

# HLA Class I Antigen Expression in Conjunctival Melanoma Is Not Associated With PD-L1/PD-1 Status

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**PURPOSE.** Antitumor T cells need expression of HLA class I molecules but can be inhibited by ligands such as programmed death ligand 1 (PD-L1). We determined expression and regulation of these molecules in human conjunctival melanoma (CM) samples, cell lines, and murine xenografts.

**METHODS.** Immunofluorescence staining was performed to examine the expression of HLA-A, HLA-B/C, and  $\beta$ -2-microglobulin (B2M) in 23 primary CM samples. HLA class I expression was compared with clinicopathologic characteristics, the presence of tumor-infiltrating leukocytes, and PD-L1/PD-1 status. The effect of interferon  $\gamma$  (IFN- $\gamma$ ) on HLA class I expression was tested on three CM cell lines using quantitative PCR and flow cytometry. Furthermore, HLA class I expression was determined in CM cell line-derived murine xenografts.

**RESULTS.** One third of tumors had positive HLA-A, HLA-B/C, and B2M expression. A positive expression was especially seen in thin and epibulbar tumors but was not associated with recurrences. HLA class I expression was correlated with M2 macrophage density and tended to associate with CD8<sup>+</sup> T-cell density but was independent of PD-L1 or PD-1 expression. IFN- $\gamma$  upregulated HLA class I expression and genes involved in HLA transcription and transportation on CM cell lines. Murine xenografts showed a comparable HLA class I expression as their respective cell lines.

**CONCLUSIONS.** Our data indicate that subsets of CM have positive HLA class I expression, and HLA class I and PD-L1/PD-1 are expressed independently. When one considers immunotherapy, one should also analyze HLA class I expression, whose downregulation can limit the efficacy of T cell-mediated therapies.

**Keywords:** conjunctival melanoma, HLA class I, immune escape, animal model, tumor infiltrating lymphocytes

Currently, a lot of attention is given to the role of the immune system in tumor growth, control, and treatment. The theory of immune surveillance, which states that the immune system can recognize and destroy tumor cells, has been widely accepted and provides targets for therapy.<sup>1</sup> The discovery of immune checkpoint regulators has transformed the treatment of many malignancies: cancer often protects itself from T-cell attack by blocking T-cell signals. Binding of programmed death ligand 1 (PD-L1) on the cancer cell to PD-1 on the T cell will suppress the T cell's function, impair cytokine production, and induce T-cell apoptosis. To reverse this immunologically tolerant state, antibodies against these immune checkpoints have been developed (e.g., targeting PD-1 or cytotoxic T-lymphocyte-associated protein 4 [CTLA4]). The results in cutaneous melanoma, bladder cancer, non-small cell

lung cancer, and mucosal melanoma are promising but restricted to subsets of patients.<sup>2-5</sup>

Expression of the HLA class I molecules is needed to recognize and lyse tumor cells by cytotoxic T cells (CTLs): CTLs can destroy cells that express the appropriate peptide/MHC class I complex.<sup>6</sup> HLA class I molecules consist of a glycoprotein heavy chain (coded in the HLA region on chromosome 6p21) and a  $\beta$ -2-microglobulin (B2M) light chain (gene located on chromosome 15).<sup>7</sup> Defects in HLA class I antigen-processing machinery (APM) components impair effective immune responses to cancer, although tumor-infiltrating lymphocytes may still present. A lack of HLA class I expression that may be associated with a worse survival<sup>8</sup> has been described in many malignancies and can either be "soft" (reversible) or "hard" (irreversible), due to various mechanisms.<sup>9</sup> A low HLA expression can often be corrected by



interferon- $\gamma$  (IFN- $\gamma$ ), thereby restoring a tumor cell's susceptibility to be lysed by T cells.

Although a lot of immunologic research in melanoma has focused on cutaneous lesions, little is known about melanoma of the conjunctiva. Conjunctival melanoma (CM) is a rare type of mucosal melanoma that originates in the melanocytes of the basal layer of the conjunctiva and accounts for 5% of all ocular melanoma.<sup>10</sup> The incidence in Caucasians is 0.8/million<sup>11</sup> and in recent years has been increasing in Western countries.<sup>12,13</sup> The appearance of CM ranges from amelanotic to heavily pigmented and CM can occur in any part of the epibulbar or nonepibulbar conjunctiva. Wide local excision with adjuvant treatment (cryotherapy, chemotherapy, brachytherapy) is the most common therapy, whereas more extensive surgical procedures are reserved as a last resort therapy.<sup>14</sup> Despite treatment, recurrence rates of 26% to 61% at 5 years after treatment have been reported,<sup>15-18</sup> with metastases occurring in 11% to 16% of patients.<sup>15,19</sup> Because limited chemotherapeutic options are available for extended or metastasized CM, these patients may benefit from the new T cell-mediated treatments. We recently described that PD-1- and PD-L1-positive cells are present in CM,<sup>20</sup> making treatment with PD-L1/PD-1 inhibitors potentially interesting. Very recently, Nivolumab (PD-1 inhibitor, Opdivo, Bristol-Myers Squibb, NY, USA) led to a successful clinical response in a CM patient with breast metastases.<sup>21</sup>

However, effective T-cell immune responses also need a proper expression of the HLA class I antigens. Currently, very little is known regarding the expression of the HLA class I antigens in CM. We studied the degree of HLA class I expression in human CM samples, CM cell lines, and animal xenografts. We found out that HLA class I expression was not associated with PD-L1/PD-1 status and detected positive HLA class I expression in only one third of primary CM.

## METHODS

### Patient Data and Selection

Patients were treated at the Leiden University Medical Center (LUMC), Leiden, The Netherlands, between 1996 and 2014. The tumor cohort included 23 primary CM for which sufficient material was available (Table 1). Tumors were examined by an experienced ophthalmic pathologist (SD). Tumor stage was assessed according to the 7th edition of the American Joint Committee on Cancer (AJCC) classification of malignant tumors (TNM) cancer staging manual.<sup>22</sup> Local recurrence was defined as a histologically proven recurrence. Metastasis was verified by histologic examination or imaging analysis. Total follow-up time was from the time of diagnosis to the last known status. The study adhered to the tenets of the Declaration of Helsinki, and the Medical Ethical Committee of LUMC agreed with this retrospective study.

### Immunohistologic Staining and Scoring

Immunofluorescence (IF) staining of HLA-A, HLA-B/C, and B2M on human paraffin-embedded CM sections was performed as previously described.<sup>19,23</sup> For immunohistochemistry (IHC) on mouse xenografts, the procedures were performed as described.<sup>24</sup> In short, primary antibodies HCA2 (mouse, anti-HLA-A, IgG1, MUB2036P, 1:50; Nordic Mubio, Uden, The Netherlands), HC10 (mouse, anti-HLA-B/C, IgG2a, MUB2037P, 1:100; Nordic Mubio), and B2M (rabbit, Z0311, 1:2000 [IHC], 1:1000 [IF]; DAKO, Glostrup, Denmark) were used for both IF and IHC staining. Isotype control staining was obtained by replacing primary antibodies with

control antibodies with respective isotypes antibodies. The detailed information of secondary antibodies is in Supplementary Table S1. IF images were taken using a Zeiss LSM 700 confocal scanning microscope (Zeiss, Breda, The Netherlands). Based on the tumor size, one to five representative images at high power (250 $\times$ ) were randomly selected from different areas for scoring. Tumor areas were distinguishable from normal tissue by DAPI (4',6-diamidino-2-phenylindole; H-1200; Vector Laboratories, Burlingame, CA, USA) staining. Two experienced independent observers (JC and NB or EJ), blinded to the clinicopathologic data, evaluated the intensity and percentage of HC10, HCA2, and B2M expression separately. In case of discrepancies, a consensus was reached by simultaneous analysis. The semiquantitative scoring system was obtained from prior studies<sup>23,25-27</sup>; intensity was scored as 0 (absent), 1 (weak), 2 (moderate), and 3 (strong), and the percentage of positive cells as 0 (0%), 1 (1% to 5%), 2 (6% to 25%), 3 (26% to 50%), 4 (51% to 75%), and 5 (76% to 100%). A single value was reached by adding intensity and percentage and was used to create three categories: negative (0 to 2), weak (3 to 6), and positive expression (7 to 8). Stromal cells served as an internal positive control. The B2M staining of one tumor was not scorable and was therefore excluded. The images of hematoxylin and eosin (HE) staining were captured using the Philips Image Management System 2.2 (Philips, Eindhoven, The Netherlands). The staining and scoring method of tumor-infiltrating lymphocytes (CD3, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>-</sup>, CD3<sup>+</sup>CD8<sup>-</sup>Foxp3<sup>+</sup>, and CD3<sup>+</sup>CD8<sup>-</sup>Foxp3<sup>-</sup> T cells, and CD68 and CD68<sup>+</sup>CD163<sup>+</sup> macrophages) was performed as previously described.<sup>20</sup> Expression of PD-L1 on tumor or stromal cells and of PD-1 was determined as described.<sup>20</sup>

### HLA Typing

HLA typing was performed at the Laboratory of the Department of Immunohematology and Blood Transfusion (IHB, LUMC) using the Gen-Probe LIFECODES HLA-SSO HLA typing assay for HLA class I typing.

### Cell Lines and Treatment

Three CM cell lines (CRMM1, CRMM2, and CM2005.1) derived from primary tumors, kindly provided by Michele Madigan (Sydney, Australia) and Sander Keijsers (Leiden University Medical Center, Leiden, The Netherlands), were used.<sup>28,29</sup> To examine the influence of IFN- $\gamma$  on HLA class I expression, CM cells were cultured with or without 100 IU/mL of IFN- $\gamma$  (ImmunoTools, Friesoythe, Germany) for 48 hours.<sup>30</sup>

### RNA Isolation and Quantitative Real-Time PCR

The isolation of total cellular RNA, complementary DNA conversion, and quantitative PCR (qPCR) was performed based on a standard protocol of our laboratory, as described previously.<sup>31</sup> Primer sequences are in Supplementary Table S2. CFX Manager 3.1 (Bio-Rad, Hercules, CA, USA) software was used to analyze the data: the Ct values of each sample were normalized against the geometric mean Ct values of housekeeping genes (*RPS11* and *RPL13*).

### Flow Cytometry

Cells were incubated with anti-HLA class I antibody (W6/32, 311414, Alexa Fluor 647; BioLegend, San Diego, CA, USA) to detect total HLA class I expression. HLA allele-specific monoclonal antibodies were obtained from the IHB Department, LUMC (Supplementary Table S3)<sup>32,33</sup> and were applied

TABLE 1. Clinicopathological Characteristics and Correlation With HLA-A, HLA-B/C, and B2M Expression

Characteristic	All cases				HLA-A (HCA2)				HLA-B/C (HC10)				B2M					
	Cases (%)	Negative Cases (%)	Weak Cases (%)	Positive Cases (%)	P	Negative Cases (%)	Weak Cases (%)	Positive Cases (%)	P	Negative Cases (%)	Weak Cases (%)	Positive Cases (%)	P	Negative Cases (%)	Weak Cases (%)	Positive Cases (%)	P	
Overall	23 (100)	4 (17)	12 (52)	7 (30)		3 (13)	13 (57)	7 (30)		3 (14)	11 (50)	8 (36)		3 (14)	11 (50)	8 (36)		
Sex																		
Male	10 (43)	1 (25)	4 (33)	5 (71)	0.28*	1 (33)	4 (31)	5 (71)	0.25*	1 (33)	3 (27)	5 (63)	0.30*	1 (33)	3 (27)	5 (63)	0.30*	
Female	13 (57)	3 (75)	8 (67)	2 (29)		2 (67)	9 (69)	2 (29)		2 (67)	8 (73)	3 (38)		2 (67)	8 (73)	3 (38)		
Age at diagnosis, y																		
≤60	11 (48)	2 (50)	6 (50)	3 (43)	1.00*	1 (33)	8 (62)	2 (29)	0.42*	1 (33)	7 (64)	3 (38)	0.51*	1 (33)	7 (64)	3 (38)	0.51*	
>60	12 (52)	2 (50)	6 (50)	4 (57)		2 (67)	5 (38)	5 (71)		2 (67)	4 (36)	5 (63)		2 (67)	4 (36)	5 (63)		
Tumor thickness	N = 21	N = 4	N = 11	N = 6		N = 3	N = 13	N = 5		N = 3	N = 11	N = 7		N = 3	N = 11	N = 7		
Median	1.2	1.9	1.4	0.2	0.03†	2.5	1.2	0.2	0.08†	2.5	1.4	0.2	0.046†	2.5	1.4	0.2	0.046†	
[range], mm	[0.1-16.0]	[0.8-16.0]	[0.2-6.0]	[0.1-2.5]		[0.8-16.0]	[0.2-6.0]	[0.1-2.5]		[0.8-16.0]	[0.2-6.0]	[0.1-2.5]		[0.8-16.0]	[0.2-6.0]	[0.1-2.5]		
Tumor LBD	N = 20	N = 4	N = 10	N = 6		N = 3	N = 12	N = 5		N = 3	N = 10	N = 7		N = 3	N = 10	N = 7		
Median	11.0	12.0	12.0	6.5	0.14†	16.0	12.0	6.0	0.06†	16.0	11.0	7.0	0.17†	16.0	11.0	7.0	0.17†	
[range], mm	[2.0-30.0]	[7.0-30.0]	[5.0-20.0]	[2.0-12.0]		[7.0-30.0]	[5.0-20.0]	[2.0-12.0]		[7.0-30.0]	[5.0-20.0]	[2.0-12.0]		[7.0-30.0]	[5.0-20.0]	[2.0-12.0]		
Location																		
Epibulbar	16 (70)	1 (25)	8 (67)	7 (100)	0.03*	1 (33)	9 (69)	6 (86)	0.30*	1 (33)	7 (64)	8 (100)	0.046*	1 (33)	7 (64)	8 (100)	0.046*	
Non-epibulbar	7 (30)	3 (75)	4 (33)	0 (0)		2 (67)	4 (31)	1 (14)		2 (67)	4 (36)	0 (0)		2 (67)	4 (36)	0 (0)		
cTNM																		
T1	16 (70)	1 (25)	8 (67)	7 (100)	0.03*	1 (33)	9 (69)	6 (86)	0.30*	1 (33)	7 (64)	8 (100)	0.046*	1 (33)	7 (64)	8 (100)	0.046*	
T2	7 (30)	3 (75)	4 (33)	0 (0)		2 (67)	4 (31)	1 (14)		2 (67)	4 (36)	0 (0)		2 (67)	4 (36)	0 (0)		
Local recurrence																		
No	13 (57)	4 (100)	7 (58)	2 (29)	0.08*	3 (100)	8 (62)	2 (29)	0.18*	3 (100)	7 (64)	3 (38)	0.20*	3 (100)	7 (64)	3 (38)	0.20*	
Yes	10 (43)	0 (0)	5 (42)	5 (71)		0 (0)	5 (38)	5 (71)		0 (0)	4 (36)	5 (63)		0 (0)	4 (36)	5 (63)		
Distant metastasis																		
No	20 (87)	4 (100)	10 (83)	6 (86)	1.00*	3 (100)	12 (92)	5 (71)	0.53*	3 (100)	10 (91)	7 (88)	1.00*	3 (100)	10 (91)	7 (88)	1.00*	
Yes	3 (13)	0 (0)	2 (17)	1 (14)		0 (0)	1 (8)	2 (29)		0 (0)	1 (9)	1 (13)		0 (0)	1 (9)	1 (13)		
Tumoral PD-L1																		
No	19 (83)	3 (75)	11 (92)	5 (71)	0.46*	2 (66)	12 (92)	5 (71)	0.31*	2 (66)	10 (91)	6 (75)	0.43*	2 (66)	10 (91)	6 (75)	0.43*	
Yes	4 (17)	1 (25)	1 (8)	2 (29)		1 (33)	1 (8)	2 (29)		1 (33)	1 (9)	2 (25)		1 (33)	1 (9)	2 (25)		
Stromal PD-L1																		
No	9 (39)	1 (25)	6 (50)	2 (29)	0.62*	0 (0)	8 (62)	1 (14)	0.051*	0 (0)	6 (55)	3 (38)	0.25*	0 (0)	6 (55)	3 (38)	0.25*	
Yes	14 (61)	3 (75)	6 (50)	5 (71)		3 (100)	5 (38)	6 (86)		3 (100)	5 (45)	5 (63)		3 (100)	5 (45)	5 (63)		
PD-1																		
No	7 (30)	1 (25)	5 (42)	1 (14)	0.59*	1 (66)	5 (38)	1 (14)	0.69*	1 (66)	5 (45)	1 (13)	0.27*	1 (66)	5 (45)	1 (13)	0.27*	
Yes	16 (70)	3 (75)	7 (58)	6 (86)		2 (33)	8 (62)	6 (86)		2 (33)	6 (55)	7 (88)		2 (33)	6 (55)	7 (88)		

Some columns/rows do not add up to 100 due to rounding. HLA scoring system is based on previous studies<sup>23</sup>; intensity was scored as 0 (absent), 1 (weak), 2 (moderate), and 3 (strong), and the percentage of positive cells as 0 (0%), 1 (1% to 5%), 2 (6% to 25%), 3 (26% to 50%), 4 (51% to 75%), and 5 (76% to 100%). A single value was given by adding intensity and percentage and was used to evaluate HLA class I expression in three categories: negative (0 to 2), weak (3 to 6), and positive (7 to 8). cTNM, clinical TNM stage, based on the first occurring conjunctival melanoma; LBD, largest basal diameter.

\* Fisher-Freeman-Halton test.

† Kruskal-Wallis test. Italic P values are ≤ 0.05.

for detecting HLA allele-specific expression. Appropriate FITC (F0315; DAKO) or RPE (R5111; DAKO) secondary antibodies were selected. Cells were collected (10,000 to 50,000 cells per live gate) using Accuri C6 or FACSCalibur cytometer (Beckton Dickinson, Vianen, The Netherlands). Results were analyzed with FlowJo software (V10.0.7; FlowJo LLC, Ashland, OR, USA).

### In Vivo Xenografts

The murine paraffin-embedded tumor sections were obtained from the stored blocks from our previous study.<sup>24</sup> The establishment of the murine model was stated as below. Briefly, nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) IL-2 receptor  $\gamma$  chain null mice were subconjunctivally injected with one of three CM cell lines. Subsequently, cells (CRMM1 and CM2005.1) obtained from established CM tumor xenografts were reinjected into the subconjunctival space of the second set of NOD/SCID mice. This in vivo passaging technique led to lung metastases (9 weeks after subconjunctival injection). The animal experiment was maintained under the guidelines of the Schepens Eye Research Institute, Boston, MA, USA, and experiments were carried out based on the guidelines for the use of animals in research of the Association for Research in Vision and Ophthalmology.

### Statistical Analysis

Data were analyzed with SPSS software version 22.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was defined as  $P \leq 0.05$ . The Fisher-Freeman-Halton test and Kruskal-Wallis test were used. Paired *t*-test (2-tailed) was used to analyze the upregulation of HLA-related genes on IFN- $\gamma$  treatment.

## RESULTS

### Frequent Downregulation of HLA Class I Expression in Primary CM

HCA2 and HC10 recognize HLA-A and HLA-B/C, respectively.<sup>34</sup> B2M is a component of the HLA class I complex.<sup>35</sup> We used triple IF staining to determine the percentage of positively-stained tumor cells and the intensity of staining on formalin-fixed, paraffin-embedded sections of 23 human primary CM (Fig. 1). Expression of HLA-A, HLA-B/C, and B2M correlated strongly (Table 2). Four (17%) cases showed no staining of HLA-A, three (13%) cases showed no expression of HLA-B/C, and three cases (14%) showed no staining of B2M (Table 1). Twelve (52%), 13 (57%), and 11 (50%) cases exhibited weak expression (scores 3 to 6) of HLA-A, HLA-B/C, and B2M, respectively, with the remaining tumors showing a positive expression of B2M, HLA-A, and HLA-B/C. Heterogeneous HLA class I expression was seen in three cases, as presented in Figure 2.

### Patient Characteristics

We correlated expression levels of HLA-A, HLA-B/C, and B2M with clinical and histopathologic data (Table 1; Supplementary Table S4). The mean age of the patients at diagnosis was 62.6 (SD 14.3) years, and 13 of the 23 patients (57%) were women. The median follow-up time was 42 months (range, 3 to 249 months). At the end of the follow-up, 10 (43%) patients had developed local recurrences, 3 (13%) had died from CM metastases, 2 (9%) had died from unknown causes, and 18 (78%) patients were alive. Local recurrence was only related

to tumor largest basal diameter ( $P = 0.046$ ) and not to tumor thickness ( $P = 0.24$ ) or TNM stage ( $P = 0.089$ ). HLA-A, HLA-B/C, or B2M expression was not correlated with largest basal diameter, local recurrence, metastasis formation, or melanoma-related survival. Epibulbar/T1 tumors had a higher HLA class I expression than nonepibulbar/T2 tumors ( $P = 0.03$ , HCA2;  $P = 0.046$ , B2M; Table 1). Thicker tumors showed a lower HLA-A and B2M expression than the thinner tumors ( $P = 0.03$  and  $0.046$ , respectively), with a trend for downregulation of HLA-B/C ( $P = 0.08$ ). Additionally, thick tumors had fewer CD68<sup>+</sup>CD163<sup>+</sup> macrophages ( $r = -0.56$ ,  $P = 0.01$ ). Expression of HLA class I was associated with the number of CD68<sup>+</sup>CD163<sup>+</sup> macrophages in the tumor area ( $P = 0.03$ , HCA2;  $P = 0.07$ , HC10;  $P = 0.02$ , B2M; Table 2) and tended to correlate with CD8<sup>+</sup> T-cell density ( $P = 0.09$ , HCA2;  $P = 0.10$ , HC10;  $P = 0.14$ , B2M; Table 2). These data suggest that macrophages and T cells play a role in stimulating HLA class I expression in CM (Table 2). Expression of PD-L1 on tumor or stromal cells and of PD-1 occurred independently of HLA class I expression (Table 1 and Cao et al.<sup>20</sup>).

### mRNA and Membrane Expression of HLA Class I in CM Cell Lines

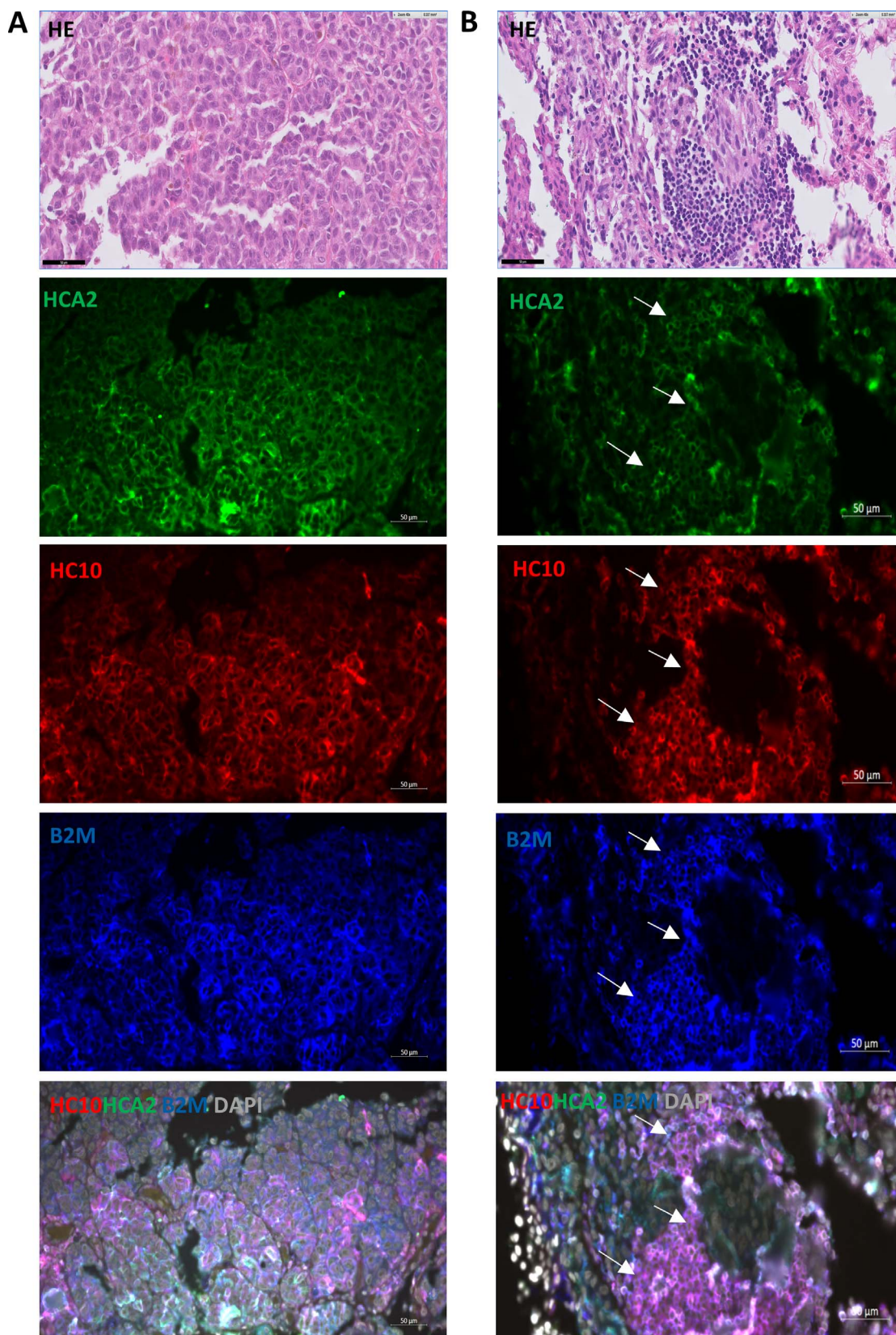
To better understand the regulation of HLA class I expression in CM, we studied three CM cell lines. For this, we first performed HLA-DNA typing (Table 3). CRMM1 genetically carries the HLA-A\*02:01 allele, which is a very attractive genotype, because thus far, most of the clinical T-cell receptor (TCR) cancer therapies are HLA-A\*02:01 restricted.

Next, we determined expression of HLA-A, HLA-B, and B2M on the three cell lines and analyzed the effect of IFN- $\gamma$ , which is a known HLA stimulator. HLA allele-specific antibodies were selected according to the results of the HLA DNA typing. Flow cytometry analysis showed that most genetically determined HLA class I antigens were expressed, except for HLA-A2 and HLA-B44 on CRMM1 and HLA-B44 on CRMM2 (Fig. 3A). IFN- $\gamma$  treatment increased the expression of most alleles, except for HLA-A2 on CRMM1 and HLA-B44 on CRMM2, indicating a hard loss of HLA antigens.

When loss of expression was observed, we set out to determine whether there was a normal activity of the transcriptional regulators (*CIITA*, *IRF1*, and *NLR5*) and genes encoding the peptide-loading machinery (*TAP1* and *TAP2*), using specific primers and qPCR. *NLR5* is essential for HLA class I transcription, whereas *CIITA* and *IRF1* are involved in HLA class I, as well as class II transcriptional regulation. *TAP1* and *TAP2* transport peptides from the cytoplasm to the endoplasmic reticulum and present these peptides to MHC class I molecules.<sup>35</sup> Figure 3B shows that the three CM cell lines presented various baseline mRNA levels of the regulator genes. mRNA expression of not only HLA-A, HLA-B, and B2M, but also of *CIITA*, *IRF1*, *NLR5*, *TAP1*, and *TAP2* was increased after IFN- $\gamma$  stimulation, indicating that these molecules were normally responsive.<sup>36</sup>

### HLA Class I Expression Was Maintained in Animal Xenografts

We previously established a CM metastasis model using in vivo passaging<sup>24</sup> and wondered whether in vivo placement of the tumor cell lines affected HLA expression. Because immunodeficient mice were used, the HLA class I expression on xenografts would not be influenced by local infiltration with T lymphocytes and environmental cytokines, thus showing the tumor's "natural" expression. IHC staining using HCA2, HC10, and B2M antibodies showed that HLA class I expression of CM cells was maintained in the murine



**FIGURE 1.** Immunofluorescence analysis of HLA class I expression. Representative pictures of triple staining show expression on CM cells in one tumor (A) and on T cells in another (B): similar membrane expression patterns are seen for the antibodies HCA2 (HLA-A, green), HC10 (HLA-B/C, red), and B2M (HLA class I, blue), respectively. White arrows indicate T cells surrounding the tumor area. Scale bar denotes 50 μm.

TABLE 2. Correlation Between Thickness, HLA Expression, and Different Types of Infiltrating Immune Cells

	HLA-A (HCA2)	HLA-B/C (HC10)	B2M	CD3	CD3 <sup>+</sup> CD8 <sup>+</sup>	CD3 <sup>+</sup> CD8 <sup>-</sup>	CD3 <sup>+</sup> CD8 <sup>-</sup> Foxp3 <sup>-</sup>	CD3 <sup>+</sup> CD8 <sup>-</sup> Foxp3 <sup>+</sup>	CD68	CD68 <sup>+</sup> CD163 <sup>+</sup>
Thickness										
<i>r</i>	-0.49	-0.50	-0.53	-0.35	-0.39	-0.32	-0.33	-0.27	-0.35	-0.50
<i>P</i>	0.02	0.02	0.01	0.12	0.08	0.16	0.15	0.23	0.12	0.02
HLA-A (HCA2)										
<i>r</i>	-	0.94	0.97	0.25	0.36	0.17	0.18	0.22	0.40	0.45
<i>P</i>	-	<0.01	<0.01	0.25	0.09	0.45	0.41	0.31	0.06	0.03
HLA-B/C (HC10)										
<i>r</i>	-	-	0.97	0.29	0.36	0.18	0.15	0.33	0.30	0.38
<i>P</i>	-	-	<0.01	0.18	0.10	0.40	0.51	0.13	0.16	0.07
B2M										
<i>r</i>	-	-	-	0.31	0.33	0.24	0.25	0.28	0.43	0.49
<i>P</i>	-	-	-	0.17	0.14	0.27	0.25	0.21	0.04	0.02

*P* ≤ 0.05 are in italics. The staining and scoring method is previously described.<sup>16</sup> *r*, 2-tailed Spearman correlation coefficient, with 23 observations.

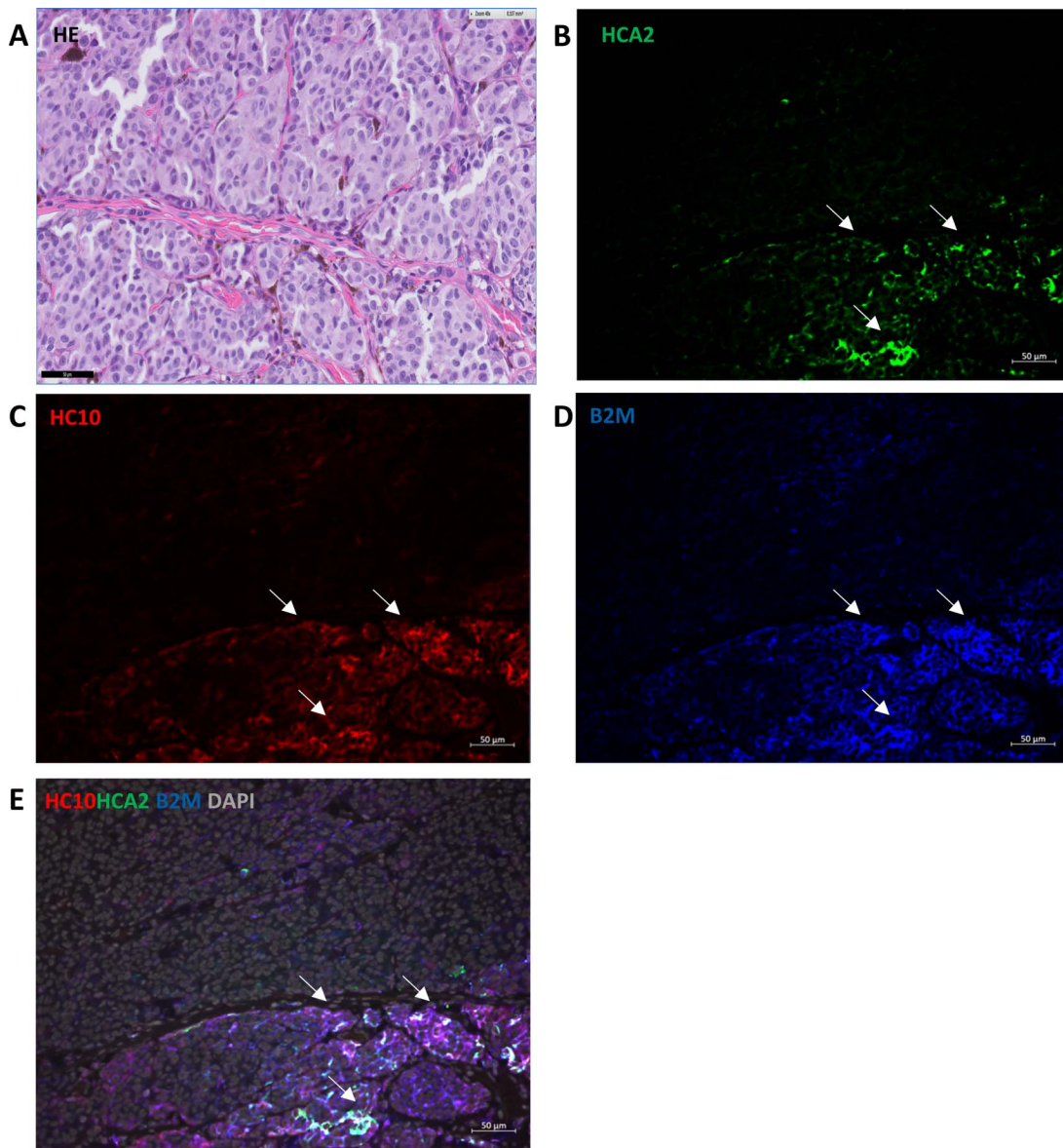


FIGURE 2. Heterogeneous expression of HLA class I antigens in a CM. Representative IF staining shows membranous HCA2 (HLA-A, green), HC10 (HLA-B/C, red), and B2M (HLA class I, blue) expression only in the lower part of the tumor area. White arrows indicate areas of positive expression. Scale bar denotes 50 μm.

TABLE 3. Results of HLA Typing in Three CM Cell Lines

Cell Line	HLA Alleles
CRMM1	A*02:01/02:07/02:09+, A*02:01/02:07/02:09+, B*44:03/44:07/44:13+, B*44:03/44:07/44:13+, C*04:01/04:07/04:09N+, C*04:01/04:07/04:09N+, DRB1*07:01/07:03/07:04+, DRB1*07:01/07:03/07:04+ DQB1*02:01/02:02/02:04+, DQB1*02:01/02:02/02:04+
CRMM2	A*03:01/03:37/03:03N+, A*30:01/30:14L/30:15+, B*40:01/40:22N/40:54+, B*44:02/44:23N/44:27+, C*03:04/03:24/03:35+, C*05:01/05:05/05:07N+, DRB1*11:01/11:04/11:02+, DRB1*13:01/13:02/13:05+, DQB1*06:03/06:07/06:14+, DQB1*06:03/06:07/06:14+
CM2005.1	A*11:01/11:05/11:07+, A*24:02/24:06/24:09N+, B*14:02/14:09/14:16+, B*35:01/35:41/35:07+, C*02:02/02:09/02:11+, C*04:01/04:09N/04:28+, DRB1*01:01/01:07/01:04+, DRB1*14:54/14:01/14:07+, DQB1*05:01/05:03/05:02, DQB1*05:03/05:02/05:07+

models in the same manner as on the cell lines in vitro (Fig. 4; Table 4). There seemed a trend toward a decrease of HLA class I expression during tumor progression (primary in vivo passaged metastasis; Fig. 4); however, HLA class I was expressed at all stages.

## DISCUSSION

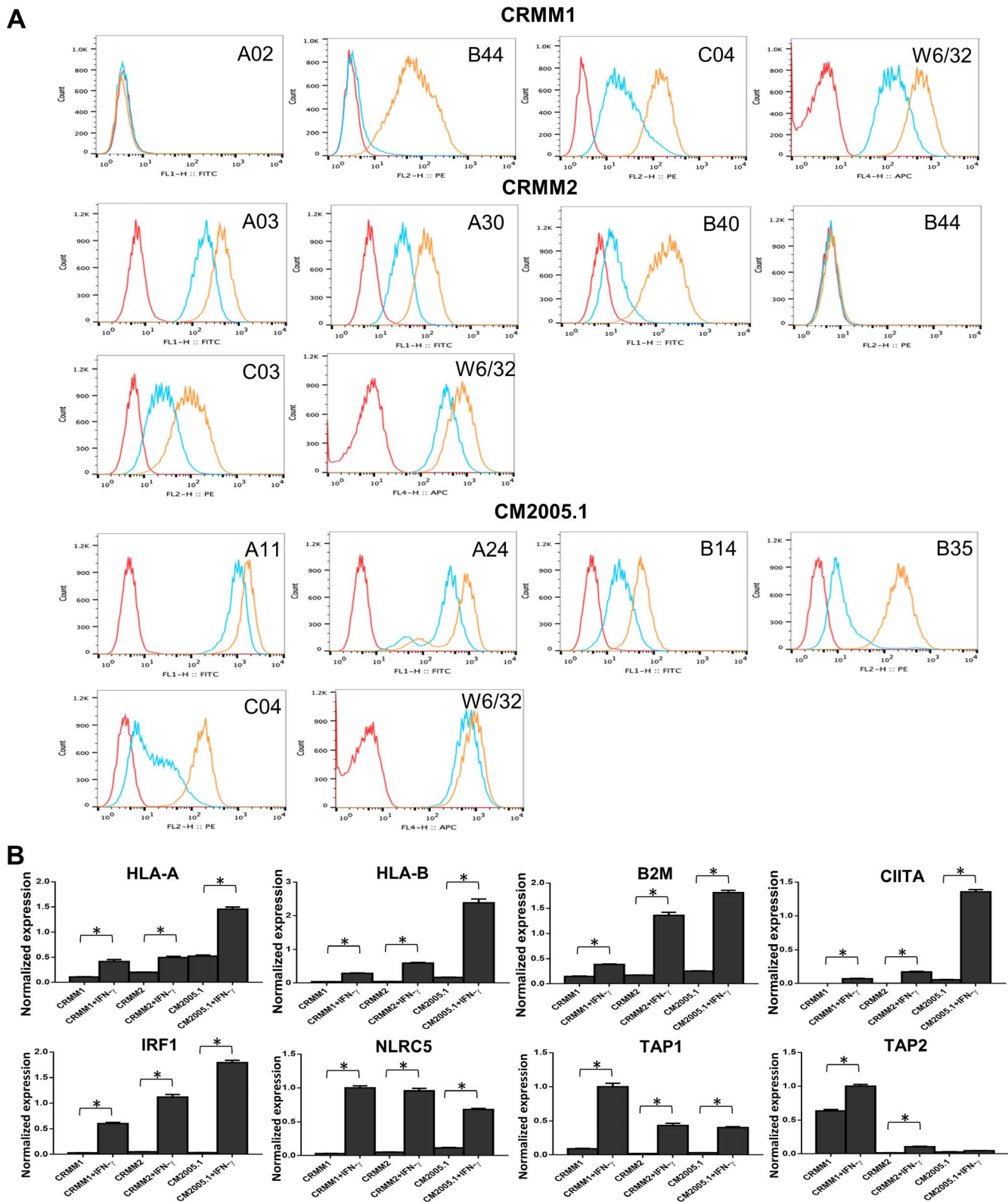
We investigated expression of HLA class I molecules in primary CM and compared HLA class I expression with clinical parameters. In only a third of CM did we observe a positive expression of HLA-A (~30%), HLA-B/C (~30%), and B2M (~36%). The presence of a negative or weak HLA class I antigen expression in approximately 70% of CM is similar to that of bladder cancer, but higher than of cutaneous melanoma and intrahepatic cholangiocarcinoma.<sup>37,38</sup> Several factors may contribute to this observed lack of HLA class I antigen expression: mutations or loss of heterozygosity (LOH) in chromosomes 6 or 15 (where B2M is located), or prior immune selection, leaving only HLA class I negative tumor cells.<sup>39</sup> We did indeed find some tumors that lacked B2M, but do not have information on mutations in this gene. An absent or low expression of HLA class I has been associated with a worse survival in patients with advanced cutaneous melanoma, cervical cancer, and rectal cancer.<sup>26,40,41</sup> We did not find a correlation between HLA class I expression and melanoma-related survival in primary CM. However, we did notice that an aberrant HLA class I expression was significantly correlated with one of the known prognostic factors known prognostic factors (i.e. tumor thickness), which is concordant with previous investigations in cervical cancer and cutaneous melanoma.<sup>42,43</sup> The association of HLA class I expression with M2 macrophages and CD8<sup>+</sup> T cells suggests a connection of IFN- $\gamma$  with HLA class I expression in the tumor. The cytokine IFN- $\gamma$ , mainly secreted by CD4<sup>+</sup> Th1, CD8<sup>+</sup> T lymphocytes, macrophages, and natural killer (NK) cells,<sup>44</sup> is known to be a key modulator of HLA expression. The data on expression and regulation of most of the HLA antigens on three unique CM cell lines, using HLA DNA typing, qPCR, and flow cytometry, indeed confirmed this theory: after IFN- $\gamma$  treatment, mRNA expression levels of CIITA, IRF1, NLRC5, TAP1, and TAP2 were increased, together with HLA class I molecules. Our finding is similar to previous investigations using uveal melanoma cell lines.<sup>36</sup> Interestingly, the flow cytometry data showed that not all alleles were expressed on the cell surface:

although the HLA-A\*02:01 and HLA-B\*44:02 alleles were detected by HLA DNA typing, they were not expressed on the cell surface of CRMM1 and CRMM2, respectively. This result may be explained by allele-specific mutations or by post translational instability due to a missing disulfide bridge.<sup>45</sup> This remains to be investigated.

To further test our theory that the presence of infiltrating immune cells plays a role in HLA expression, we looked at murine xenografts that were obtained by injection of three CM cell lines into the subconjunctiva of NOD/SCID-mice (with impaired T, B, and NK cells). We noticed that primary tumors of the xenograft showed a similarly low HLA class I expression as the cell lines (Figs. 3, 4). In addition, a trend toward gradually decreased HLA class I expression in the xenografts after in vivo passaging and on a lung metastasis was seen (Fig. 4). A lack of IFN- $\gamma$  production may be responsible for this finding, as lack of lymphocytic infiltration leads to low IFN- $\gamma$  production. In a study on uveal melanoma, HLA class I expression was similarly weaker in SCID murine xenografts than in their original freshly obtained tumors, which had shown high HLA class I expression.<sup>35</sup> However, another study shows an opposite outcome: in a mouse fibrosarcoma model, a HLA class I negative tumor clone led to HLA class I-positive spontaneous lung metastasis in immunodeficient mice that lacked T cells.<sup>46</sup>

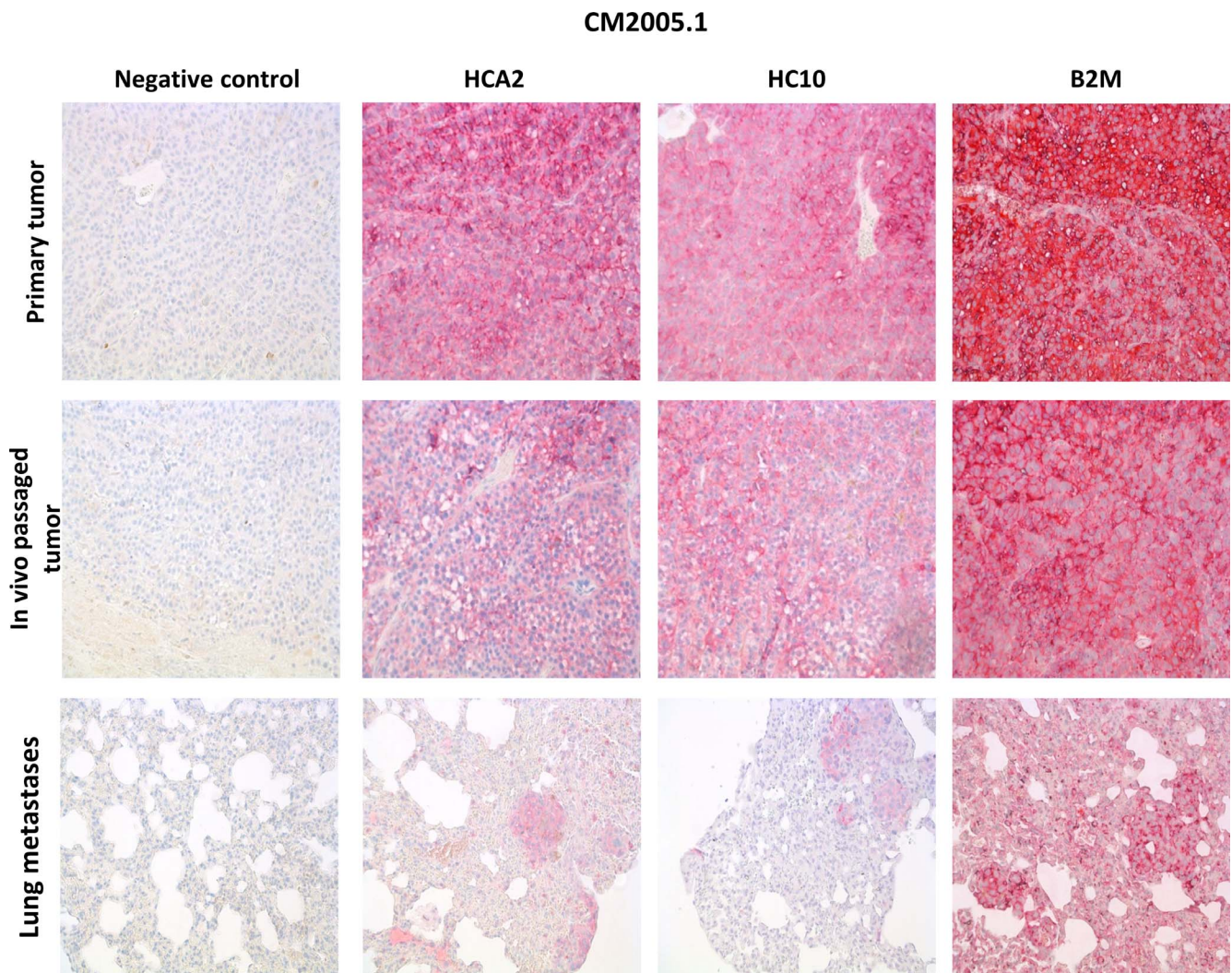
TCR-based immunotherapies against cutaneous melanoma have also been studied extensively in the last decade, using CD8<sup>+</sup> T cells, which recognize the tumor-associated antigens of, among others, tyrosinase, MAGE-A1 (melanoma-associated antigen A1), MART-1 (melanoma antigen recognized by T cells), and glycoprotein 100 (gp100).<sup>47</sup> In this context, a proper HLA class I expression is needed because metastatic cutaneous melanoma with a low HLA class I expression was resistant to T cell-based immunotherapy and progressed.<sup>48,49</sup> A recent study shows that mutations in *B2M* and *IFN* genes contribute to the recurrences of four metastatic cutaneous melanoma patients after anti-PD-1 (pembrolizumab) therapy.<sup>50</sup> These findings strongly suggest that both “soft” (reversible) and “hard” (irreversible) types of absence of HLA class I expression can influence the efficacy of T cell-mediated immunotherapy. Our flow cytometry tests showed that some of the HLA antigens were not expressed, even in the presence of IFN- $\gamma$ , whereas other antigens showed a higher expression following exposure to IFN- $\gamma$ . This indicates that both the soft and the hard type of downregulation of HLA expression occurs in CM. In our cohort, PD-L1/PD-1 was not expressed on the same tumors as the HLA class I antigens. However, it is possible that HLA class I has predictive value concerning a tumor’s response to immune checkpoint blockade therapy. Recent studies have shown that PD-L1 positivity is not a perfect predictor for anti-PD-L1/PD-1 treatment, because the overall response rate using PD-L1/PD-1 inhibitors is 48% for patients with PD-L1-positive tumors, contrary to 15% in those with PD-L1-negative tumors.<sup>51</sup> That HLA antigens are important was recently shown: a greater diversity in HLA class I genotype prolongs the patients’ survival after immune blockade treatment, possibly because the heterozygous HLA class I molecules present more tumor-specific mutant antigens (neoantigens) to CD8<sup>+</sup> T cells.<sup>52</sup>

Although our study contains only a small cohort from one medical center, to the best of our knowledge, this is the first comprehensive view of HLA class I expression of CM in vivo and in vitro. The downregulation of HLA class I may contribute to the mechanisms of immune escape, and limit effective T-cell immune responses. When designing the immune-therapeutic strategies to treat CM, one should evaluate tumor cell HLA class



**FIGURE 3.** IFN- $\gamma$  upregulates protein and gene expression of HLA class I and increases mRNA levels of several HLA regulators in CM cell lines. Cells were treated with or without IFN- $\gamma$  (100 IU/mL) exposure for 48 hours. (A) Representative histograms show the surface expression of HLA class I (W6/32) and specific HLA allele antigens of CRMM1, CRMM2, and CM2005.1 cell lines. Histograms with red, blue and brown line represent unstained, baseline expression, and expression after IFN- $\gamma$  stimulation, respectively. (B) The mRNA expression of HLA genes (*HLA-A*, *HLA-B*, *B2M*), HLA transcriptional regulators (*CIITA*, *IRF1*, *NLRC5*), and genes encoding the peptide-loading machinery (*TAP1*, *TAP2*) were variably increased after IFN- $\gamma$  stimulation. \* $P < 0.05$ , using paired *t*-test.





**FIGURE 4.** Murine xenografts maintain their HLA class I expression. Representative images present the membranous expression of HLA-A (HCA2), HLA-B/C (HC10), and B2M in a CM2005.1 murine model. Cells from cell line CM2005.1 were first placed subconjunctivally, transferred to the subconjunctival space of a second set of mice and spread systemically, giving rise to lung metastases. Images were taken at  $\times 200$  magnification.

**TABLE 4.** HLA Class I Expression in CM Murine Xenografts

Material	Staining Intensity/% Positive Tumor Cells		
	HLA-A (HCA2)	HLA-B/C (HC10)	B2M
CRMM1			
Primary tumor*	2/2 (Weak)	2/2 (Weak)	3/3 (Weak)
In vivo-passaged tumor†	0/0 (Negative)	2/1 (Weak)	2/2 (Weak)
Lung metastasis‡	0/0 (Negative)	0/0 (Negative)	0/0 (Negative)
CRMM2			
Primary tumor*	2/3 (Weak)	1/2 (Weak)	3/4 (Positive)
CM2005.1			
Primary tumor*	3/3 (Weak)	3/2 (Weak)	3/5 (Positive)
In vivo-passaged tumor†	3/1 (Weak)	3/2 (Weak)	3/4 (Positive)
Lung metastasis‡	1/3 (Weak)	2/3 (Weak)	2/3 (Weak)

The staining intensity scoring system is described in the Methods.

\*  $n = 3$ .

†  $n = 2$ .

‡  $n = 1$ .

I expression and potentially enhance it to improve the efficacy of T cell-based immunotherapy.

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