Checkpoint inhibition enhances cell contacts between CD4+ T cells and Hodgkin-Reed-Sternberg cells of classic Hodgkin lymphoma

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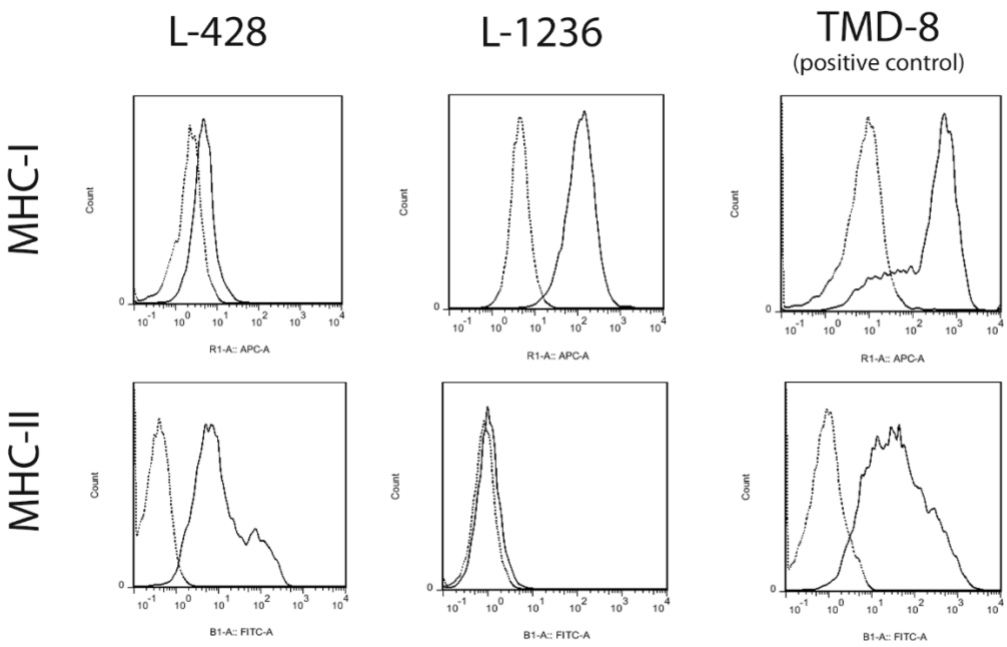
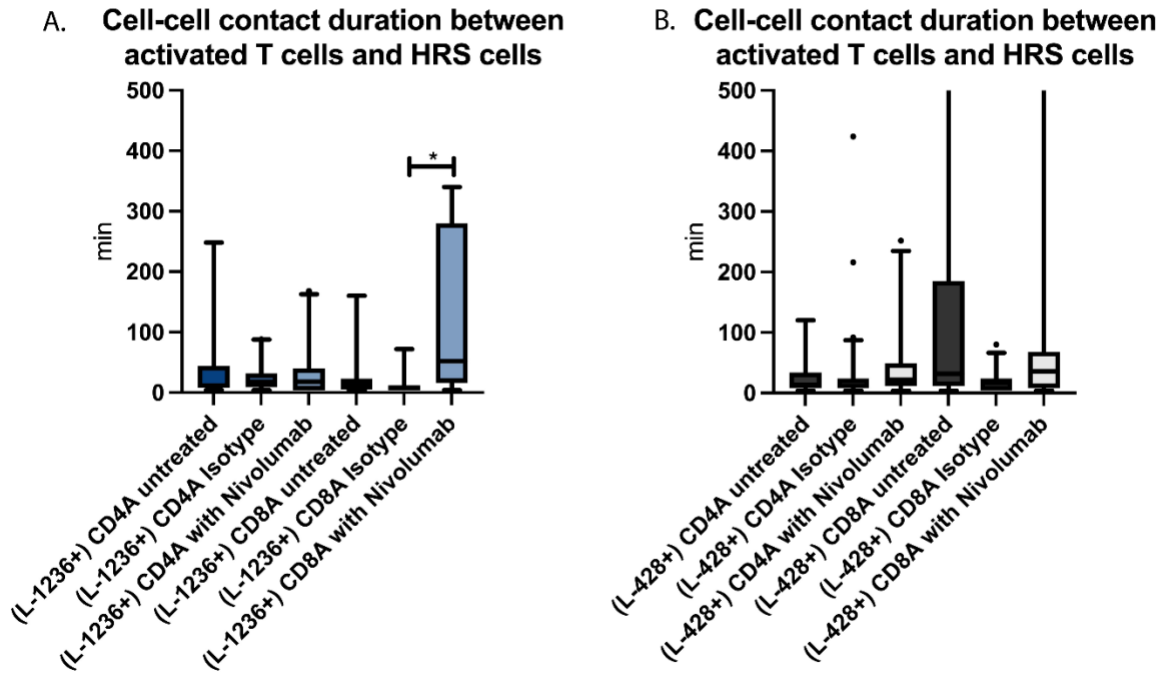
Supplementary Figures and Methods

Supplementary Figures

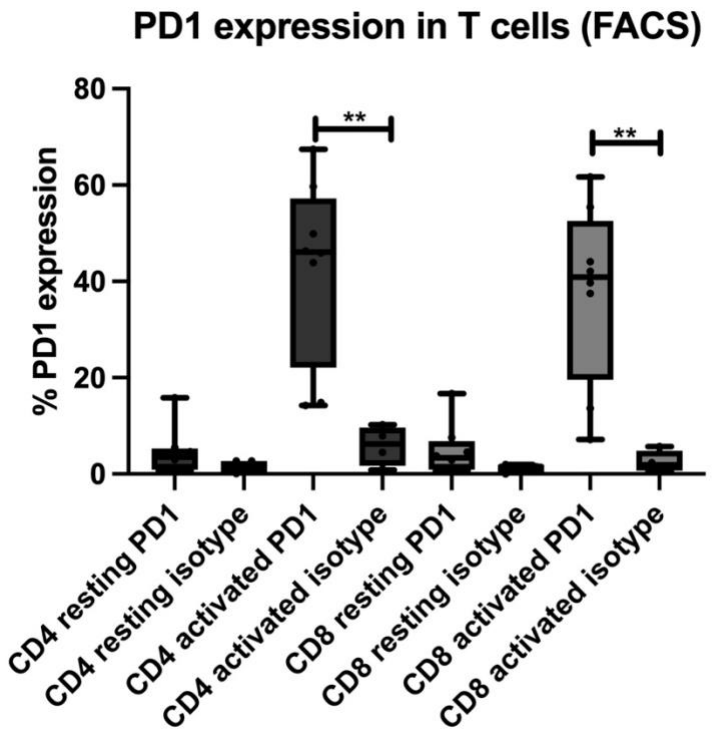


Suppl. Figure 1. MHC-I and –II expression in classic Hodgkin lymphoma cell lines L-428 and L-1236
Upper row: MHC-I expression as determined by FACS (bold line) versus isotype control (dotted line). The diffuse large B-cell lymphoma cell line TMD-8 was used as positive control.
Bottom row: MHC-II expression as determined by FACS (bold line) versus isotype control (dotted line). The diffuse large B-cell lymphoma cell line TMD-8 was used as positive control.

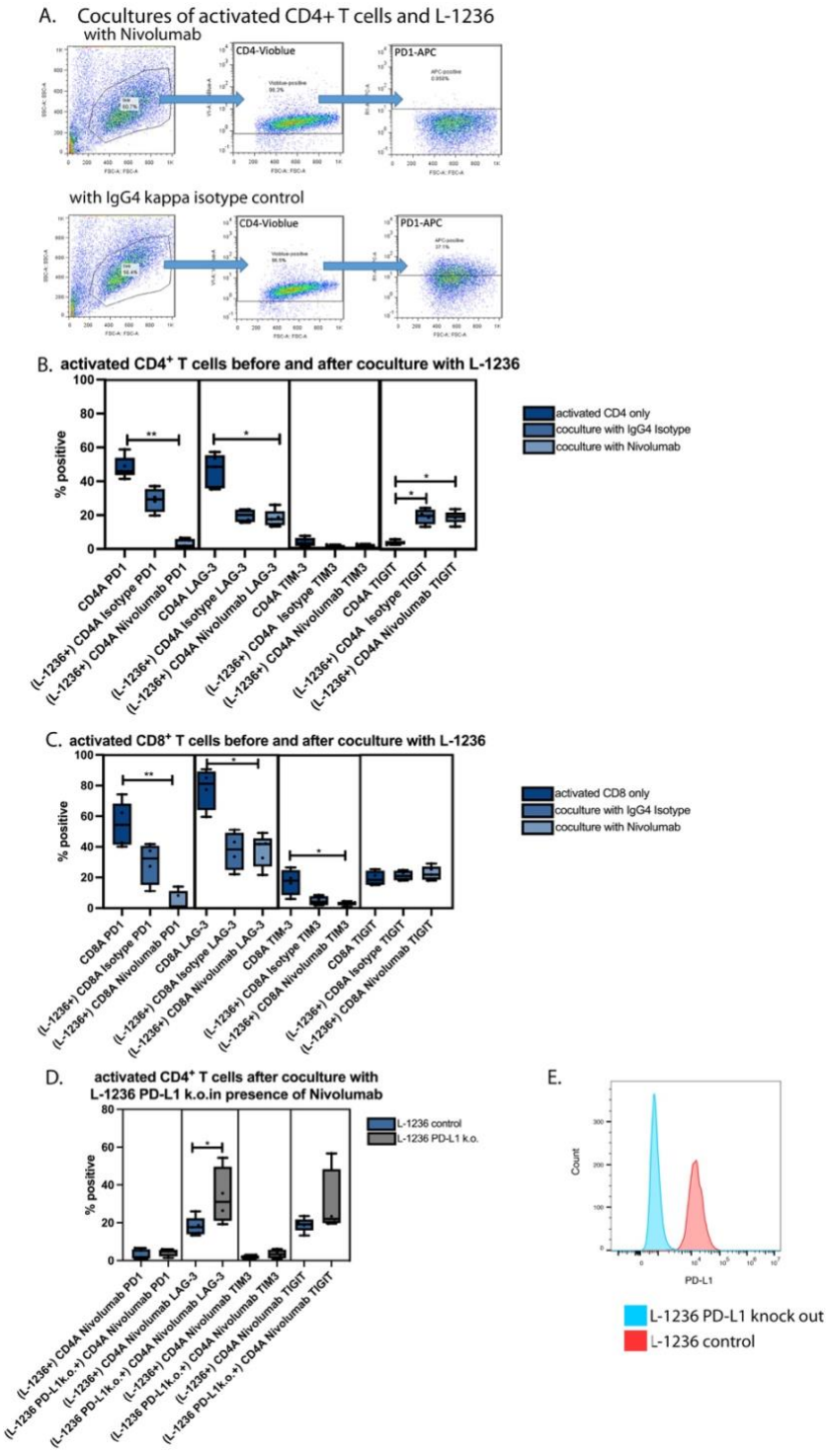


Suppl. Figure 2. Duration of cell-cell contacts between Hodgkin-Reed-Sternberg (HRS) cells and activated T cells

A. Duration of cell-cell contacts between HRS cells of cell line L-1236 and activated $CD4^+$ or $CD8^+$ T cells with or without Nivolumab or with IgG4 kappa isotype control
   * $p<0.05$, Kruskal-Wallis test
B. Duration of cell-cell contacts between HRS cells of cell line L-428 and activated $CD4^+$ or $CD8^+$ T cells with or without Nivolumab or with IgG4 kappa isotype control

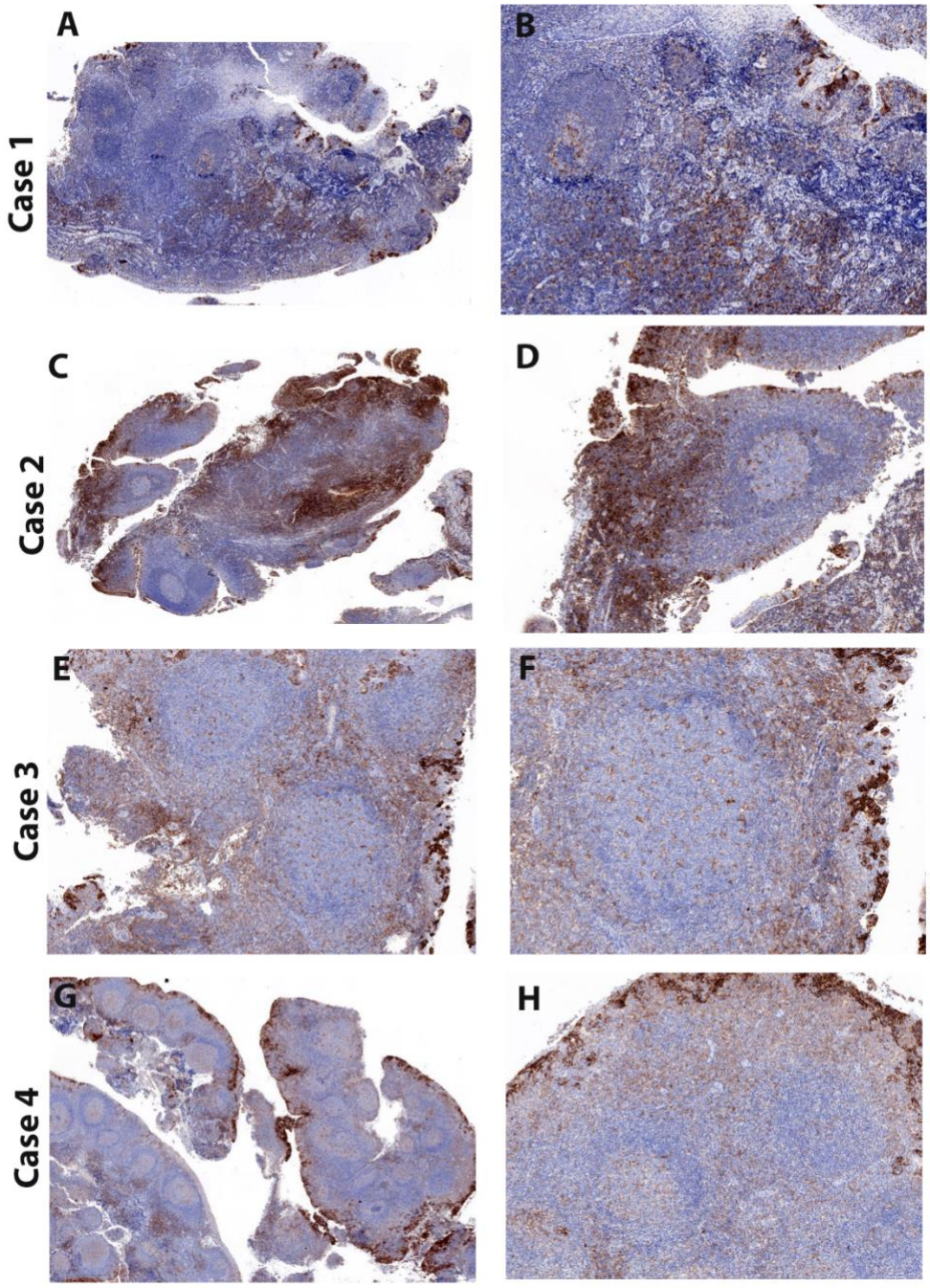


Suppl. Figure 3. PD1 expression by flow cytometry in T cells
PD1 expression by flow cytometry in resting and activated $CD4^+$ and $CD8^+$ T cells used in microchannel experiments, compared to isotype control

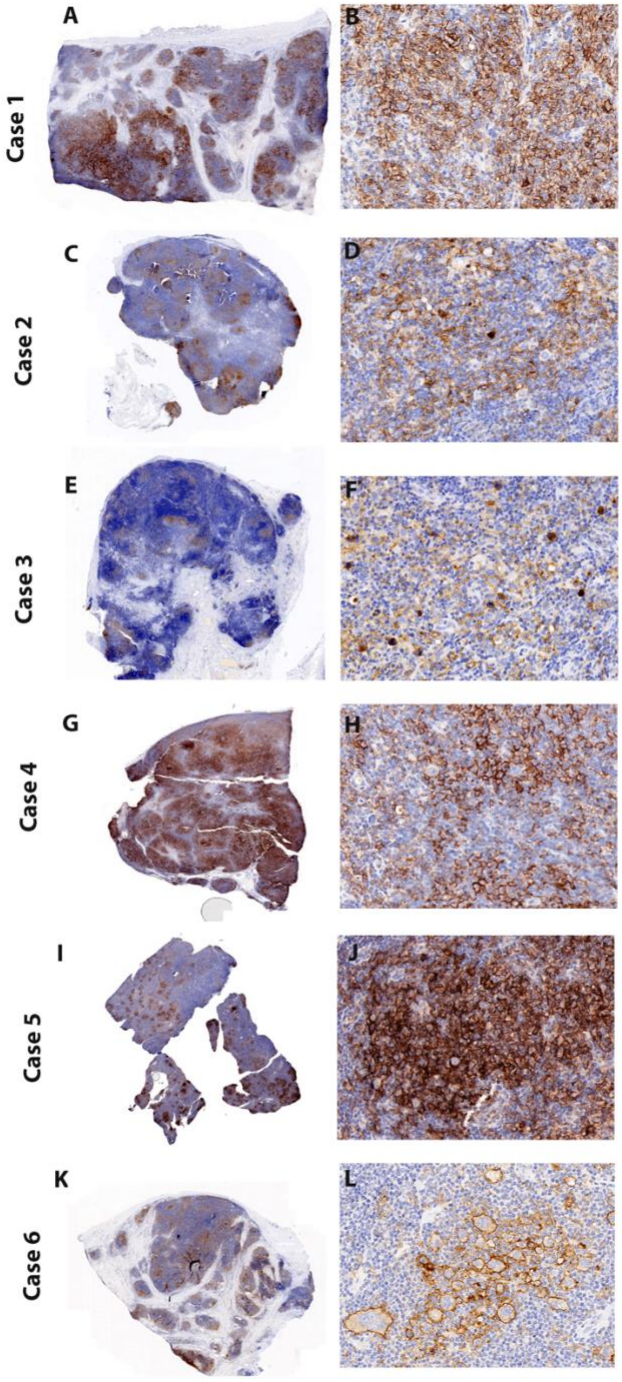


Suppl. Figure 4. Expression of the immune checkpoint proteins PD1, LAG-3, TIM-3 and TIGIT in an *in vitro* coculture model of classic Hodgkin lymphoma

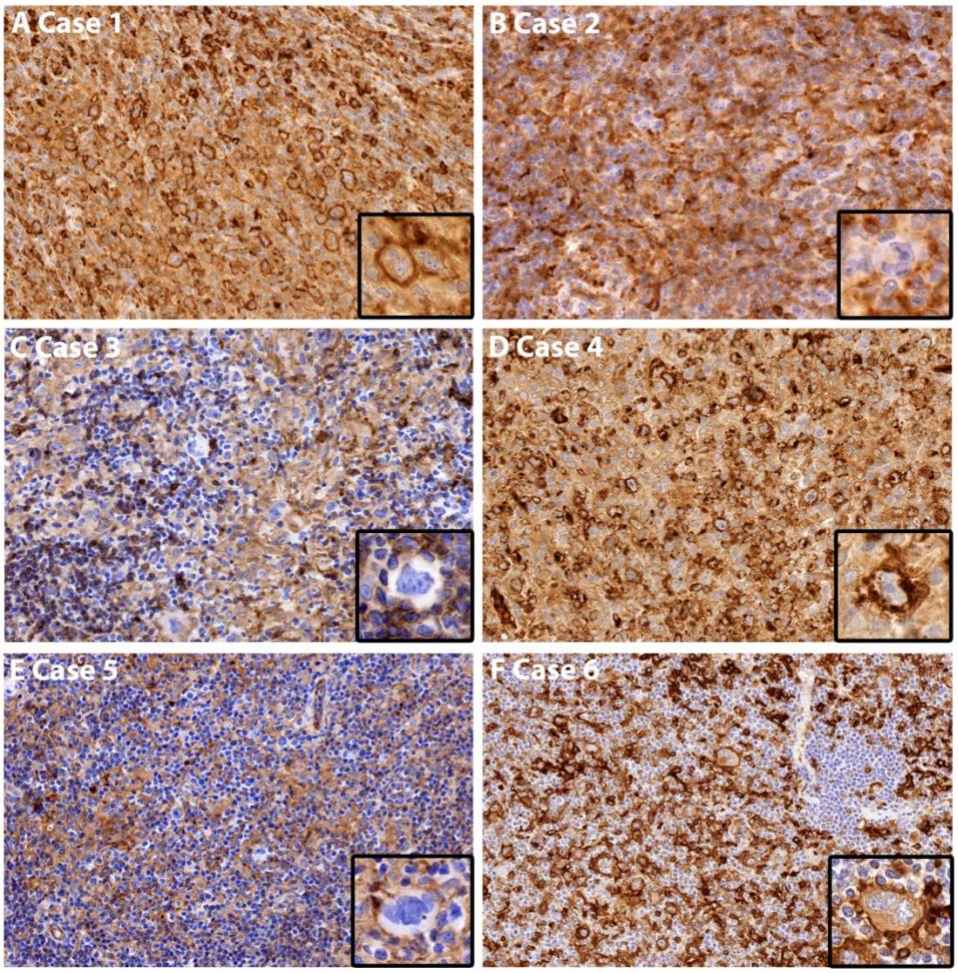
A. Examples of a 20:1 overnight coculture of activated $CD4^+$ T cells and HRS cells from the L-1236 cell line in presence of Nivolumab or IgG4 kappa isotype control antibody. Gating strategy for the assessment of immune checkpoint protein expression
B. Expression of PD1, LAG-3, TIM-3 and TIGIT in activated $CD4^+$ T cells alone and after overnight coculture with HRS cells from the L-1236 cell line in presence of Nivolumab or the IgG4 kappa isotype control antibody.
C. Expression of PD1, LAG-3, TIM-3 and TIGIT in activated $CD8^+$ T cells alone and after overnight coculture with HRS cells from the L-1236 cell line in presence of Nivolumab or the IgG4 kappa isotype control antibody.
D. Expression of PD1, LAG-3, TIM-3 and TIGIT in activated $CD4^+$ T cells after overnight coculture with HRS cells from a PD-L1 knockout L-1236 cell line or the L-1236 control cell line in presence of Nivolumab.
E. PD-L1 expression in the PD-L1 knockout L-1236 cell line and the L-1236 control cell line at baseline.



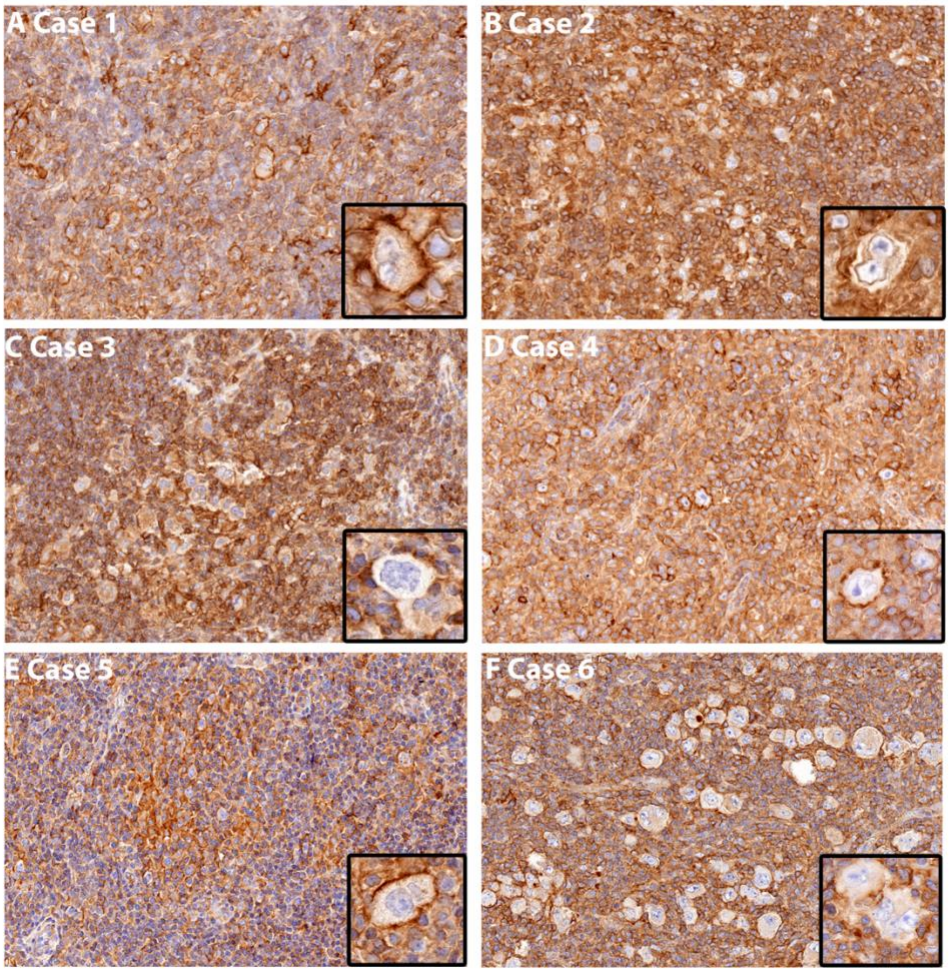
Suppl. Figure 5. PD-L1 expression in hyperplastic lymphatic tissue from the pharyngeal tonsil. Images from four representative of 13 donors.
A,C,E,G: 40-fold magnification of four representative examples of hyperplastic lymphoid tissue investigated by live cell imaging. B,D,F,H: 100-fold magnification of the same cases.



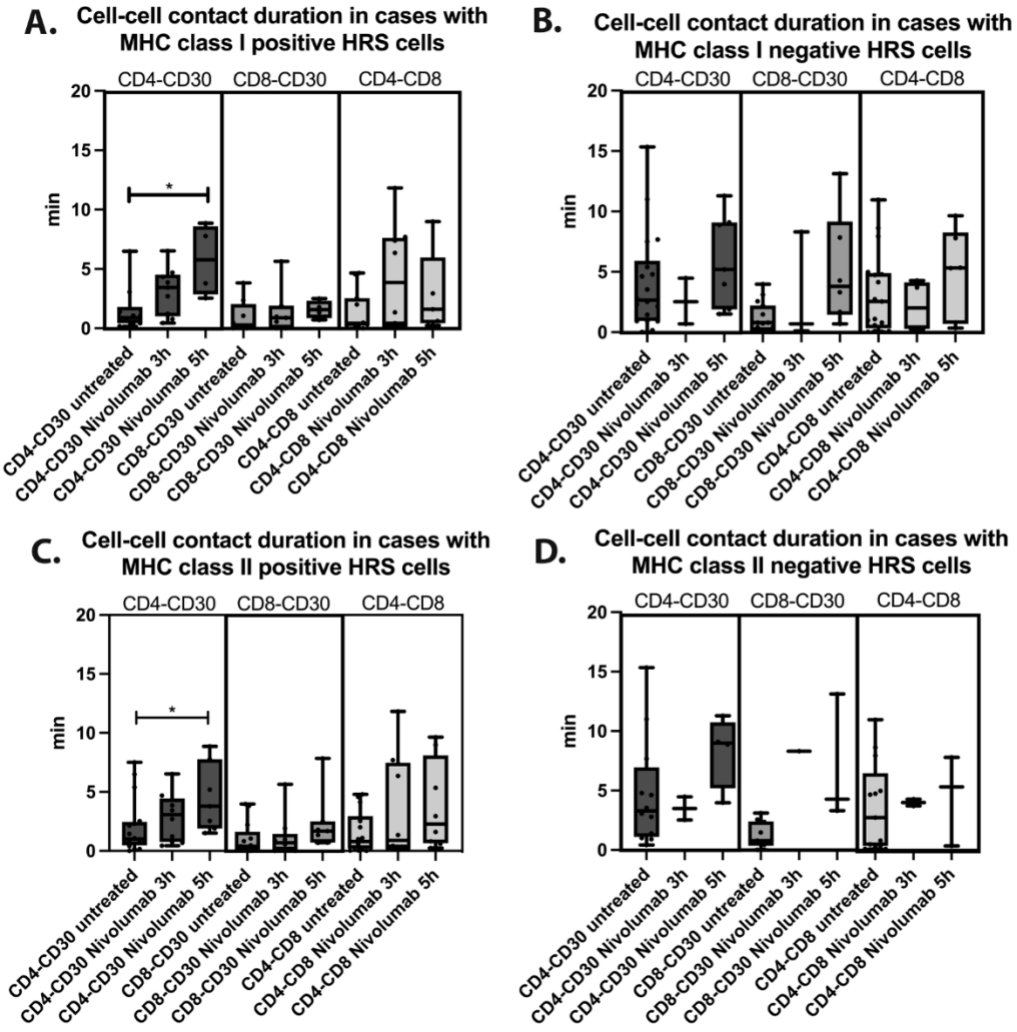
Suppl. Figure 6. PD-L1 expression in primary cases of classic Hodgkin lymphoma studied by live cell imaging.
A, C, E, G, I, K: 10-fold magnification of PD-L1 staining of the five cases investigated by live cell imaging.
B, D, F, H, J, L: 300-fold magnification of the same cases.



Suppl. Figure 7. MHC-II (HLA-DPB1) expression in the six classic Hodgkin lymphoma cases investigated by live cell imaging
A.-F. Cases 1-6 25 x magnification. Insert: Representative Hodgkin-Reed-Sternberg cells in 60x magnification.



Suppl. Figure 8. MHC-I (HLA-ABC) expression in the six classic Hodgkin lymphoma cases investigated by live cell imaging
A.-F. Cases 1-6 25 x magnification. Insert: Representative Hodgkin-Reed-Sternberg cell in 60x magnification.



Suppl. Figure 9. Cell-cell contact duration according to MHC expression in Hodgkin-Reed-Sternberg (HRS) cells of the classic Hodgkin lymphoma cases investigated.

A. Cell-cell contact duration between $CD4^+$ T cells and HRS cells, $CD8^+$ T cells and HRS cells and $CD4^+$ and $CD8^+$ T cells in MHC I positive cHL cases (n= 3), each dot represents the mean of one movie
B. Cell-cell contact duration between $CD4^+$ T cells and HRS cells, $CD8^+$ T cells and HRS cells and $CD4^+$ and $CD8^+$ T cells in MHC I negative cHL cases (n= 3), each dot represents the mean of one movie
C. Cell-cell contact duration between $CD4^+$ T cells and HRS cells, $CD8^+$ T cells and HRS cells and $CD4^+$ and $CD8^+$ T cells in MHC II positive cHL cases (n= 3), each dot represents the mean of one movie

D. Cell-cell contact duration between $CD4^+$ T cells and HRS cells, $CD8^+$ T cells and HRS cells and $CD4^+$ and $CD8^+$ T cells in MHC II negative cHL cases (n= 3), each dot represents the mean of one movie

Suppl. Table 1. MHC I and II expression by immunohistochemistry in classic Hodgkin lymphoma cases investigated by live cell microscopy
NS: Nodular sclerosing subtype, MC: Mixed cellularity subtype

Case number	Subtype	MHC-I (HLA-ABC)	MHC-II (HLA-DPB1)
1	NS, EBV-	+	+
2	NS, EBV-	-	-
3	NS, EBV-	-	-
4	MC, EBV+	+	+
5	MC, EBV+	+	-
6	NS, EBV-	-	+

Supplementary Methods

Flow cytometry of classic Hodgkin lymphoma (cHL) cell lines

Non-transduced cHL cell lines were stained with antibodies against PD-L1 (Clone 29E.2A3 PE, Biolegend, San Diego, CA, USA), HLA-ABC (Clone G46-2.6, APC, ThermoFisher Scientific, Waltham, MA, USA) HLA-DR, DP, DQ (Clone Tu39 FITC, BD Biosciences,Heidelberg, Germany), and isotype controls and analyzed by fluorescence activated cell sorting (FACS) in a MACSQuant (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cocultures of activated T cells with L-1236

After 48h of activation with dynabeads human T-Activator CD3/CD28 (Gibco, Thermofisher Scientific), activated $CD4^+$ or $CD8^+$ T cells were seeded in a 24-well plate with L-1236 cells (or L-1236 PD-L1 knockout cells) at a ratio of 20:1. Overnight culture was performed in the presence of Nivolumab or IgG4 isotype control antibody (10 µg/ml). After 24 h, cells were stained with either CD4-Vioblue or anti-CD8-Vioblue (both Miltenyi Biotec) and the following antibodies:
CD223 (LAG-3) Antibody, anti-human, APC, REAfinity (130-119-567), CD366 (TIM-3) Antibody, anti-human, APC, REAfinity (130-119-781), TIGIT Antibody, anti-human, APC, REAfinity (130-116-815), REA Control Antibody (S), human IgG1, APC, REAfinity (130-113-434, all Miltenyi), APC anti-human CD279 (PD-1) Antibody, clone EH12.2H7 (Biolegend) and Isotype APC Mouse IgG1, κ Isotype Ctrl Antibody MOPC-21 (both Biolegend).

Cells were gated on live population and the respective $CD4^+$ or $CD8^+$ T cell population and expression of the respective checkpoint proteins was assessed (Suppl. Figure 4A).

PD-L1 knockout L-1236 cell line

The PD-L1 knockout L-1236 cell line was obtained from Prof. Roland Schmitz, Gießen:

Vector construction

pLVX-TRE3G-Cas9-Hygro was created by removing the puromycin resistance gene from pLVX-TRE3G (Clontech Laboratories) with BfuAI/KpnI and replacing it with a synthetic Hygro construct. Cas9 was isolated using PCR from LentiCrispr v2 (Addgene #52961) and cloned into pLVX-TRE3G -Hygro digested with BamHI using Gibson cloning. SgRNA sequences targeting CD274 (ACATGTCAGTTCATGTTCAG) or AAVS1 control (GGGGCCACTAGGGACAGGAT) were cloned into pLenti guide dsRed (Addgene #128055) using BsmBI.

Generation of Doxycycline-inducible Cas9 expressing L-1236 clones

L-1236 cells were transduced with pLVX-Tet3G (Clontech Laboratories). 72 hours post-transduction, cells underwent selection with G418. Selected cells were transduced with pLVX-Tre3G-Cas9-Hygro. After 3 days of infection, transduced cells were split and incubated with hygromycin. A fraction of the selected pLVX-Tre3G-Cas9-Hygro cells were transduced with a sgRNA vector targeting nonessential surface markers such as ICAM1. 250 ng/mL of doxycycline was added to induce Cas9 and initiate genetic ablations for 8-11 days. The surface expression of the target gene was measured by FACS. After functional validation of Cas9, single-cell clones were generated by using limiting dilution from the pool of Cas9-expressing cells.

Lentivirus production and transduction

To generate lentivirus, HEK293FT cells (ThermoFisher) were cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin. One day before transfection, HEK293FT cells were seeded in a 10 cm tissue culture dish at 60% confluency. Cells were transfected using TransIT-293 transfection reagent (Mirus). For each dish, 50 µl serum-free OptiMEM (Gibco) was mixed with 6 µg sgRNA vector, 4.5 µg psPax2 (Addgene #12260) and 1.5 µg pMD2.G (Addgene #12259). 27 µl TransIT-293 transfection reagent was diluted with 950µl OptiMEM, incubated for 5 min at room temperature, and combined with the mixture of plasmids. This transfection mixture was incubated for 30 min at room temperature and then added dropwise to the cells. Virus-containing supernatant was collected 72h post-transfection. Cell debris was removed by

centrifugation at 500xg for 10 min followed by filtration of the supernatant through a 0.45µm low-protein binding membrane (Millipore Steriflip HV/PVDF). Viral supernatant was concentrated by incubation with Lent-X concentrator (Takara) for at least 3h at 4°C. Virus was concentrated 100x, resuspended in sterile PBS, aliquoted, and frozen at -80°C. L-1236 doxycycline-inducible Cas9 cell line was transduced with lentivirus, and after 3 days of transduction, dsRed was measured using FACS. 250 ng/mL doxycycline was added for 8-11 days to induce Cas9. Cells were passaged every three days with fresh medium containing doxycycline and the surface expression of the targeted gene (CD274, clone 29E.2A3) was measured by FACS. Transduced post-doxycycline cells underwent two rounds of FACS sorting.