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

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.

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.

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 ($P < 0.01$).

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 counteracting 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.

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 (MAP) 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 antitumor 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 β2-microglobulin (*B2M*; ref. 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, β2m defects were preferentially associated with HLA class I loss in hereditary colorectal cancers (Lynch syndrome and MAP), whereas 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 immunotherapeutic approaches (15, 16). On the other hand, evidence for T-cell–mediated antitumor immune responses could support the development of prophylactic vaccination strategies based on peptides that are frequently mutated in the aforementioned 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). Patients with Lynch syndrome have an increased lifetime risk of developing colorectal cancer, as well as other cancer types, when compared with 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 patients with Lynch syndrome 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: 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.

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 (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 (Figures 1C and 1D). 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 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

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

			Infiltrate density, n cells/mm2							
			CD3+/CD8−		CD8+ T cells (CD3+/CD8+)		Activated CD8+ T cells (CD3+/CD8+/GZMB+)		CD3−/CD8−/GZMB+	
HLA class I status	Tumors, n (%)	% β2M loss	Median	Mean	Median	Mean	Median	Mean	Median	Mean
Positive	15 (17)	—	6.13	20.78	39.10	62.86	16.85	31.96	1.03	3.30
Heterogeneous	17 (19)	26	8.73	19.56	72.88	111.9	51.74[a]	67.97	1.86	3.78
Negative	58 (64)	59	11.98[a]	23.51	60.58	90.39	35.33[a]	54.38	4.96[a]	23.03

[a] Medians differ significantly from the HLA class I–positive group.

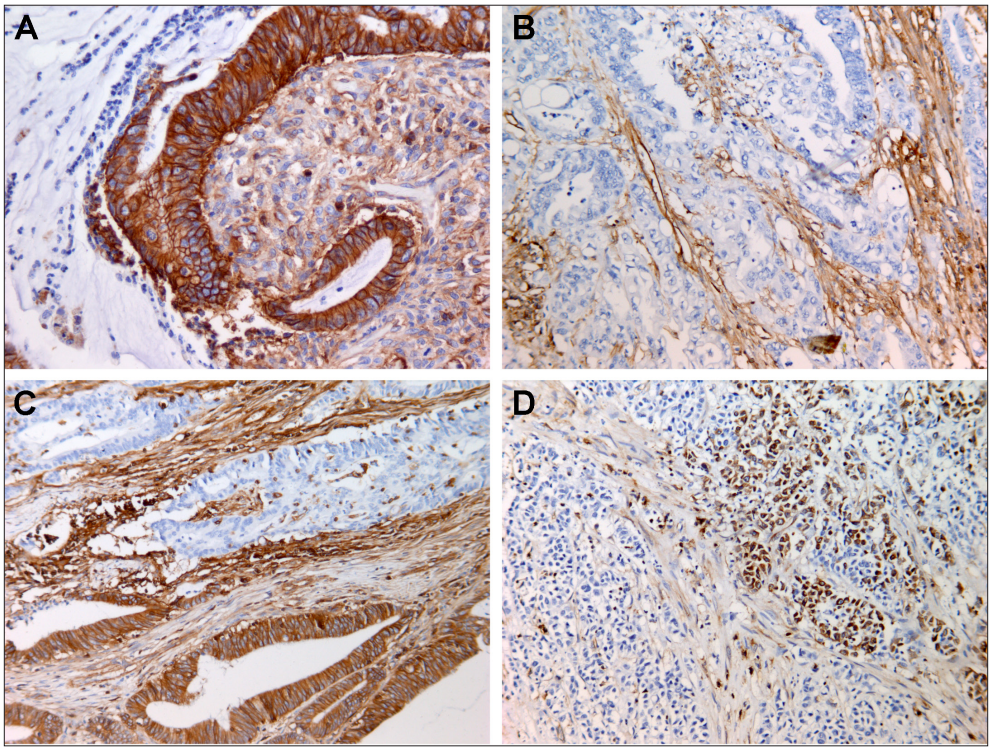

Figure 1. Three different HLA class I phenotypes were observed in Lynch colorectal carcinomas. Tumors were either HLA class I positive, with clear membranous expression of the HLA class I molecules (A); HLA class I negative, with total absence of HLA class I expression in tumors cells and respective positive internal control provided by stromal cells (B); and HLA class I heterogeneous, with HLA class I positive and negative tumor *foci* (C, D).

cells), and CD3+/CD8+/GZMB+ (activated CD8+ T cells; Figures 2A–D). No significant difference was detected between distinct HLA class I phenotypes for total CD8+ T-cell density (Figures 3A). However, HLA class I–negative and heterogeneous tumors were

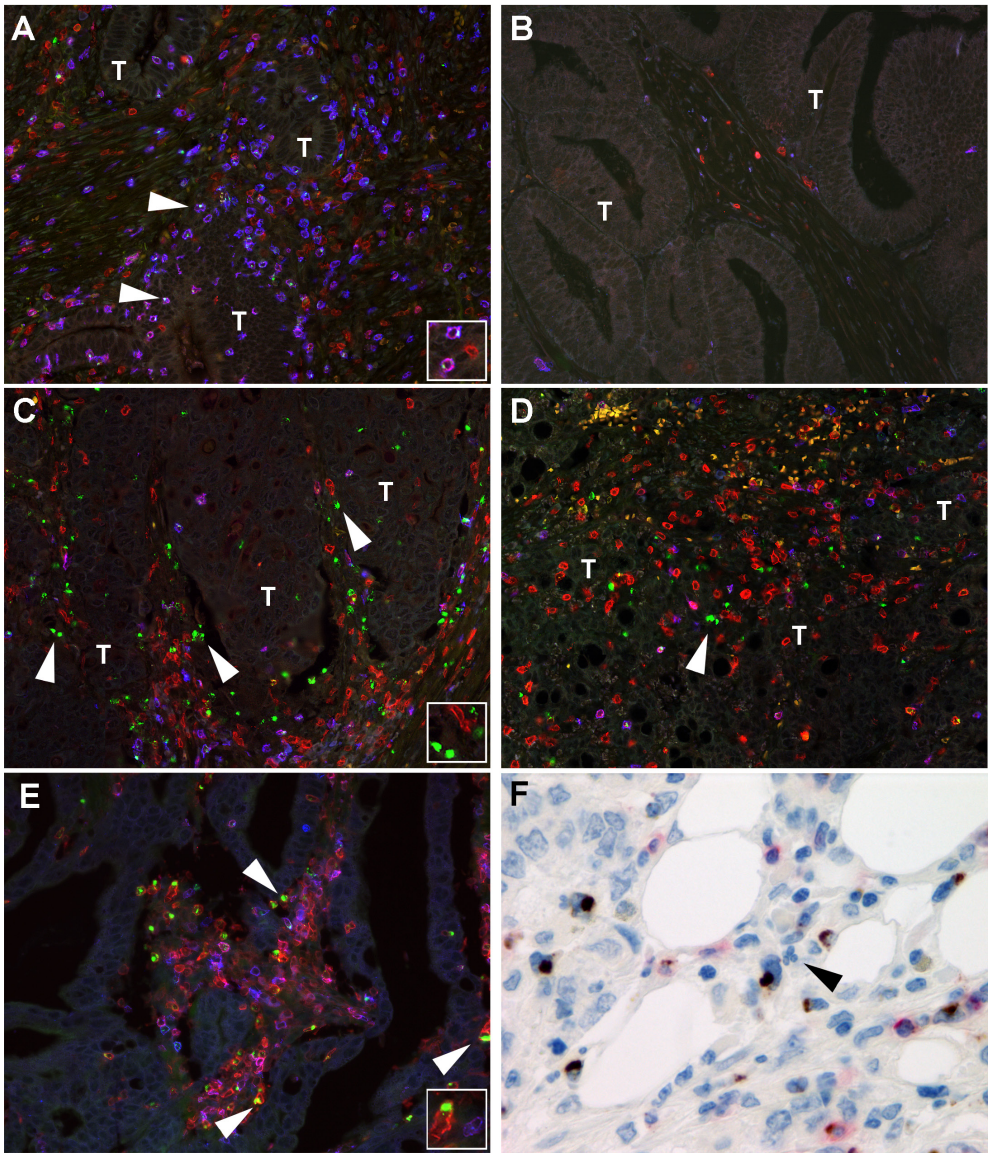

Figure 2. Density of lymphocyte infiltration in tumor fields (T) was determined by means of triple immunofluorescence. Antibodies against CD3 (red), CD8 (blue), and GZMB (green) were simultaneously used to identify CD3+/CD8– cells (presumably CD4+ or γδ T cells), total CD8+ T cells (CD3+/CD8+), and activated CD8+ T cells (CD3+/CD8+/GZMB+; A–D). CD4+ or γδ T cells are represented in red, whereas CD3+/CD8+ cells (purple) correspond to CD8+ T cells. GZMB positivity in CD3+/CD8+ cells (green/white) reveals activated CD8+ T cells (A, arrowheads). Density of lymphocyte infiltration varied greatly among tumors (A and B). A large number of GZMB-positive cells, without colocalization of CD3 and CD8, were detected in some tumors (C and D, arrowheads). We attempted to characterize those cells by using 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, arrowheads). By means of bright-field double staining, we excluded that CD8/GZMB+ cells were multinucleated granulocytes (F, arrowhead).

more densely infiltrated by activated CD8+ T cells than HLA class I–positive tumors (P = 0.02 and P = 0.04, respectively; Figure 3B). 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; Figure 3C). 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 (Figures 2C 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; Figure 3D).

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

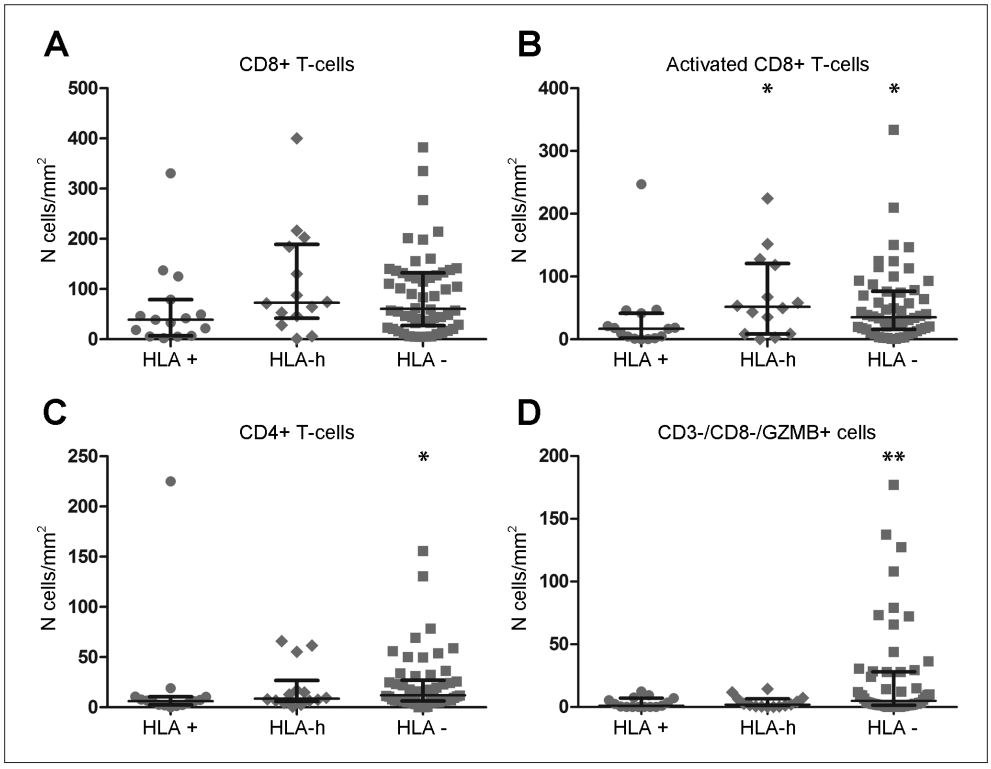

Figure 3. Total CD8+ T cells were not distributed differently between tumors with different HLA class I phenotypes (A). Activated CD8+ T cells were more frequent both in HLA class I–negative (HLA–) and heterogeneous (HLA-h) tumors than HLA class I–positive cases (B). CD3+/CD8– (CD4+ and γδ T cells) were 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). *, $P < 0.05$; **, $P < 0.01$.

cells was gradually smaller with increasing tumor stage ($P < 0.05$; ANOVA, Figure 4A). This difference was most striking when the analysis was restricted to HLA class I–negative tumors ($P = 0.002$; Figure 4B). The same trend was observed for activated CD8+ T cells in all tumors ($P = 0.04$) and HLA class I–negative cases ($P = 0.004$; Figures 4C 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–

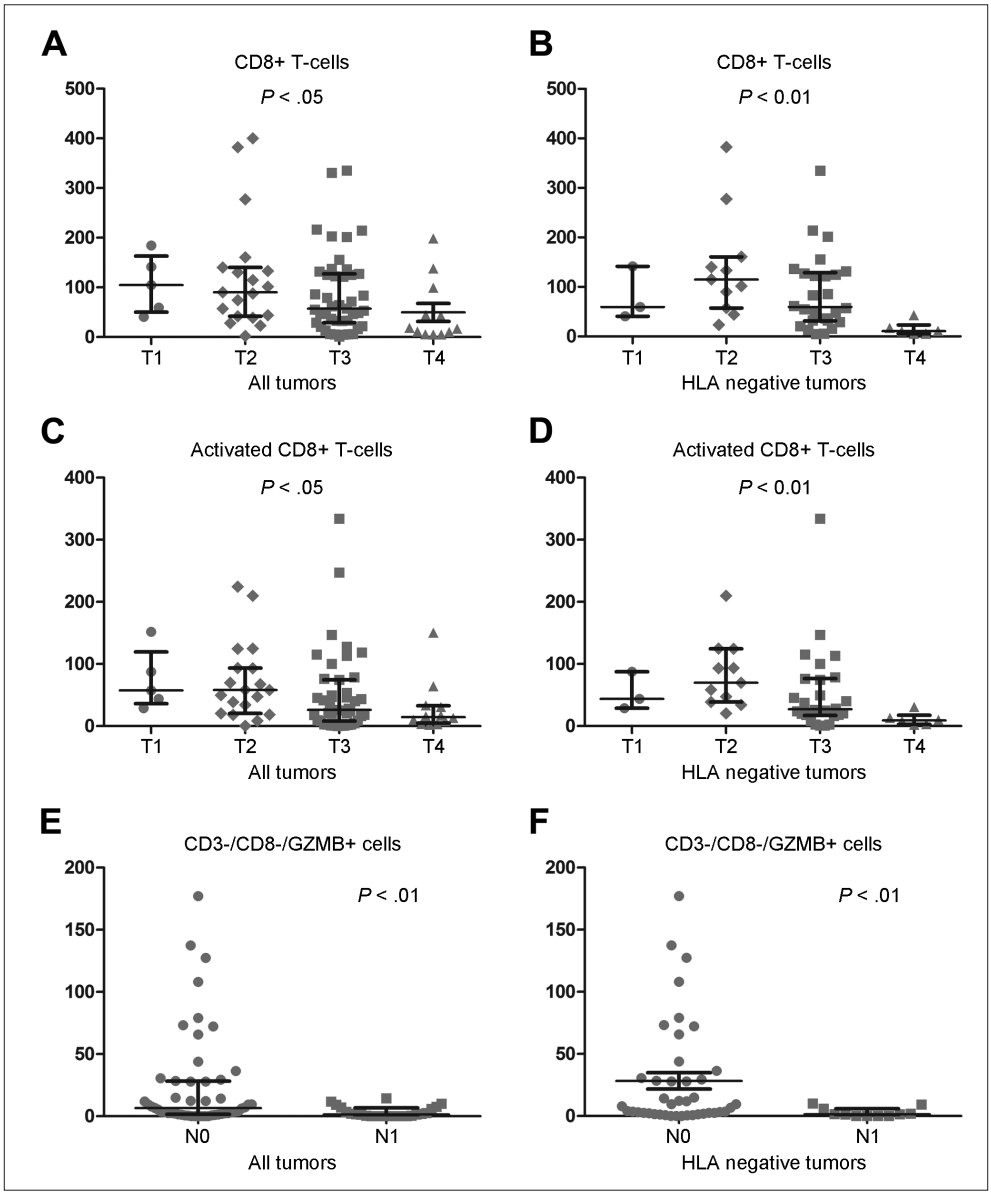

Figure 4. The density of lymphocytic infiltration was related to tumor stage both for the primary tumor (T) and lymph node status (N). Total CD8+ T cells and activated CD8+ T cells were more frequent in earlier stages of the primary tumor (A–D). The elusive CD3–/CD8–/GZMB+ cell population was almost exclusively present in lymph node–negative (N0) carcinomas (E and F).

negative carcinomas ($P < 0.01$; Figures 4E 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 Table 2).

The only marker that clearly associated with the CD3−/CD8−/GZMB+ cell population was CD45 (Figure 2E), thus confirming the hematopoietic nucleated lineage of these cells. CD56+ cells were rare and located nearby blood vessels (Figure 5A). CD16 cells were abundant but did not colocalize with GZMB positivity (Figure 5B). 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 (Figure 5C). 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 (Figures 2F and 5D).

This CD45+/CD8−/CD56−/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 (Figures 2F and 5D).

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

Table 2. Description of primary antibodies utilized for the characterization of tumor infiltrating lymphocytes.

Antigen	Antibody	Host/Isotype	Dilution	Company	Antigen expression	Secondary antibody [a]
CD3	ab828	Rabbit polyclonal	1:200	Abcam	T cells	goat anti-rabbit IgG-Alexa Fluor 546
CD8	4B11[b]	Mouse IgG2b	1:200	Leyca Mycrosystems	CD8+ T cells	goat anti-mouse IgG2b-Alexa Fluor 647
CD8	c8/144B	Mouse IgG1	1:400	DAKO	CD8+ T cells	goat anti-mouse IgG1-Alexa Fluor 546
Granzyme B	GrB-7	Mouse IgG2a	1:400	DAKO	Cytotoxic T cells; NK cells	goat anti-mouse Ig2a-Alexa Fluor 488
CD2	AB75	Mouse IgG1	1:10	DAKO	T and NK cells	goat anti-mouse IgG1-Alexa Fluor 546
CD16	DJ130c	Mouse IgG1	1:200	Ab Serotech	NK cells, neutrophils, monocytes, macrophages	goat anti-mouse IgG1-Alexa Fluor 546
CD45	2B11+PD7	Mouse IgG1	1:100	DAKO	Hematopoietic nucleated cells except plasma cells	goat anti-mouse IgG1-Alexa Fluor 546
CD56	123C3	Mouse IgG1	1:100	DAKO	NK cells, activated T cells	goat anti-mouse IgG1-Alexa Fluor 546
CD57	HNK-1 hybridoma	Mouse IgM	1:20	ATCC	NK cells, T cells	goat anti-mouse IgM-Alexa Fluor 546
CD68	KP1	Mouse IgG1	1:200	DAKO	Monocytes, macrophages	goat anti-mouse IgG1-Alexa Fluor 546
CD117	A4502	Rabbit polyclonal	1:50	DAKO	Mast cells, lymphoid progenitors	goat anti-rabbit IgG-Alexa Fluor 546
NKp46	195314	Mouse IgG2b	1:100	R&D systems	NK cells	goat anti-mouse IgG2b-Alexa Fluor 647
TCR-γ	γ3.20	Mouse IgG1	1:25	Thermo Fisher	γδ T cells	goat anti-mouse IgG1-Alexa Fluor 546

[a] All secondary antibodies were employed at a 1:200 dilution and purchased from Invitrogen; Rb - rabbit; Mo - Mouse; Go – goat. [b] The c8/144B anti-CD8 clone was only used in combination with the NKp46 marker due to isotype matching between latter that the anti-CD8 4B11 clone.

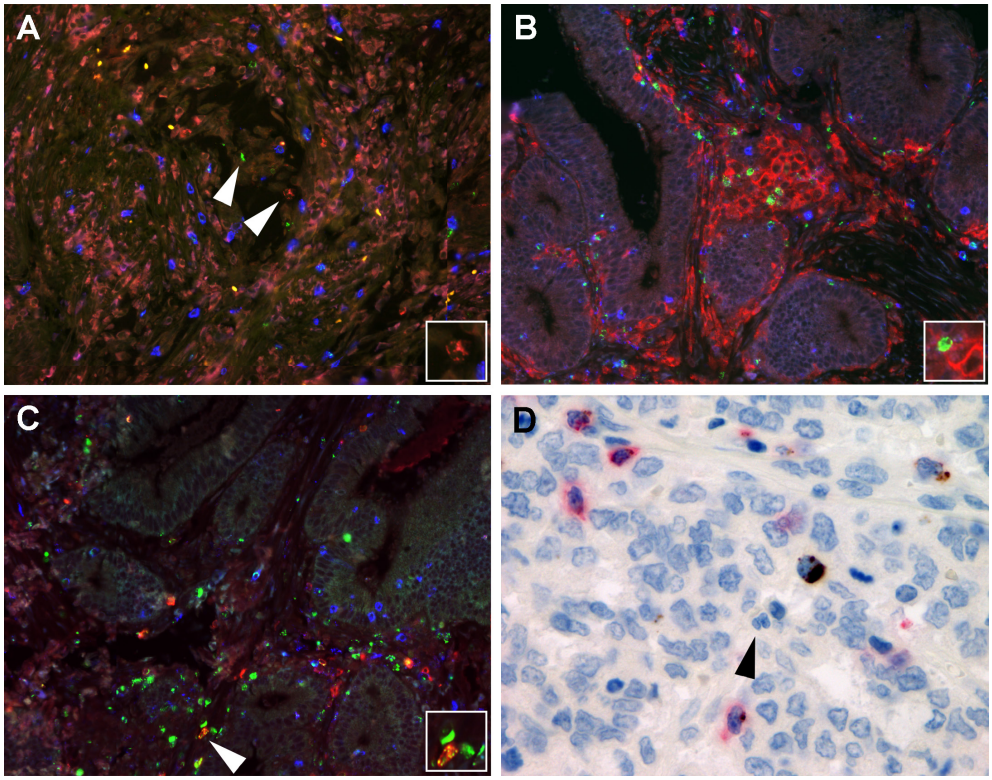

Figure 5. The characterization of the CD3-/CD8-/GZMB+ cell population was made through the employment of various markers (red) together with CD8 (blue) and GZMB (green). CD8-/GZMB+ cells failed to co-localize with CD56 (A, arrows), CD16 (B) and CD57 (C). Few CD57+ cells displayed GZMB positivity (C, arrow). By means of bright-field double-staining we excluded that CD8-/GZMB+ cells were multi-nucleated granulocytes (D, arrow).

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 affluence 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 (27). 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 (28, 29). 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 (30). Colorectal tumor cells were also shown to express HLA class II and might thereby promote the migration

and activation of CD8+ T cells (31). Michel and colleagues described that approximately one third of microsatellite instability-high (MSI-H) colorectal tumors presented membranous expression of HLA class II (32). 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 (33). They, thus, constitute an important component of the innate immune system, responsible for dealing with cells lacking important host markers–missing self hypothesis (34). Accordingly, NK cells have been regarded as promising vectors for the treatment of HLA class I–negative cancers (35). 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 (36). Expression of HLA-G, an NK cell inhibitory ligand, was previously associated with a worse prognosis in colorectal cancer (37). 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 (36). 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 (38, 39). The makeup and magnitude of the immune responses observed in patients were previously associated with the clinical prognosis and staging of colorectal cancer (40). Moreover, Galon and colleagues proposed that the type, density, and location of immune cells were better predictors of patient outcome than staging (41). 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.

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 (42). 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 (32, 33); 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. 33, 34; 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 polyclonal antibody A0072 (Dako). The following day, primary antibody binding was detected with the BrightVision Poly-HRP (horeradish 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+ 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 mmol/L EDTA solution (pH 9) instead. Tissue sections were incubated overnight with a mixture of anti-CD8 [4B11, immunoglobulin G2b (IgG2b)], and anti-Granzyme B (GZMB; GrB-7, IgG2a) antibodies. Further detail on the primary antibodies is provided in Table 2. 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 carried out with 3,3'-diaminobenzidine (Dako) and Fast Red (Roche Applied Science) according to the manufacturer's instructions.

Immunofluorescence

Characterization of lymphocytic infiltration in Lynch carcinomas was carried out with triple immunofluorescence. After deparaffinization and rehydration of the 4-μm tissue sections, heat-mediated antigen retrieval with a 1 mmol/L EDTA solution (pH 9) and blockage of nonspecific antibody binding with 10% normal goat serum (Dako) were carried out. A solution containing 3 primary antibodies was applied to the tissue sections overnight. The next day, a mixture

of fluorescently labeled secondary antibodies was used to detect primary antibody binding. A detailed list of primary and secondary antibodies used is provided in Table 2. 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, -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).

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