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Supplementary Materials for

Colocalization of Inflammatory Response with B7-H1 Expression in Human Melanocytic Lesions Supports an Adaptive Resistance Mechanism of Immune Escape

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The PDF file includes:

Supplementary Materials and Methods

Immunohistochemistry (IHC)

We showed previously that B7-H1 protein could be detected on the cell surface of various cancers by IHC on frozen tissue sections with the mAb 5H1 [5]. For archived formalin-fixed paraffin embedded (FFPE) slides, however, an additional re-naturation step is required for this mAb [27]. We compared the staining pattern and sensitivity of these two approaches. To do so, immunodeficient NOD-SCID IL2Rgamma^{null} mice were inoculated with B7-H1-transfected human 624-mel cultured melanoma cells to induce progressively growing tumors. Tumor nodules were harvested and bisected: half of the material was embedded in OCT compound and frozen, and the other half was fixed in formalin and embedded in paraffin. As shown in Figure S4, similar membrane staining patterns were observed in both frozen and FFPE sections. Although the sensitivity of the methods is generally comparable, we observed slightly less intensity for staining in FFPE than frozen sections.

For the purpose of this retrospective study, archival FFPE specimens were cut into 5μm sections and mounted on glass slides. IHC for B7-H1 was performed using a murine anti-human B7-H1 mAb (clone 5H1, isotype mouse IgG1) [5] at a concentration of 2 ug/ml according to a standard protocol (See Supplementary Material). After deparaffinization, antigen retrieval was performed using a Tris-EDTA buffer, pH 9.0 at 120^{o}C for 10 minutes in a Decloaking Chamber (Biocare Medical). Endogenous peroxidase, biotin and proteins were blocked (CAS system K1500, DAKO; Avidin/biotin Blocking Kit, SP-2001, Vector Laboratories; Serotec Block ACE). The primary antibody was applied and allowed to incubate at 4^{o}C for 20 hours. Secondary antibody (biotinylated anti-mouse IgG1, 553441 BD) at a concentration of 1 ug/ml was applied for 30 minutes at room temperature. The signal was then developed with amplification according to the manufacturer's protocol (CAS system K1500, DAKO). Sections were counterstained with hematoxylin, dehydrated in graded ethanol and cleared in xylene, and a coverslip was applied.

Positive control specimens for B7-H1 IHC were created by transfecting cultured human melanoma 624-mel cells [5], which do not constitutively express B7-H1, with a recombinant plasmid encoding full length human B7-H1. Cell surface B7-H1 expression on cultured cells

was confirmed with flow cytometry. NOD-SCID IL2Rgamma^{null} mice were inoculated with the B7-H1-transfected 624-mel cells. Xenografted tumors were harvested and preserved as both fresh-frozen and paraffin-embedded specimens. A B7-H1 fusion protein was used to competitively block binding of the 5H1 mAb to tissue sections, confirming the specificity of 5H1 (Figure S4C). Animal studies were approved by the Institutional Review Board at Johns Hopkins University and adhered to the NIH Guidelines for the Care and Use of Laboratory Animals.

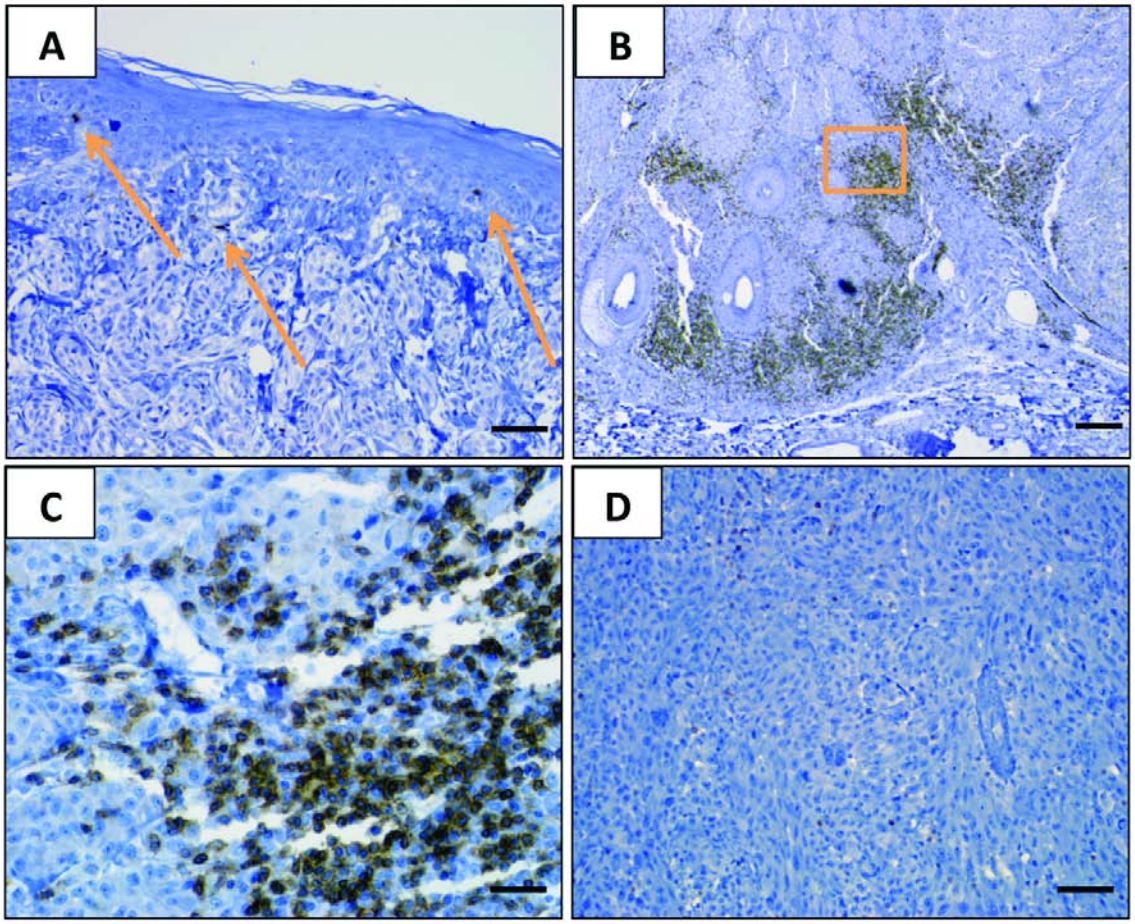
IHC for B7-H1 was also performed on select slides using a rabbit polyclonal Ab to B7-H1 designated 4059 (ProSci) as previously described [50]. When we compared the staining pattern of mAb 5H1 and polyclonal Ab 5049 on FFPE specimens, 5H1 staining showed a clear membranous staining pattern, whereas Ab 4059 demonstrated diffuse cytoplasmic staining (Figure S5). Non-specific isotype controls using purified mouse IgG1 or rabbit IgG1 were performed for each case stained with either the 5H1 mAb or 4059 polyclonal Ab, respectively. Western blot analysis indicated that both antibodies specifically bound a purified human recombinant B7-H1/IgG fusion protein (Figure S6A), but in lysates of melanoma cells the Ab 4059 detected multiple proteins in addition to a 50 kDa protein corresponding to the expected mass of glycosylated B7-H1 (Figure S6B). Therefore, mAb 5H1 was used for all IHC experiments in the current study.

Laser capture microdissection (LCM) and quantitative RT-PCR for IFN-γ

Formalin-fixed human melanoma biopsy specimens and cultured cell pellets were embedded in paraffin blocks. Cultured cells included the human melanoma cell line 1359-mel, and isolated normal donor CD3+ T cells (Pan T Cell Isolation Kit II, Miltenyi Biotech) that were activated for 48 hr by anti-CD3 and -CD28 mAbs (1 ug/ml each). Fresh normal donor peripheral blood mononuclear cells (PBMCs), including lymphocytes and monocytes, were isolated from leukapheresis specimens by density centrifugation. Cells in suspension were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), resuspended in 50-100 ul of PBS, dispensed atop solidified 2% agarose plugs in microcentrifuge tubes, and spun for 5-7 min at 1500 rpm. Formalin was slowly added to each pellet, and samples were allowed to sit at room temperature for at least 24 hr before embedding in paraffin blocks.

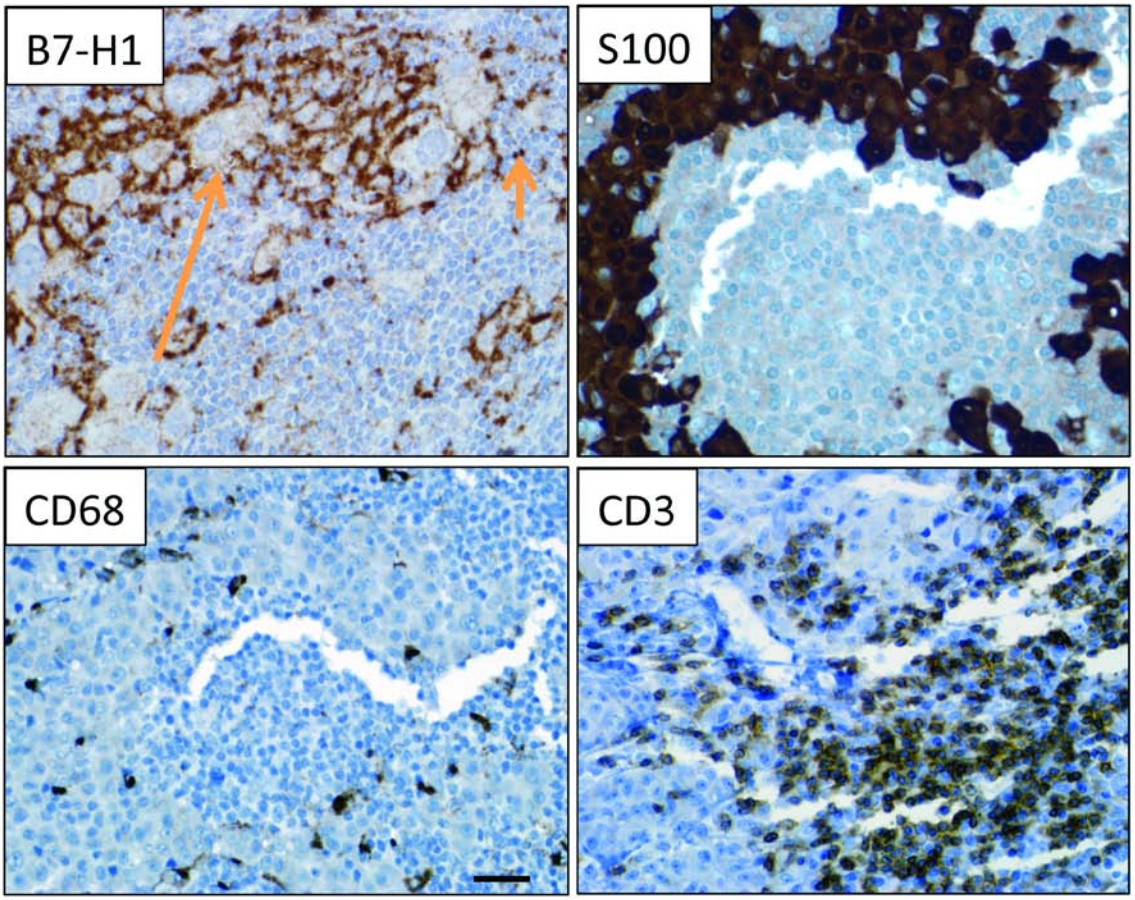
Specimens were cut into 7μm sections on Arcturus PEN membrane glass slides (Applied Biosystems). Slides were de-paraffinized and stained with a modified hematoxylin and eosin (H&E) protocol. LCM was performed using a Leica LMD6000 Laser Capture Micro-dissection microscope. In melanoma biopsies that were B7-H1(-), central areas containing non-necrotic tumor cells were dissected with intent to avoid surrounding stromal elements. In tumors that were B7-H1+, areas of expression were identified by IHC on neighboring sections and tumor cells were excised along with associated infiltrating immune cells (Figure S7). Total RNA was isolated from LCM specimens using the High Pure RNA Paraffin Kit (Roche) with the following modifications: residual PEN membrane pieces were removed by centrifugation after adding binding buffer, and a DNAse incubation step was added to remove contaminating genomic DNA. Total RNA concentration was determined using a NanoDrop spectrophotometer. Fifty ng of total RNA from each specimen was reverse-transcribed in a 20 ul reaction volume using qScript cDNA SuperMix (Quanta Biosciences) per protocol. One ul from each RT reaction was combined with 5ul TaqMan Universal Master Mix II (Applied Biosystems), 3.5ul Molecular Grade Water, and 0.5 ul of primer/probe preparations specific for either human IFN-γ (Applied Biosystems), or CD45 (PTPRC) to standardize to myeloid-derived cells (Applied Biosystems). PCR reactions were run in triplicate on 384-well plates using a 7900 HT Fast Real Time PCR system (Applied Biosystems). Cycle thresholds were determined using a manual cutoff of 0.04. Triplicate C_T averages and standard errors of the mean are reported.

Supplementary Figure 1

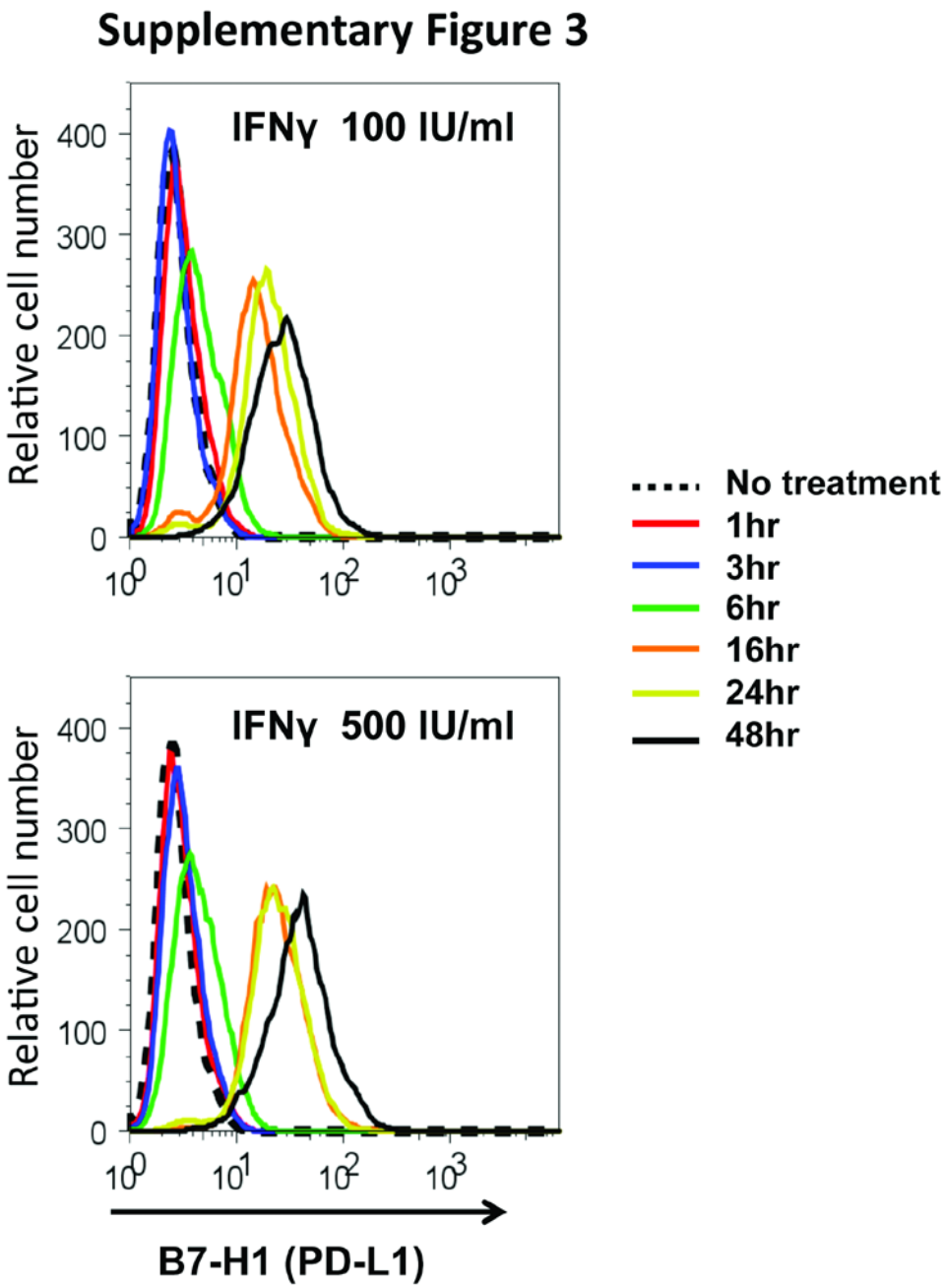


Supplementary Figure 1. Geographic patterns of $CD3^+$ TILs corresponding to the patterns of B7-H1 expression in cases shown in Fig. 1. (A) Rare CD3 TILs identified by IHC in a benign nevus that was B7-H1(-), indicated by arrows. 200x original magnification (size bar 50 um). (B) A 'moderate' infiltrate of CD3+ TILs at the advancing edge of an invasive primary melanoma, nodular histologic subtype, associated with B7-H1+ tumor cells (see Figure 1B). 40x original magnification (size bar 200 um). (C) 400x original magnification (20 um size bar) of the boxed area shown in panel B. **(D)** Only singular CD3+ TILs were observed, predominantly in perivascular areas and not infiltrating among tumor cells, in a diffusely B7-H1+ melanoma metastasis. 200x original magnification (size bar 50 um).

Supplementary Figure 2

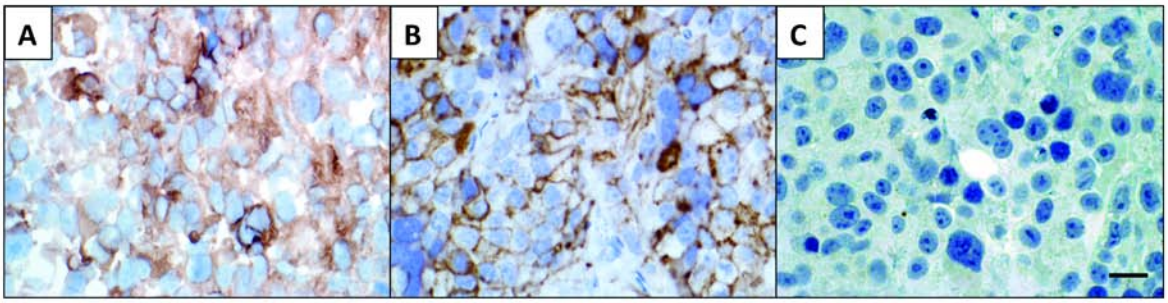


Supplementary Figure 2. Immunohistochemical characterization of cell types and architecture at the interface of B7-H1 expression and immune infiltrates in a melanoma lesion. All panels 400x original magnification (size bar 20 um). B7-H1 expression is present on the surface of tumor cells (long arrow) and infiltrating lymphocytes (short arrow). S100 immunostaining highlights melanoma cells aggregating in nests. CD68 highlights macrophages/histiocytes accompanying TILs. CD3+ TILs comprise the majority of cells in the immune infiltrate. Lymphocytes are distinguished from melanoma cells by much smaller size and nuclear homogeneity. The nesting architecture of S100+ melanoma cells is distinguished from the singular arrangement of CD3+ lymphocytes and CD68+ histiocytes.



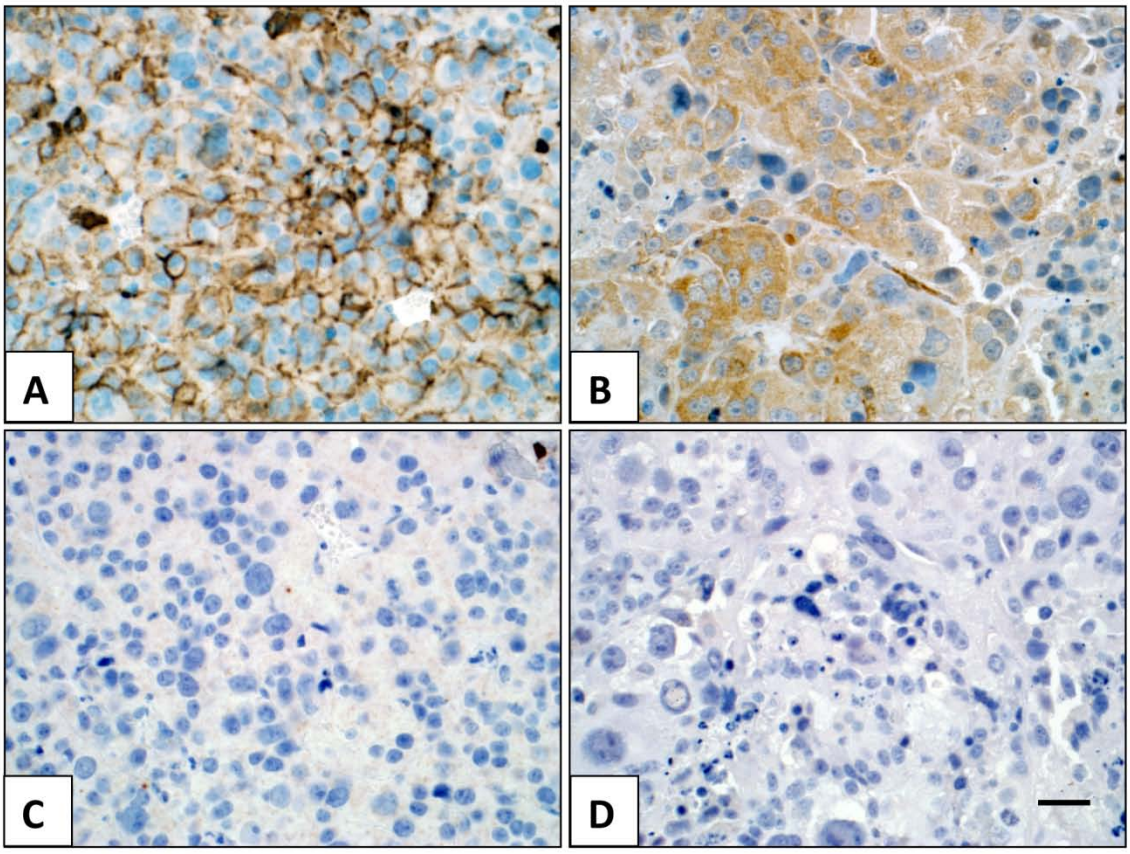
Supplementary Figure 3. Kinetics of B7-H1 induction by IFN-γ in cultured human melanoma cells. The cultured cell line 1102-mel was incubated in the presence of 0, 100, or 500 international units (IU)/ml of IFN-γ (Biogen) for the indicated time periods. Then, adherent cells were harvested with trypsin at timed intervals, and B7-H1 expression was determined by flow cytometry with the anti-human B7-H1 mAb MIH1 (eBioscience Inc.). These results show induction of B7-H1 expression as early as 6 hr after IFN-γ exposure in vitro.

Supplementary Figure 4



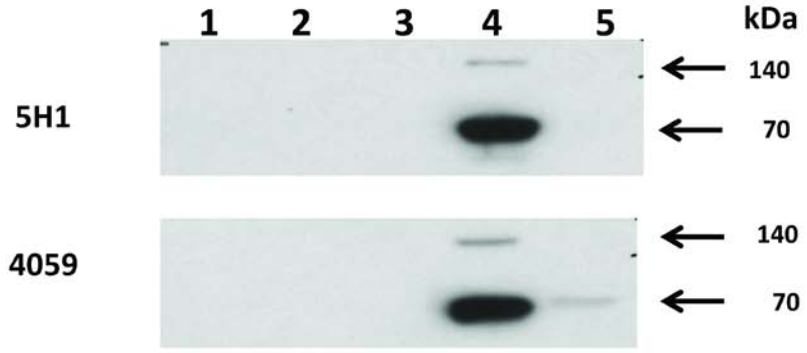
Supplementary Figure 4. Comparison of B7-H1 detection in fresh and FFPE tissues using mAb 5H1. NOD-SCID IL2Rgamma^{null} mice were inoculated with B7-H1 transfected 624-mel cells. Tumor was harvested and bisected: half was embedded in OCT compound and frozen, and half was formalin-fixed and paraffin-embedded. IHC for B7-H1 using the 5H1 mAb shows a similar expression pattern in both (A) frozen and (B) paraffin-embedded tissues. Prevalence of cell surface staining and staining intensity in the paraffin-embedded section is slightly less than in the frozen specimen. (C) B7-H1 fusion protein was used to competitively block binding of the 5H1 mAb, confirming specificity for B7-H1. 400x original magnification (size bar 20 um), all panels.

Supplementary Figure 5

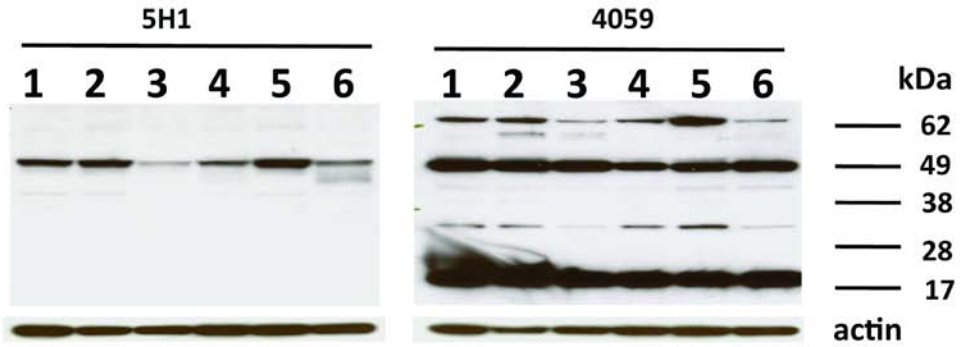


Supplementary Figure 5. Comparison of B7-H1 detection by the mAb 5H1 versus the polyclonal antibody 4059. IHC for B7-H1 was performed using mAb 5H1 or polyclonal Ab 4059, as described in Methods, on FFPE specimens of xenografted B7-H1 transfected 624-mel. (A) Predominantly membranous staining was detected with the 5H1 mAb. (B) Ab 4059 showed diffuse cytoplasmic staining with rare, focal membranous staining. Isotype staining controls using (C) purified mouse IgG1 or (D) rabbit IgG1 were performed for 5H1 or 4059, respectively. 400x original magnification (size bar 20 um), all panels. Evaluation of cell surface expression by IHC mandates employing a mAb such as 5H1, which is capable of detecting cell surface B7-H1 in FFPE specimens. The 5H1 mAb cannot stain FFPE sections without a protein re-naturing process, indicating that 5H1 binds a conformation-dependent epitope on B7-H1.

Supplementary Figure 6A

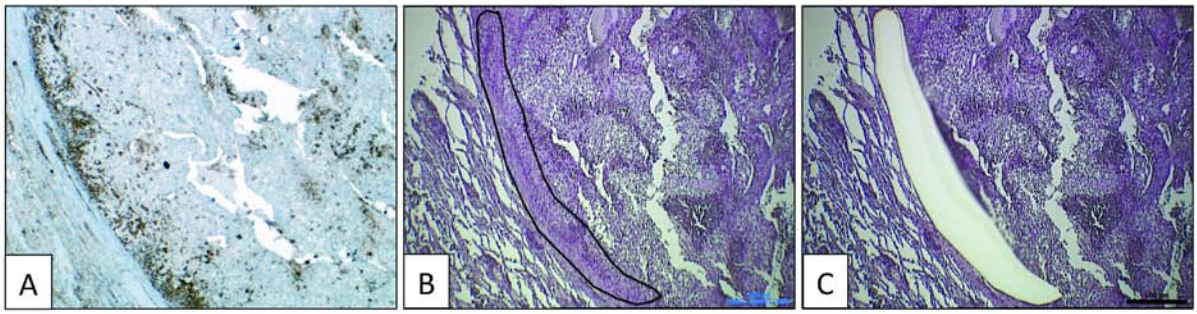


Supplementary Figure 6B



Supplementary Figure 6. Comparative specificities of anti-B7-H1 mAb 5H1 and polyclonal antibody 4059 by Western blotting. (A) Both antibodies are specific for a 70 kDa recombinant human B7-H1/IgG fusion protein. Lane 1, human IgG1; lane 2, hB7.1/IgG; lane 3, hB7.2/IgG; lane 4, hB7-H1/IgG; lane 5, hB7-DC/IgG. (B) In melanoma cell lysates, mAb 5H1 specifically detects a 50 kDa band consistent with glycosylated B7-H1, whereas polyclonal Ab 4059 detects additional proteins of various molecular weights, which were not further characterized. Lane 1, cultured human melanoma 537-mel, whole cell lysate; lane 2, membrane fraction of lysate; lane 3, cytosolic/nuclear fraction of lysate; lanes 4-6, same as lanes 1-3, performed on 537-mel cells cultured with IFN-γ 500 IU/ml x 72 hr.

Supplementary Figure 7



Supplementary Figure 7. B7-H1^{+} tumor and associated TILs sampled by laser capture microdissection. In tumors that were B7-H1+, (A) areas of expression were identified by IHC, and on neighboring sections, areas of tumor cells along with associated infiltrating immune cells were demarcated (B) and excised (C). Size bar 500 um for all panels.

Supplementary Table 1. B7-H1 expression by melanocytes and infiltrating immune cells in 54 in situ and invasive primary melanomas does not correlate with pT or TNM stage

	B7-H1(-)	**B7-H1(+)**	**Adjusted inflam. Score <5**	**Adjusted inflam. Score ≥5**
pT Stage				
pis (n=11)	5	6	5	6
1a (n=11)	6	5	3	8
2a (n=3)	2	1	1	2
2b (n=1)	1	0	0	1
3a (n=8)	6	2	6	2
3b (n=4)	3	1	3	1
4a (n=10)	6	4	3	7
4b (n=6)	6	0	4	2
pT1 and pT2 (n=15)	9	6	4	11
pT3 and pT4 (n=28)	21	7	16	12
TNM stage				
0 (n=11)	5	6	5	6
IA (n=11)	6	5	3	8
IB (n=3)	2	1	1	2
IIA (n=7)	6	1	5	2
IIB (n=9)	7	2	6	3
IIIA (n=2)	1	1	1	1
IIIB (n=5)	4	1	2	3
IIIC (n=4)	2	2	1	3
IV (n=2)	2	0	1	1