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

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Abstract

Although checkpoint molecules like CTLA-4 and PD1 have been described several years ago, checkpoint inhibitors such as nivolumab (an anti-PD-1 antibody) have only recently been used to treat classic Hodgkin lymphoma (cHL). Several studies have shown convincing therapeutic effects of nivolumab in cHL. However, the mechanism of action of nivolumab in cHL is not fully understood. The aim of this study was to monitor changes in cell motility and cell contacts after administration of nivolumab to an *in vitro* model of cHL as well as to native hyperplastic lymphoid tissue and native human tissue from cHL. In both tissue and *in vitro*, CD4+, CD8+, CD30+ and CD20+ cell velocities were unchanged after nivolumab incubation. In contrast, in primary cHL tissue, the duration of cell contacts between CD4+ T cells and Hodgkin-Reed-Sternberg cells was significantly increased after 5 hours of nivolumab treatment, and the number of contacts with HRS cells was also slightly increased for CD4+ T cells (not significant), suggesting that CD4+ T cells in particular contribute to the cytotoxicity observed as a result of nivolumab therapy. There was no change in the duration of cell contacts in the hyperplastic lymphoid tissue after nivolumab incubation. In conclusion, we show here for the first time by imaging of native lymphoma tissue an enhanced interaction of CD4+ T cells and Hodgkin-Reed-Sternberg cells in cHL after nivolumab administration.

Introduction

The human immune system is a sophisticated structure that controls most types of infectious agents as well as developing malignant cells. However, on relatively rare occasions malignant cells manage to escape this highly efficient control system, leading to the development of a neoplasm. The first immune checkpoints, such as CTLA-4 and PD1, were discovered 20 years ago.[1-3] Only in recent years therapies targeting these specific checkpoints have been developed. Checkpoint inhibitors are very effective, especially in tumors with a high mutational burden and expression of tumor-specific neo-antigens.[4] Among lymphoid-derived tumors, classic Hodgkin lymphoma (cHL) is a neoplasm that often shows a good outcome after checkpoint inhibitor therapy.[5-11] Patients with *de novo* early unfavorable cHL showed an overall survival rate of 100% after a median follow-up of 41 months in the NIVAHL trial.[8] However, the exact mechanism that leads to the dissolution of Hodgkin-Reed-Sternberg (HRS) cells remains so far unknown.

In order to exert their anti-tumor activity, T cells must form a productive conjugate with their targets. In solid tumors, an inability of T cells to migrate and reach cancer cells has been described resulting in an “immune-excluded” tumor profile, which may explain why anti-PD1 antibodies do not always give the expected results.[12] Although T-cell motility is a critical factor influencing the success of immunotherapy, there is limited experimental data exploring the potential links between PD1 blockade and T-cell migration, particularly in a tumor context.

In vitro co-culture experiments provided the first evidence

that PD1 controls T-cell motility and interaction time between T cells and antigen-presenting cells.[13,14] In brief, it has been demonstrated that engagement with PD1 reduces T-cell receptor-induced signaling, thereby preventing T cells from establishing stable contact with antigen-presenting cells. Conversely, when PD1 binding to its ligand was blocked, T cells were able to regain stable contact with antigen-presenting cells. Intravital two-photon microscopy of a murine melanoma model showed that PD1 blockade restored stabilized conjugates between T cells and tumor cells.[15]

As the efficacy of nivolumab in the treatment of cHL has now been demonstrated in multiple studies, the aim of the present study was to further elucidate the direct effect of nivolumab on $CD4^+$ and $CD8^+$ lymphocytes, under reactive/inflammatory conditions and in cHL.

Methods

In vitro model

For the cHL *in vitro* model, the cHL cell lines L-428 (CVCL_1361) and L-1236 (CVCL_2096) were purchased from DSMZ (Braunschweig, Germany). Cell lines were transduced to express Life-Act GFP as previously described[16] for better visualization in microfluidics microchannels.

$CD4^+$ and $CD8^+$ T cells were purified from peripheral blood by magnetic-activated cell sorting (MACS) using the untouched $CD4^+$ and $CD8^+$ T-cell isolation kits (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity of the isolated cells was confirmed by fluorescence-activated cell sorting to be >94% for $CD4^+$ T cells (CD4-Vioblue antibody, Miltenyi Biotec) and >89% for $CD8^+$ T cells (CD8-PE/Cy7-antibody, Clone SK1, Biolegend, San Diego, CA, USA). T cells were either kept in culture overnight with interleukin (IL)-2 supplementation to recover from the MACS procedure or activated with dynabeads human T-activator CD3/CD28 (Gibco, Thermofisher Scientific, Waltham, MA, USA) for 48 hours and then seeded at $2x10^5$ cells in a microchannel punch. Nivolumab or an IgG4κ isotype control antibody (MedChemExpress, Monmouth Junction, NJ, USA) was added at a dose of 10 μg/mL to the cell culture medium that was used to resuspend the cells. The other punch on the opposite side of the 4x10 μm microchannels was loaded with $2x10^7$ cells of either L-428 or L-1236 cell lines (see Figure 1A). Time-lapse images were taken every 4 minutes (min) overnight. The velocity of the cells was measured as previously published.[16] The duration and number of cell-cell interactions was manually analyzed. In order to assess the expression of immune checkpoint proteins on activated T cells, T cells were co-cultured overnight with the L-1236 cell line or an L-1236 *PD-L1* knockout cell line at a ratio of 20:1. The expression of PD1, LAG-3, TIM-3 and TIGIT before and after co-culture was determined by flow cytometry (*Online Supplementary Appendix*).

Suggestions for text editing were generated by artificial intelligence (DeepL). All suggestions were checked by the authors for accuracy.

Live cell imaging

For 4D imaging, thick sections from 13 cases of hyperplastic lymphatic tissue from the pharyngeal tonsil and six cases of cHL were analyzed as previously described.[17] CHL cases 1-3 and 6 were nodular sclerosing subtype and Epstein-Barr virus (EBV)-negative, the fourth and fifth case were EBV-positive mixed cellularity (*Online Supplementary Table S1*). From the fifth case only movies in the untreated condition could be obtained due to limited tissue size.

The study was conducted according to theDeclaration of Helsinki and informed consent was obtained from all patients. The ethic committee of Goethe University Hospital agreed on the study (Nr20-376aV). The tissue was stained with fluorescent antibodies against CD4 (T-helper cells, clone SK3, NovaFluor Blue 585, ThermoFisher Scientific), CD8 (cytotoxic T lymphocytes, clone SK1 AlexaFluor 647, Biolegend), CD20 (B cells, clone L26, AlexaFluor 488, ThermoFisher Scientific), and CD30 (clone BerH2, sc-19658 AF488, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). In each case, nivolumab was added at a dose of 100 μg/mL on top of the tissue slices and then overlayed with RPMI medium. In the cases of hyperplastic lymphoid tissue an IgG4κ isotype antibody was added as negative control in addition to the untreated condition.

Results

$CD4^+$ resting T cells show prolonged cell contacts with classic Hodgkin lymphoma cell line L-1236 in microfluidic microchannels after incubation with nivolumab

In order to model the effects of nivolumab on T cells and HRS cells, we first used a model system with microfluidic microchannels, where the different cell types (i.e., T cells *vs*. HRS cells) can enter the microchannels from opposite sides (Figure 1A). cHL cell lines L-428 and L-1236 were used, which both show expression of PD-L1 (Figure 1B) and loss of expression of major histocompatibility complex (MHC) class I (L-428) or II (L-1236; *Online Supplementary Figure S1*) as frequently observed in HRS cells in primary cHL cases.[18] In general, activated T cells moved at a higher velocity (mean 3.51-5.01 μm/min; Figure 1D) when compared with resting T cells (mean 1.93-4.3 μm/min; Figure 1C). There was no systematic difference in velocity when either nivolumab or the IgG4κ isotype control antibody was added to the cell culture medium.

The duration of cell-cell contacts with HRS cells of the L-1236 cell line was significantly increased for resting $CD4^+$ T cells in the presence of nivolumab when compared to the IgG4κ isotype control (mean 96.00 *vs*. 15.64 min; Figure

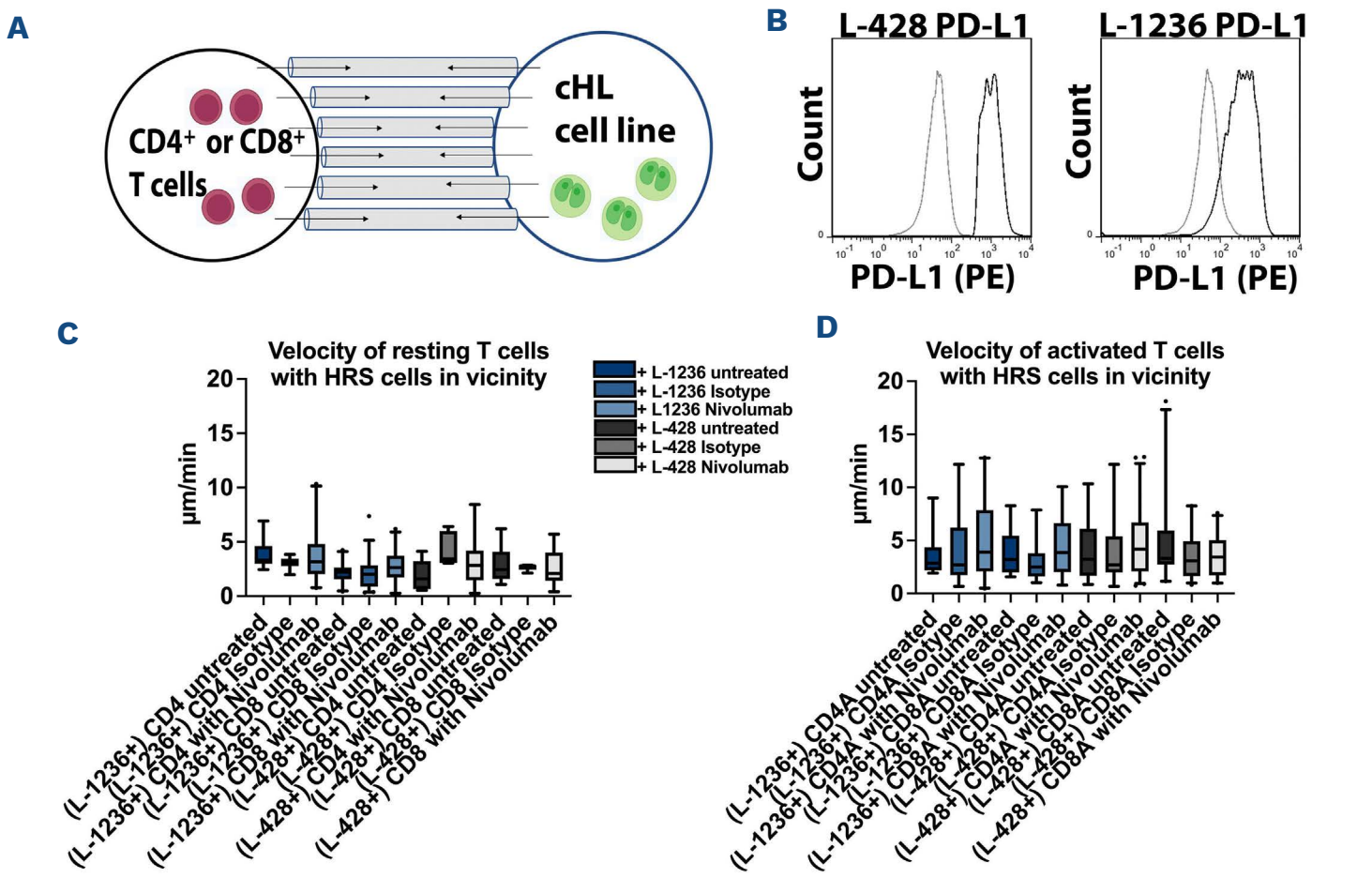

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Figure 1. Effects of nivolumab on T-cell motility in microchannels in the presence of Hodgkin-Reed-Sternberg cells. (A) Schematic overview of experimental setup with 4x10 μm microchannels connecting 2 reservoirs containing either CD4+ or CD8+ T cells (left side) and classic Hodgkin lymphoma (cHL) cell lines (right side). (B) PD-L1 expression in the cHL cell lines L-428 and L-1236 (black) compared with isotype control (grey). (C) Velocity of resting CD4+ and CD8+ T cells in microchannels with and without nivolumab or with IgG4κ isotype control in presence of cHL cell lines. (D) Velocity of activated CD4+ and CD8+ T cells in microchannels with and without nivolumab or with IgG4κ isotype control in presence of cHL cell lines. (E) Duration of cell-cell contacts between Hodgkin-Reed-Sternberg (HRS) cells of cell line L-1236 and resting CD4+ or CD8+ T cells with or without nivolumab or with IgG4κ isotype control. (F) Duration of cell-cell contacts between HRS cells of cell line L-428 and resting CD4+ or CD8+ T cells with or without nivolumab or with IgG4κ isotype control. (G) Example of several short duration contacts between a resting CD4+ T cell and a HRS cell without nivolumab (HRS cell green, CD4+ T cell red). (H) Example of a long duration contact between a resting CD4+ T cell and a HRS cell with nivolumab (HRS cell green, CD4+ T cell red).

1E, G, H); *P*<0.01, Kruskal-Wallis test). In addition, contacts with activated CD8+ T cells were significantly longer compared to the IgG4κ isotype control (mean 121.00 *vs*. 15.84 min; *Online Supplementary Figure S2*; *P*<0.01, Kruskal-Wallis test). In the L-428 cell line there was no effect of nivolumab treatment on the duration of cell-cell contacts (Figure 1F; *Online Supplementary Figure S2* for activated T cells).

Nivolumab treatment of activated T-cell co-cultures has no effect on the expression of LAG-3, TIM-3 and TIGIT, while PD1 expression becomes undetectable

Expression of the immune checkpoint proteins PD1, LAG-3, TIM-3 and TIGIT was assessed by flow cytometry in activated CD4+ and CD8+ T cells before and after overnight co-culture with the cHL cell line L-1236. The aim was to determine if the expression of other checkpoint proteins is altered after nivolumab treatment. Activated T cells were used because relevant PD1 expression was only observed in activated T cells (*Online Supplementary Figure S3*). Both PD1 and LAG-3 expression was reduced in the co-cultured CD4+ and CD8+ T cells in the presence of the IgG4κ isotype control antibody (*Online Supplementary Figure S4*). However, there was no difference in LAG-3 expression between nivolumab and IgG4κ isotype treated co-cultures. Using a PD-L1 knockout L-1236 cell line for the T-cell co-cultures, LAG-3 expression was significantly higher in the activated CD4+ T cells compared to the activated CD4+ T cells co-cultured with the L-1236 control cell line (33.88 *vs*. 18.04% LAG-3 expression; *Online Supplementary Figure S4D*, E; *P*<0.05, Mann-Whitney test), while all other checkpoint proteins were unchanged when using *PD-L1* knockout L-1236 (*Online Supplementary Figure S4*).

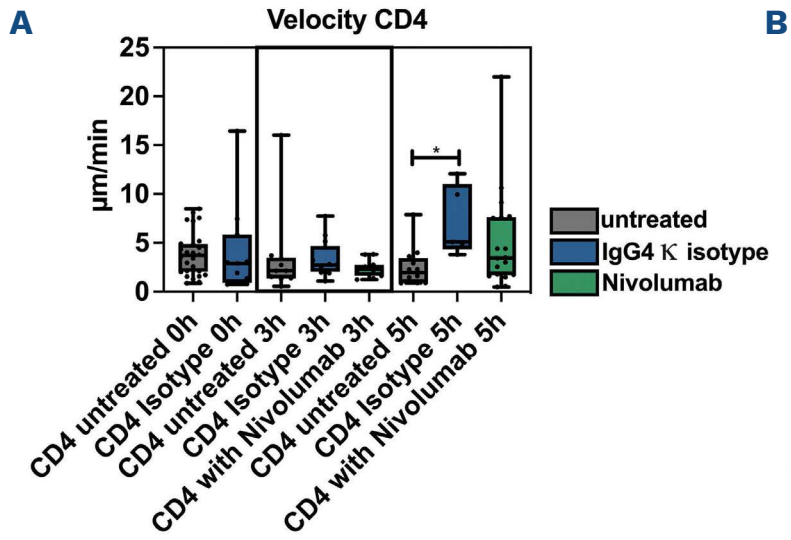
Duration of T-cell contact with B cells is not affected in normal lymphoid tissue after nivolumab treatment

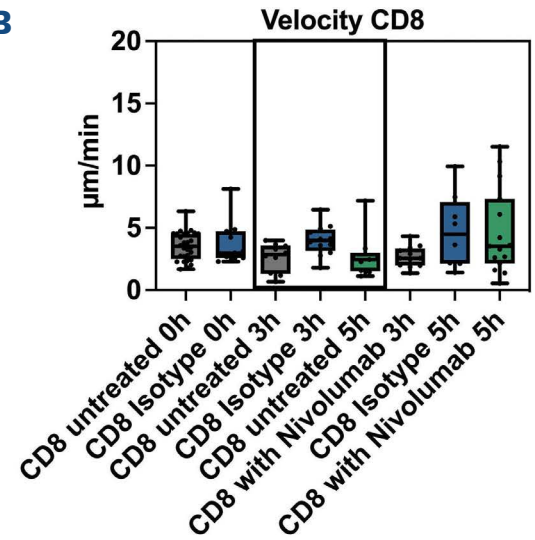
We then examined the motility of CD4+ and CD8+ T cells and CD20+ B cells in native thick sections of native pharyngeal tonsil hyperplastic tissue, as we also observed PD-L1 expression in normal lymphoid tissue (*Online Supplementary Figure S5*). Time-lapse movies were taken immediately and after 3 and 5 hours. While the velocity of CD4+ and CD8+ T cells as well as CD20+ B cells was almost unchanged at 3 and 5 hours (h) with and without nivolumab, generally T cells were significantly faster when compared with B cells (mean 3.77 and 3.40 μm/min for CD4+ and CD8+ T cells *vs*. 2.39 μm/min for B cells; *P*<0.05, Kruskal-Wallis test with Dunn´s *post hoc* test for multiple comparisons). Similarly, there were no changes in track length or displacement for CD4+, CD8+ T cells or B cells after nivolumab incubation (*data not shown*). After 5 h of incubation with the IgG4κ isotype control antibody, the velocity of CD4+ T cells was significantly higher compared to the untreated tissue slices (*P*<0.05, Kruskal-Wallis test; Figure 2A). This difference may be attributed to the limited sample size and the different areas of imaging, whether within or outside of follicles. In all other settings there was no effect on velocity of neither nivolumab nor the IgG4κ isotype control antibody.

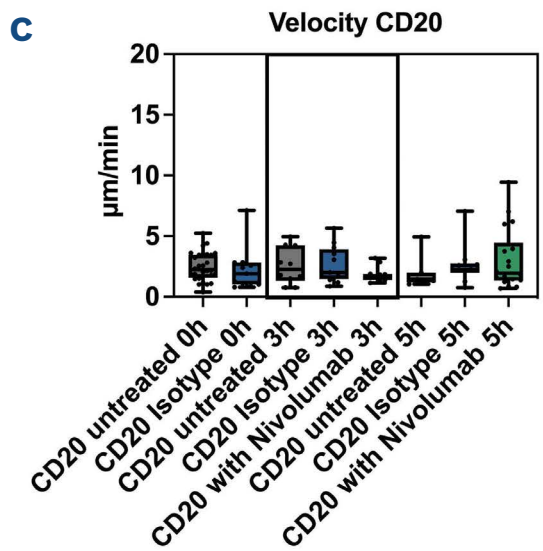
Cell-cell contacts in untreated tissue were shortest when CD8+ cells were involved (CD8-CD20 mean 1.30 min, CD4-CD8 mean 1.65 min), whereas the duration of CD4-CD20 cell contacts under baseline conditions was longer (mean 3.72 min) and had a higher variance, reflecting the physiologic interaction of CD4+ T cells and CD20+ B cells in follicles and interfollicular areas. Overall, there was no clear effect of nivolumab on cell contact duration in normal lymphoid tissue (Figure 2D-F). However, CD4-CD20 cell contacts were significantly longer after 5 h of IgG4κ isotype control antibody incubation compared to the nivolumab-treated tissue slices (5.08 *vs*. 0.74 min; *P*<0.01, Kruskal-Wallis test with Dunn's *post hoc* test for multiple testing; Figure 2D). This may be due to the limited number of samples and different areas of imaging.

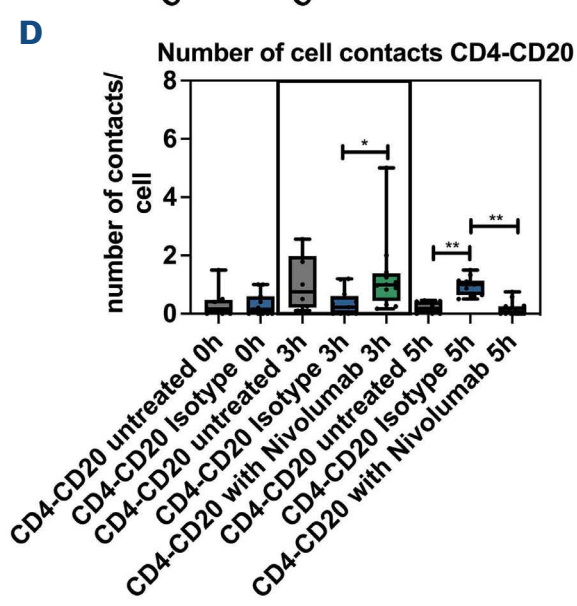
Hodgkin-Reed-Sternberg cells in classic Hodgkin lymphoma present enhanced cell-cell contacts with CD4+ T cells after nivolumab treatment

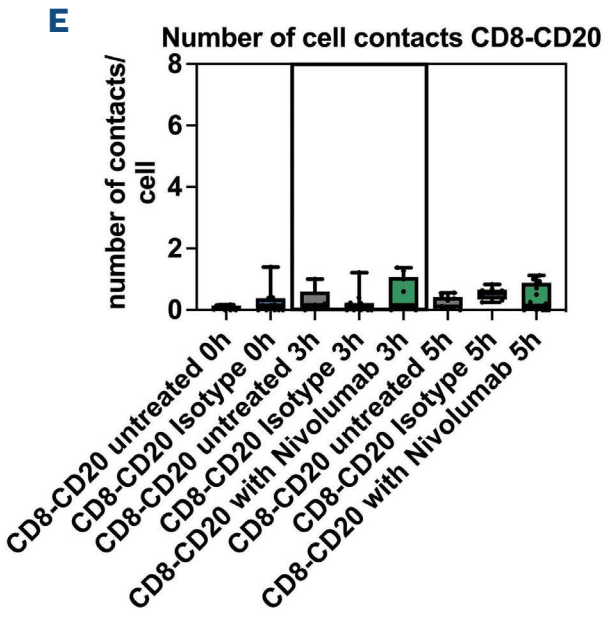
Next, we incubated thick slices of native tissue from cHL-affected lymph nodes with nivolumab and took movies at baseline and after 3 and 5 h of nivolumab incubation. All cases had PD-L1-positive HRS cells and bystander cells (*Online Supplementary Figure S6*). The velocity of CD4+ T cells (2.87-4.94 μm/min), CD8+ T cells (3.54-4.07 μm/min) and HRS cells (1.94-3.13 μm/min) was largely unchanged after 3 and 5 h of nivolumab incubation (Figure 3A). Similarly, track length and displacement were essentially unchanged in all cell types after 3 and 5 h of nivolumab incubation (*data not shown*). The number of cell-cell contacts be-











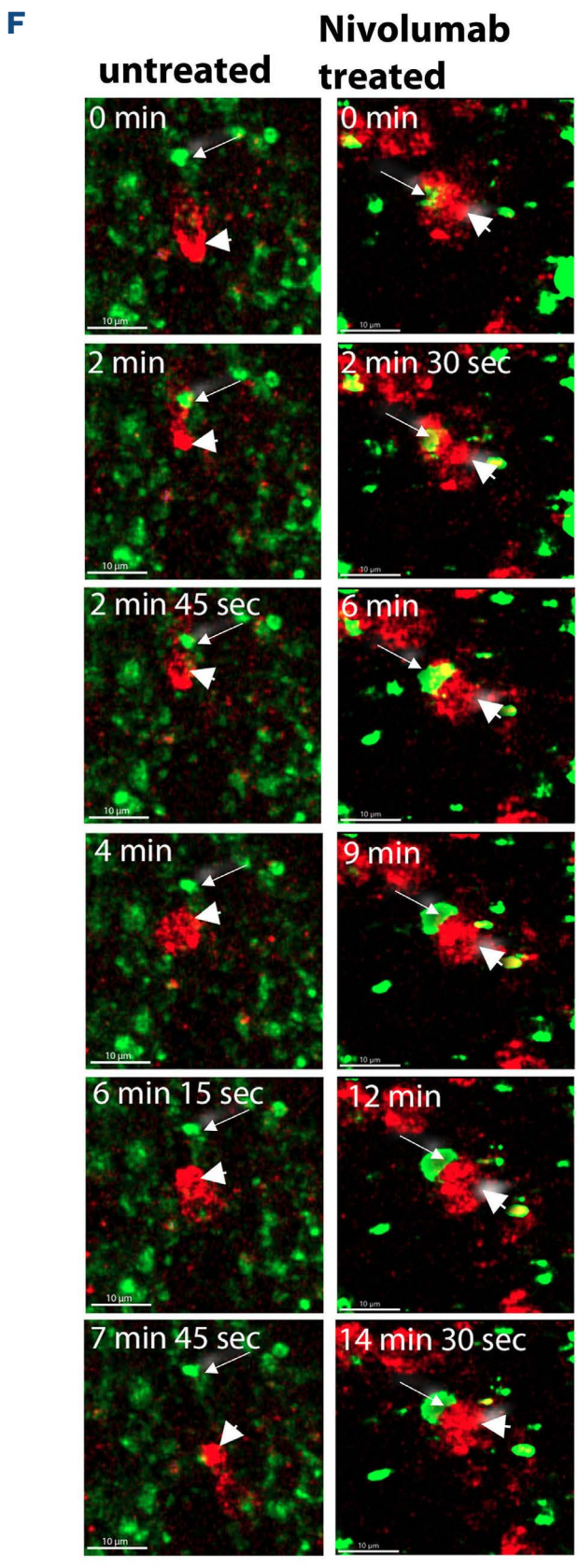


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Figure 2. Cell velocity and duration of cell-cell contacts in hyperplastic lymphoid tissue. Each dot represents the mean of a movie. Typically, 3 movies per condition were obtained. (A) Velocity of CD4+ T cells in hyperplastic lymphoid tissue (from 13 donors) with and without nivolumab or IgG4κ isotype control antibody; *P<0.05, Kruskal-Wallis test with Dunn´s *post hoc* test for multiple testing. (B) Velocity of CD8+ T cells in hyperplastic lymphoid tissue (from 13 donors) with and without nivolumab or IgG4κ isotype control antibody. (C) Velocity of CD20+ B cells in hyperplastic lymphoid tissue (from 13 donors) with and without nivolumab or IgG4κ isotype control antibody. (D) Duration of cell-cell contacts between CD4+ T cells and CD20+ B cells in hyperplastic lymphoid tissue (from 13 donors) with and without nivolumab or IgG4κ isotype control antibody; *P<0.05, Kruskal-Wallis test with Dunn´s *post hoc* test for multiple testing. (E) Duration of cell-cell contacts between CD8+ T cells and CD20+ B cells in hyperplastic lymphoid tissue (from 13 donors) with and without nivolumab or IgG4κ isotype control antibody. (F) Examples for a short cell contact (left) between a CD8+ T cell (arrow head) and a CD20+ B cell (arrow, green cell) and longer duration cell contact (right) between a CD8+ T cell (arrow head, red cell) and a CD20+ B cell (arrow, green cell); CD8+ T cells labeled in red, CD20+ B cells labeled in green.

tween CD4+ T cells and HRS cells was increased after 5 h of nivolumab incubation (mean 1 contact/CD4+ T cell/movie after 5 h of nivolumab *vs*. mean 0.45 contact/CD4+ T cell/movie at baseline, not significant; Figure 3B). No differences were seen in the number of cell contacts between CD8+ T cells and HRS cells or between CD4+ and CD8+ T cells after nivolumab incubation (Figure 3B). Regarding the duration of cell-cell contacts, a significant increase in the duration of cell-cell contacts between CD4+ T cells and CD30+ HRS cells was observed after 5 h nivolumab incubation (Figure 3C, E; after 5 h of nivolumab mean 5.89 min *vs*. untreated mean 3.20 min; P<0.05, Kruskal-Wallis test with Dunn´s *post hoc* test for multiple comparisons). At the same time, the duration of cell contacts between CD8+ T cells and CD30+ HRS cells was significantly increased after 5 h of nivolumab incubation (mean 3.73 min *vs*. mean 1.11 min in untreated setting; P<0.05, Kruskal-Wallis test with Dunn´s *post hoc* test for multiple comparisons). There was no difference in the duration of cell contacts between CD4+ and CD8+ cells in the untreated *versus* nivolumab-treated groups (Figure 3C).

When only cHL cases with MHC I- or II-positive HRS cells were considered (*Online Supplementary Table S1*; *Online Supplementary Figures S7-9*), the increase in cell-cell contact duration between CD4+ T cells and HRS cells remained significant after 5 h of nivolumab treatment. Differences in the duration of CD8+ and HRS cell contacts were abolished, when only the subgroups of MHC I- or II-positive or -negative cases were considered.

When examining the duration of cell contact between CD4+ and CD8+ T cells and HRS cells after 5 h of nivolumab incubation, it was found that there was a significantly longer duration of contact between CD4+ T cells and HRS cells in MHC II-negative cHL cases compared to MHC II-positive cHL cases (mean 8.31 min in MHC II-negative cases *vs*. 4.51 min in MHC I-positive cases; P<0.05, Mann-Whitney test; Figure 3D). The duration of contacts between CD8+ T cells and HRS cells was also longer in cHL cases with MHC I-negative HRS cells (mean 5.15 min in MHC I-negative *vs*. 1.59 min in MHC I-positive cHL cases, not significant). Figure 4 shows examples (from case 1) where long tracks with little displacement were seen in most cell types after 5 h of nivolumab treatment.

Discussion

This is the first study to demonstrate the dynamic effects of the anti-PD1 antibody nivolumab on cell motility and cell-cell interactions of T cells, B cells and HRS cells in cHL using live cell imaging. Previously observed differences in the cell dynamics of immune cells[17] were confirmed in this study, with T cells moving significantly faster than B cells in hyperplastic lymphoid tissue.

As PD1 has been described to be expressed on non-malignant CD4+, CD8+ and CD20+ cells,[19] we would potentially expect to see effects in all these cell types as PD-L1 was almost ubiquitously expressed in the tissue (*Online Supplementary Figures S5, S6*). However, overall cell motility was not altered after nivolumab incubation in either microchannels, hyperplastic lymphoid tissue or primary cHL tissue, suggesting that there is no direct effect of nivolumab on cell motility.

While the duration of cell-cell contacts between CD4+ T cells and B cells in in hyperplastic lymphoid tissue was unchanged after nivolumab treatment, resting CD4+ T cells in microchannels showed significantly longer cell-cell contacts with HRS cells of the cHL cell line L-1236 as well as CD4+ T cells with HRS cells in primary cHL tissue. Although the microchannel system is more artificial than imaging of primary tissue, this suggests that the situation in cHL is generally different from that in hyperplastic lymphoid tissue. While data from the literature suggests that the interferon (IFN)γ pathway is active in both hyperplastic tonsillar tissue[20] and cHL,[21,22] there are several other differences between hyperplastic lymphoid tissue and cHL, such as the composition of the microenvironment, the frequent lack of MHC-I and -II expression in HRS cells, the tumor mutation burden, which has been observed to be on average 3.57/Mb in cHL nodular sclerosing type,[23] as well as differences in chemokine secretion in the neoplastic HRS cells of cHL. In this regard, tumor mutational burden, microsatellite instability, expression of tumor neoantigens, epigenetic silencing, expression of chemokines and receptors such as CCR5, CXCL9 and CXLC13, have been described to be associated with response to checkpoint inhibitor therapy.[24-26] These findings may explain the divergent observation of cell-cell interaction after nivolumab treatment in our study and also the large variance between samples, as each patient

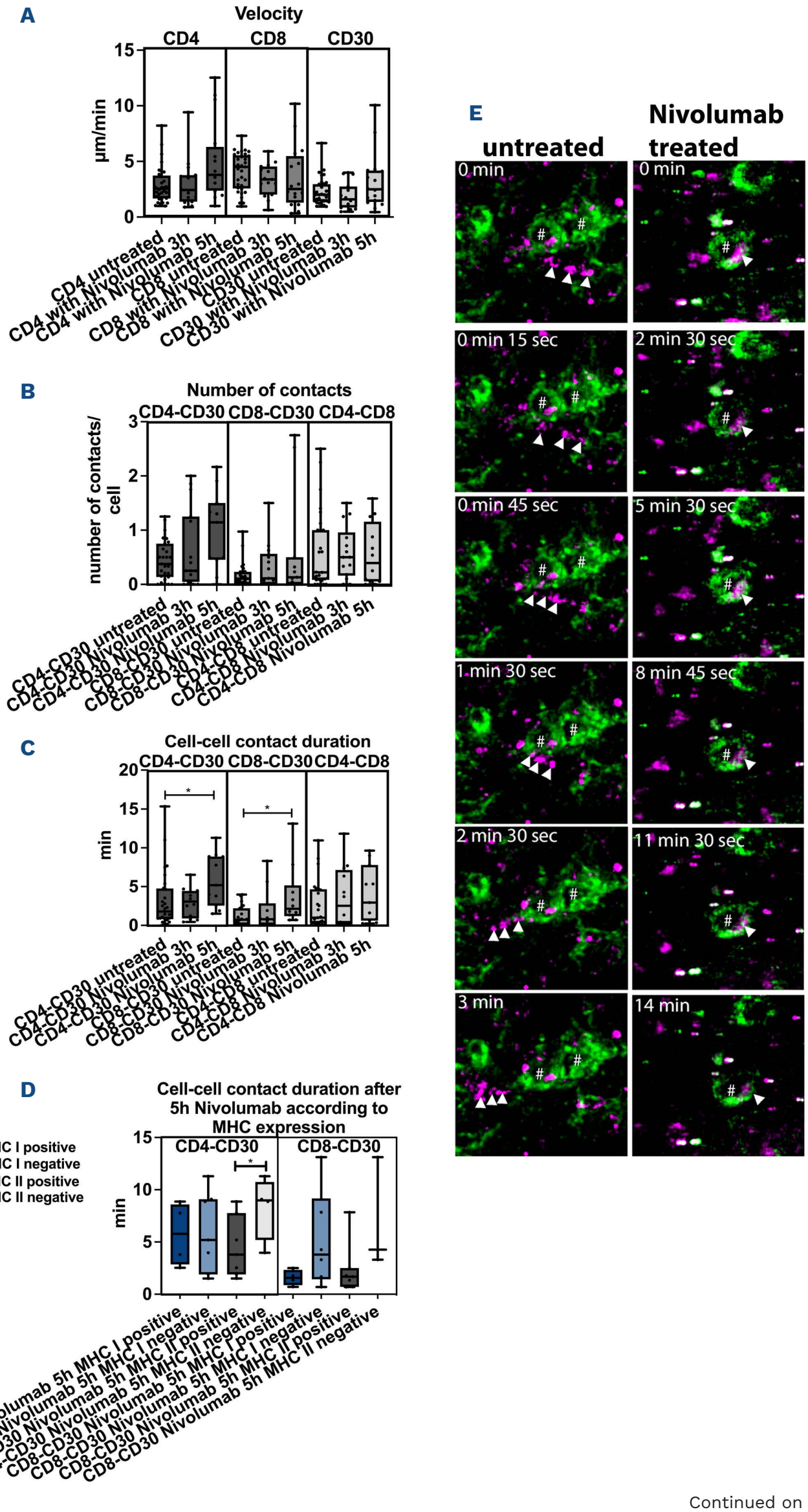

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Figure 3. Nivolumab incubation extends cell-cell contacts between CD4⁺ T cells and Hodgkin-Reed-Sternberg cells in classic Hodgkin lymphoma. The study analyzed 6 cases of classic Hodgkin lymphoma (cHL), including 5 cases in both untreated and nivolumab-treated conditions, and 1 case in untreated condition only. Each dot represents the mean of a movie. Typically, 3 movies per condition were obtained. (A) Velocity of CD4⁺ and CD8⁺ T cells as well as CD30⁺ Hodgkin-Reed-Sternberg (HRS) cells in cHL in untreated condition (0 hours [h]) and after incubation with nivolumab for 3 h or 5 h. (B) Number of cell-cell contacts between individual CD4⁺ T cells/movie and CD30⁺ HRS cells, individual CD8⁺ T cells/movie and CD30⁺ HRS cells as well as CD4⁺ and CD8⁺ T cells in cHL in untreated condition (0 h) and after incubation with nivolumab (3 h and 5 h). (C) Duration of cell-cell contacts between CD4⁺ T cells and CD30⁺ HRS cells, CD8⁺ T cells and CD30⁺ HRS cells as well as CD4⁺ and CD8⁺ T cells in cHL in untreated condition (0 h) and after incubation with nivolumab (3 h and 5 h). **P*<0.05, Kruskal-Wallis test with Dunn´s *post hoc* test for multiple testing. (D) Duration of cell-cell contacts between CD4⁺ T cells and CD30⁺ HRS cells as well as CD8⁺ T cells and CD30⁺ HRS cells after 5 h nivolumab incubation. Cases were stratified based on major histocompatibility complex (MHC) I and II expression in the HRS cells. **P*<0.05, Kruskal-Wallis test with Dunn´s *post hoc* test for multiple testing. (E) Examples for a short cell contact (left) between 3 CD4⁺ T cells (arrow heads) and a CD30⁺ HRS cell (#) in untreated condition and long duration cell contact (right) between a CD4⁺ T cell (arrow head) and a CD30⁺ HRS cell (#); CD4⁺ T cells labeled in pink, CD30⁺ HRS cells labeled in green; confocal micrsopscopy images 40x magnification.

or tissue has individual factors that influence the efficacy of checkpoint inhibition. HRS cells from cHL strongly secrete various chemokines such as CXCL9, CCL5, CCL17 and CCL22 and thus attract both CD4⁺ and CD8⁺ T cells.[21,27-29] While an inferior outcome after checkpoint inhibition was observed in patients with loss of MHC I protein expression in HRS cells of cHL[30], we found rather prolonged cell contacts of CD8⁺ T cells and HRS cells in cHL with MHC I loss (not significant). Similarly, the duration of cell-cell contacts between CD4⁺ T cells and HRS cells was significantly longer when MHC II expression was absent in HRS cells. This suggests that the effects of nivolumab are, to some extent, independent of MHC expression.

The *in vitro* cHL model can be extended in the future to study T-cell activation by visualizing intracellular Ca^{2+} signaling and measuring T-cell cytotoxicity. The importance of CD4⁺ T cells in mediating the effects of nivolumab has previously been demonstrated in several studies, including the significant expansion of the CD4⁺ T-cell receptor repertoire in the peripheral blood of patients responding to nivolumab treatment.[31] In addition, lymph node biopsies taken 1 week after start of nivolumab therapy showed little change in the frequency of CD4⁺ and CD8⁺ T cells in the cHL microenvironment, despite the frequent disappearance of neoplastic CD30⁺ HRS cells.[32]

Another study found that clonally expanded T cells in un-

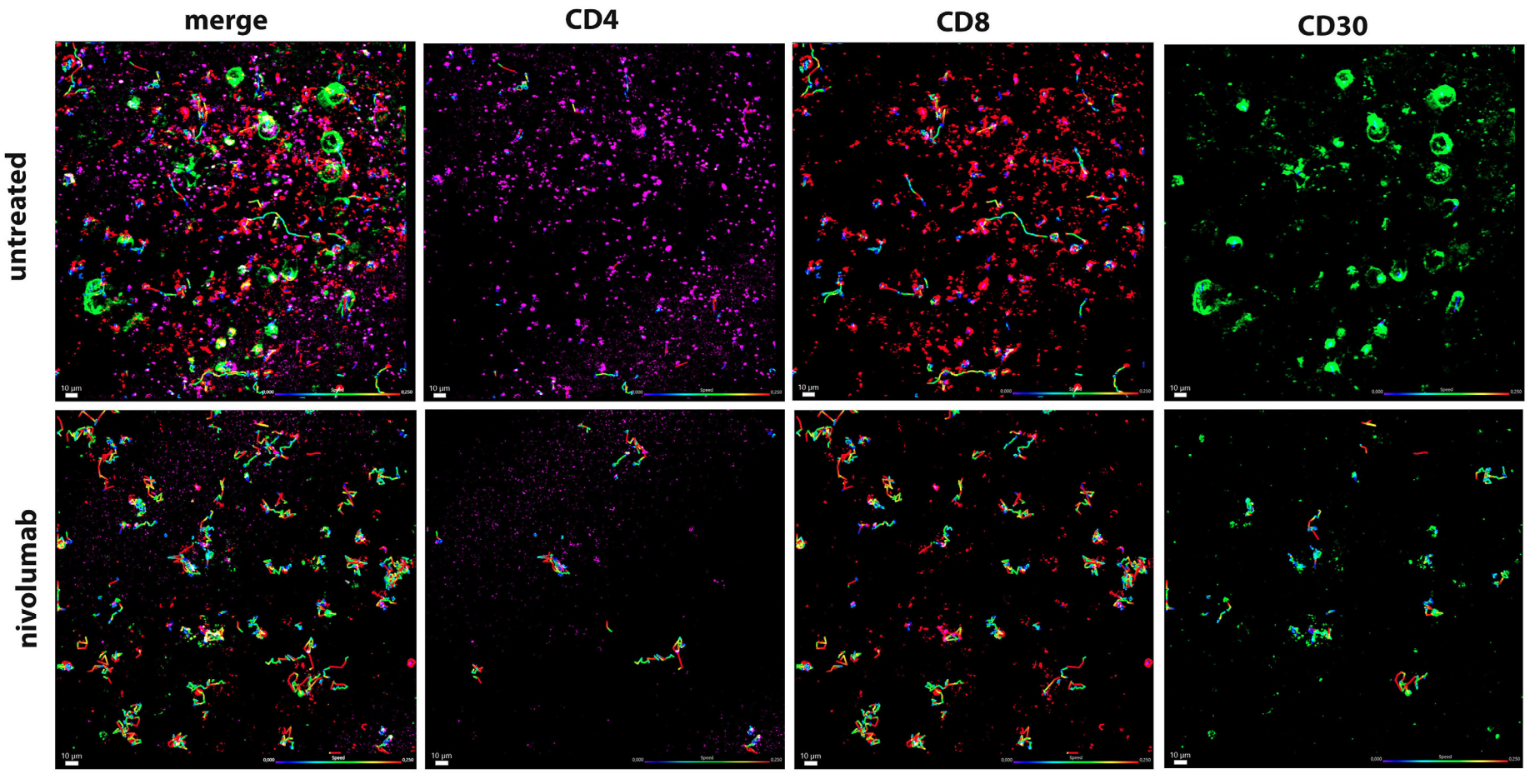

Figure 4. Cell tracks in classic Hodgkin lymphoma without and with nivolumab. Top row shows images (merged, CD4 pink, CD8 red, CD30 green) and the respective tracks of the cells in a 15-minute (min) movie of untreated classic Hodgkin lymphoma (cHL) tissue. Bottom row shows images (merged, CD4 pink, CD8 red, CD30 green) and the respective tracks of the cells in a 15-minute movie of cHL tissue from the same case after incubation with nivolumab for 5 hours. Scale bars are in ym/sec, with each individual scale bar 10 μM.

treated cHL lymph nodes were rare, were exclusively CD8+, had a non-naïve immune phenotype and only a minority of them expressed classical immune checkpoint molecules.[33] Another study showed that MHC II expression on the HRS cells was predictive of response to nivolumab treatment whereas no such correlation was observed for MHC I expression on HRS cells.[18] One hypothesis for how nivolumab might work is that subsets of rosetting CD4+ T cells, which contribute to the growth support of the HRS cells,[34] move away from the HRS cells, resulting in the removal of growth support. Immunological synapses were observed between HRS cells and rosetting T cells in this regard.[35]

The phenotype of CD4+ T cells with regard to LAG-3, TIM-3 or TIGIT expression did not change after nivolumab incubation in our *in vitro* co-culture model of cHL. On the one hand, this model has the limitation that CD4+ and CD8+ T cells were purified from peripheral blood and did not undergo long-term co-culture with HRS cells. On the other hand, the phenotype of CD8+ T cells after checkpoint inhibition in a melanoma patient, studied on single-cell level in the literature, also did not change with respect to LAG-3, TIM-3 or TIGIT expression.[36] Co-inhibition of other checkpoint proteins may therefore be an additional therapeutic option. In general, most studies, including ours, have focused on the effects of nivolumab on the activity of CD4+ or CD8+ T cells. Effects on other cell types such as histiocytes, macrophages and NK cells have not been studied in cHL and may provide explanations for the remaining unanswered questions about the effect of nivolumab. In a mouse model, it was observed that anti-PD1 antibody can be captured from CD8+ T cells by macrophages leading to resistance to checkpoint inhibition.[37] In conclusion, nivolumab altered cell-cell interactions between CD4+ T cells and CD30+ HRS cells in cHL. The significant prolongation of cell-cell contacts between CD4+ T cells and HRS cells may be one component of the successful mode of action of nivolumab in cHL. In this study, the effects of nivolumab were found to be independent of the expression of MHC I or II on HRS cells. Future studies, analyzing the correlation between nivolumab-induced changes in motility, cell-cell contacts and outcome after therapy are warranted.

Disclosures

No conflicts of interest to disclose.

Contributions

KY performed research, data analysis, interpreted data, wrote manuscript. SS, SG, HS, ASSD and AGL performed research, contributed essential material, analyzed data, interpreted data, and critically revised the manuscript. ED, M-LH, HY and RS performed data interpretation, contributed essential material, and critically revised the manuscript. SH developed the concept of study, acquired funding, analyzed and interpreted data, and wrote manuscript.

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Data-sharing statement

Imaging data are available from the corresponding author on reasonable request.

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