ cells were non-lobated and easily discriminated from polymorphonuclear granulocytes (Figure 1 F and Supplementary Figure 2 D). This CD45+/CD8-/CD56-/GZMB+ cell population presented consistently throughout the staining procedures a higher amount of GZMB+ granules, when compared to activated CD8+ T-cells. Morphologically, these cells also appeared to be considerably larger than T lymphocytes (Figure 1 F and Supplementary Figure 2 D).
Discussion:

We characterized the quality and density of lymphocytic infiltrate, in a cohort of Lynch colorectal cancers, in the context of the tumor’s HLA class I expression status and clinicopathological stage. We aimed to establish a relation between the make-up of the host’s immune response and the tumor’s HLA class I phenotype in order 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, as compared to 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 affluence of activated CD8+ T-cells. It is less evident why tumors that lost HLA class I expression remain 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 positive tumor cells. The elevated number of CD3+/CD8- cells (CD4+ and γδ T-cells) in HLA class 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 et al. described that approximately one third of 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 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 the primary tumor, and its clinicopathological stage. Furthermore, different immune cell populations related to the tumor’s local invasive behavior or with its disseminating capacity. Our results support a role for CD8+ T-cells in counteracting the local invasion of cancer cells through the mucosa 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 combination of immunological responses might contribute to the improved survival of Lynch syndrome carriers when compared to patients with sporadic colorectal cancer (42, 43). The makeup and magnitude of the host’s immune response was previously associated with the clinical prognosis and staging of colorectal cancer (44). Moreover, Galon et al. 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 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 Lynch syndrome patients, with recurrent tumor antigens, could elicit an early and robust CD8+ T-cell immune response (17, 18). Such 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 tumor’s lymph node status, 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.
Acknowledgments:

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

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<th>N tumors</th>
<th>% B2M loss</th>
<th>Median</th>
<th>Mean</th>
<th>Median</th>
<th>Mean</th>
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<td>39,10</td>
<td>62,86</td>
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<td>31,96</td>
<td>1,03</td>
<td>3,30</td>
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<tr>
<td>Heterogeneous</td>
<td>17 (19%)</td>
<td>26%</td>
<td>8,73</td>
<td>19,56</td>
<td>72,88</td>
<td>111,9</td>
<td>51,74*</td>
<td>67,97</td>
<td>1,86</td>
<td>3,78</td>
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<td>Negative</td>
<td>58 (64%)</td>
<td>59%</td>
<td>11,98*</td>
<td>23,51</td>
<td>60,58</td>
<td>90,39</td>
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<td>54,38</td>
<td>4,96*</td>
<td>23,03*</td>
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* - Medians differ significantly from the HLA class I positive group.
Figure 1 – Density if lymphocyte infiltration in tumor fields (T) was determined by means of triple immunofluorescence. Antibodies against CD3 (red), CD8 (blue) and GZMB (green) were simultaneously employed to identify CD3+/CD8- cells (presumably CD4+ or γδ T-cells), total CD8+ T-cells (CD3+/CD8+) and activated CD8+ T-cells (CD3+/CD8+/GZMB+) (A – D). CD4+ or γδ T-cells are represented in red, while CD3+/CD8+ cells (purple) correspond to CD8+ T-cells. GZMB positivity in CD3+/CD8+ cells (green/white) reveals activated CD8+ T-cells (A, arrows). Density of lymphocyte infiltration varied greatly among tumors (A, B). A large number of GZMB positive cells, without co-localization of CD3 and CD8, were detected in some tumors (C, D, arrows). We attempted to characterize those cells by employing a set of markers in combination with CD8 (blue) and GZMB (green). CD45 (red) was the only marker that clearly associated with the CD8-/GZMB+ cells (E, arrow). By means of bright-field double-staining we excluded that CD8/GZMB+ cells were multinucleated granulocytes (F, arrow).

Figure 2 – Total CD8+ T-cells were not distributed differently between tumors with different HLA class I phenotypes (A). Activated CD8+ T-cells were more frequent both in HLA class I negative (HLA -) and heterogeneous (HLA-h) tumors, when compared to HLA class I positive cases (B). CD3+/CD8- (CD4 + and γδ T-cells) were also more frequent in HLA class I negative tumors (C). GZMB+ cells lacking CD3 or CD8 markers were characteristic for HLA class I negative carcinomas (D). (* - P < .05; ** - P < .01).

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, independently of the tumor’s HLA class I expression status (A - D). The elusive CD3-/CD8-/GZMB+ cell population was almost exclusively present in lymph node negative (N0) carcinomas (E, F).
Figure 1

A

B

C

D

E

F

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Figure 3

(A) CD8+ T-cells

\[ P < .05 \]

(B) CD8+ T-cells

\[ P < .01 \]

(C) Activated CD8+ T-cells

\[ P < .05 \]

(D) Activated CD8+ T-cells

\[ P < .01 \]

(E) CD3-/CD8-/GZMB+ cells

\[ P < .01 \]

(F) CD3-/CD8-/GZMB+ cells

\[ P < .01 \]
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