Complex Immune Evasion Strategies in Classical Hodgkin Lymphoma

Frederik Wein, Marc A. Weniger, Benedikt Höing, Judith Arnolds, Andreas Hüttmann, Martin-Leo Hansmann, Sylvia Hartmann, and Ralf Küppers

Abstract

The cellular microenvironment in classical Hodgkin lymphoma (cHL) is dominated by a mixed infiltrate of inflammatory cells with typically only about 1% Hodgkin and Reed/Sterberg (HRS) tumor cells. T cells are usually the largest population of cells in the cHL microenvironment, encompassing T helper (Th) cells, regulatory T cells (Tregs), and cytotoxic T cells. Th cells and Tregs presumably provide essential survival signals for HRS cells. Tregs are also involved in rescuing HRS cells from antitumor immune responses. An understanding of the immune evasion strategies of HRS cells is not only relevant for a characterization of the pathophysiology of cHL but is also clinically relevant, given the current treatment approaches targeting checkpoint inhibitors. Here, we characterized the cHL-specific CD4+ T-cell infiltrate regarding its role in immune evasion. Global gene expression analysis of CD4+ Th cells and Tregs isolated from cHL lymph nodes and reactive tonsils revealed that Treg signatures were enriched in CD4+ Th cells of cHL. Hence, HRS cells may induce Treg differentiation in Th cells, a conclusion supported by in vitro studies with Th cells and cHL cell lines. We also found evidence for immune-suppressive purinergic signaling and a role of the inhibitory receptor-ligand pairs B- and T-cell lymphocyte attenuator–herpesvirus entry mediator and CD200R–CD200 in promoting immune evasion. Taken together, this study highlights the relevance of Treg induction and reveals new immune checkpoint-driven immune evasion strategies in cHL. Cancer Immunol Res; 5(12); 1122–32. ©2017 AACR.

Introduction

Classical Hodgkin lymphoma (cHL) is one of the most frequent lymphomas in the Western world. The Hodgkin and Reed/Sterberg (HRS) tumor cells originate from germinal center (GC) B cells (1) but have lost most attributes typical of B cells (2). HRS cells retain expression of surface molecules that are relevant for a crosstalk with CD4+ T cells, including CD40, CD80, and CD86. HRS cells also express CD58 and major histocompatibility complex (MHC) II in some cHL (3). CD4+ T cells are usually the most abundant cell type in the cHL infiltrate, whereas HRS cells often account for only about 1% of cells in the tissue (4). HRS cells are typically surrounded by CD4+ T cells, a phenomenon called “rosetting.” These CD4+ T cells may not only provide survival signals to HRS cells, but also shield them from an attack by tumor-specific cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. HRS cells are dependent on CD4+ T cells: these T cells always accompany HRS cells, even when HRS cells disseminate into other organs, and HRS cells do not survive in immunodeficient mice and only very rarely in vitro (5).

In immune responses, T helper (Th) cells can differentiate into Th1 cells that support cytotoxic immune reactions or into Th2 cells that are involved in humoral immune responses and stimulate B cells. HRS cells secrete CCL5, CCL17, and CCL20 that attract Th2 cells, and Th2 cells are indeed frequent in the cHL microenvironment (6, 7). However, a further study indicated that Th1 cells predominate in the cHL tissue (8). HRS cells also attract natural regulatory T cells (nTreg) by secretion of the CCL5, CCL17, and CCL20 chemokines (9). HRS cell lines also induced a regulatory phenotype in naïve T cells (induced Treg; iTreg) upon cocultivation (10), although the suppressive potential of these T cells was not verified. The attraction and induction of Tregs may be one mechanism by which HRS cells escape elimination by CTLs and NK cells, but other factors likely contribute to immune evasion. For example, HRS cells secrete immunosuppressive cytokines, such as IL10 and TGFβ, express CD95, which can induce apoptosis in CD95L-positive CTLs, and PD-L1 and PD-L2, which inhibit the activity of PD1+ CTLs (3).

We aimed to study the interplay between HRS and CD4+ T cells by analyzing gene expression and differentiation stages of cHL-infiltrating Th cells and Tregs in comparison with these T-cell subsets from reactive lymphoid tissues. We identified and analyzed four components of the interaction between HRS cells and T cells: (i) generation of iTregs by HRS cells, (ii) immunosuppressive CD200R–CD200 interactions, (iii) immunosuppressive B- and T-cell lymphocyte attenuator (BTLA)–herpesvirus entry mediator (HVEM) interactions, and (iv) purinergic signaling in the cHL microenvironment. Our results confirmed the influence of HRS cells on T-cell differentiation and function. We found more complexity in the immune escape mechanisms of HRS cells than previously thought.
Materials and Methods

Cell culture and T-cell isolation

The cHL cell lines L428, KM-H2, L1236, SUP-HD1, and HD-LM2, the GC B cell-type diffuse large B cell lymphoma (GCB-DLBC) cell lines SU-DHL4 and SU-DHL6, and primary human CD3+ and CD4+ T lymphocytes were cultured in RPMI-1640 (PAN-Biotech GmbH) medium supplemented with 10% fetal calf serum (FCS; Biochrom AG). The cHL line U-HO1 was cultured as above but in IMDM (PAN-Biotech) with 10% FCS. Cell lines were obtained from the DSMZ. Cell lines were routinely tested for mycoplasma contamination and authenticated by short tandem repeat analysis (Promega). Cell culture media were supplemented with 1% penicillin-streptomycin (Invitrogen). Peripheral blood (PB) T cells were isolated from full blood donations or buffy coats by Ficoll density gradient centrifugation and subsequent CD3- or CD4-directed MACS strategies (Miltenyi Biotec) to a purity of more than 98%. For untouched isolation of CD4+ T cells the Human CD4+ T-cell Enrichment Kit (StemCell Technologies) was used. Untouched CD4+ CD25-depleted T cells were isolated by a subsequent depletion of CD25+ T cells using anti-CD25-MicroBeads (Miltenyi Biotec). Positive selection of CD25+ T cells from CD4+ T cells was used to enrich that population as a reference for Tregs in some experiments.

Isolation of T-cell subsets for global gene expression analysis

Fresh lymph node specimens from patients with cHL and tonsils from children or adolescents undergoing routine tonsillectomy were collected with donors’ informed consent, as approved by the local ethics committee of the Medical School of the University of Duisburg-Essen. Mononuclear cells were obtained following density centrifugation. Cell suspensions were stained with anti-CD4-APC, anti-CD25-FITC, and anti-CD127-PE antibodies (all from BD Biosciences) to identify CD4+CD25+CD127− Th cells and CD4+CD25−CD127+ Tregs. T-cell populations were subsequently isolated by cell sorting using a FACS Vantage SE with Digital DiVa option cell sorter (Becton Dickinson).

Gene expression profiling

Integrity of RNA isolated from T cells was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples were prepared from five samples each of tonsillar and cHL lymph node Th cells and Tregs for genechip analysis with the Ovation Pico WTA System Kit V2 (NuGen) according to manufacturer’s instructions and analyzed on Affymetrix GeneChip Human Gene ST arrays. After quality assessment in the Affymetrix Expression Console, one technical outlier was removed from the data set. The remaining 19 arrays were further analyzed in PartekGS, using the RMA algorithm for signal summarization, background correction and normalization. Differential gene expression was analyzed with ANOVA and gene set enrichment analysis (GSEA) was performed using the MSigDB database v5.2. The genechip data have been submitted to the Gene Expression Omnibus database under accession number GSE102693. Gene expression profiling data of primary, microdissected HRS cells, cHL cell lines, and GC B cells were available through an earlier study (11).

RNA isolation, cDNA synthesis, and real-time reverse transcription (RT)-PCR

Total RNA was prepared using the peqGOLD MicroSpin Total RNA Kit (Peglab). Total RNA of 5 × 10^6 – 5 × 10^7 cells were reverse transcribed using the High capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems) using predesigned Applied Biosystems TaqMan Gene Expression Assays (Hs01060665_g1: IRF4; Hs01056533, or specific primers and the SYBR Green PCR Table Master Mix (Sigma; Supplementary Table S1).

Flow cytometry

For flow cytometry analysis using a FACS-Canto cytometer (Becton Dickinson), cells were stained with the following antibodies from BD Biosciences: anti-CD4-APC (RPA-T4), anti-CD25-FITC (M-A251), anti-CD73-PE (AD2), anti-CD127-PE (HI7R-M21), and for isotype control mouse anti-IgG2b/APC/FITC; from eBioscience anti-CD39-PE, anti-CD73-PE-Cy7, from BioLegend anti-CD8-APC, anti-CD272-PE (BTLA; MH126), anti-CD200-PE (OX-104), anti-CD200R-PE (OX-108), and from Miltenyi Biotec anti-CD270-PE (HVEM). For all intracellular stainings or a combination of surface and intracellular staining, cells were treated with the FIX & PERM Cell Fixation and Cell Permeabilization Kit (Thermo Fisher), using the following antibodies: anti-FOXP3-PE (259D/C7; BD), anti-CTLA4/CD152-PE (14D3; eBioscience), and anti-IL-2-PE (N7.48A; Miltenyi Biotec), anti-TNFR-FITC (CA2, Miltenyi Biotec), anti-IFNy-FITC (45-15; Miltenyi Biotec). For data analysis, BD FACS Canto software or the FlowJo software was used.

T-cell activation assays

Intracellular cytokine staining was performed after addition of phorbol-12-myristate-13-acetate (PMA; 50 ng/μL), ionomycin (1 μg/μL), and Brefeldin A (10 μg/μL) for 4.5 hours at 37°C to stimulate single or HRS cell cocultured T cells. Prior to stimulation, cells were cultured with or without HRS cells in 12-well cell culture dishes using 2 × 10^5 HRS cells and 2 × 10^5 T cells and treated with a blocking anti-CD200R antibody (OX108; anti-human CD200R) and for control a mouse anti-IgG2b kappa (both functional grade purified from eBioscience) in a final concentration of 10 μg/mL or a selective A2A adenosine receptor antagonist (SCH58261; TOCRIS) in a concentration of 2 μmol/L to block extracellular adenosine (eADO) and A2AR interaction. The corresponding concentration of DMSO was used in controls without antagonist. In these assays, 0.5 mmol/L ATP was added to allow generation of eADO.

For the carbonylfluorescein-succinimidyl-ester (CFSE)-based suppression assay (Biolegend) HRS cell-primed T cells were tested for immunosuppressive activity in cocultures with proliferating autologous CD3+ responder cells (RC). Prior to coculture with cHL cell line L428 for 72 hours at a HRS cell: T-cell ratio of 1:10 T cells was negatively enriched for CD4+ and depleted of CD25+ cells, as described above. Subsequently, primed T cells were separated from L428 cells via cell sorting and added to CFSE-labeled autologous CD3+ RC (1 × 10^6/well) in wells of flat-bottom 96-well plates at optimized cell ratio of 1:1. To induce proliferation, cells were
stimulated with plate-bound anti-CD3 monoclonal antibody (1 µg/100 µl/well; ebioscience), soluble monoclonal anti-CD28 (5 µg/ml; ebioscience), and 400 IU/ml IL2 (ebioscience) for 4 days. Protein G (5 µg/ml; Sigma-Aldrich) was added to crosslink the stimulating antibodies. Subsequently, cells were harvested and stained with anti-CD8 prior to flow cytometry to discriminate fast proliferating CFSElow/negative RC and unlabeled CD4+ cells, and to specifically monitor CD8+ RC.

Statistical methods

Data are presented as mean ± standard deviation. Experimental results were compared using the Student 2-tailed t test for paired samples or the unpaired t test for unequal sample sizes. Significant P values are indicated by an asterisk (*, P < 0.05).

Results

Characterizing cHL-infiltrating T cells by gene expression profiling

To identify characteristics of cHL-infiltrating T cells, we compared global gene expression profile of CD4+ T cells from five cHL lymph nodes with the profiles of corresponding T cells from five reactive tonsils by Affymetrix genechip analysis. CD4+ Treg and Th cells were isolated according to their surface expression pattern of CD25 and CD127 (14). CD4+CD25+/CD127+ cells isolated from cHL lymph node biopsies were termed cHL-Tregs, and CD4+CD25-CD127high cells were termed cHL-Th cells. Tonsillar T-cell subsets with the respective expression patterns are termed tons-Th cells and tons-Tregs. By unsupervised hierarchical clustering, tons-Th cells clustered separately from cHL-Th cells, and tons-Tregs clustered separately from cHL-Tregs (Fig. 1A). This indicates that there are consistent differences in gene expression for each of the two T-cell subsets from reactive tissues and cHL lymph nodes. However, in supervised comparisons of the T-cell subsets from the different sources no probe set was significantly differentially expressed (P value ≤ 0.05 and a false discovery rate [FDR] ≤ 20%). This may indicate a substantial variation in gene expression at the level of single genes.

To confirm differential expression of selected candidate genes, qRT-PCR was performed with additional cHL and tonsillar T-cell samples. We focused on genes with a potential role in HRS/T-cell interactions. Increased amounts of FOXP3 and CTLA4 mRNA in both T-cell populations confirmed successful Treg isolation (Fig. 1B). We also observed higher CTLA4 transcript levels in cHL-Th than in tons-Th cells. Upregulation of IRF4 in cHL-Th cells suggests that naïve CD4+ cells are polarized toward Th2 and Tregs rather than Th1 cells (15). In both CD4+ T-cell subsets from cHL, qRT-PCR also validated high expression of hepatoma-derived growth factor-related protein 3 (HDGFRP3; Fig. 1B), which contributes to tumor resistance, likely by inactivating reactive oxygen species (16). CD26 expression levels were reduced in cHL-Tregs, whereas transcript amounts of CD38 and autoxatin (ATX) were increased (Fig. 1B). These three factors are involved in purinergic signaling. Moreover, two receptors with immunosuppressive functions, namely BTLA and CD200R, showed higher transcript levels in cHL-Tregs than in tons-Tregs (Fig. 1B).

Performing GSEA, 356 and 732 publicly available gene sets were significantly (P < 0.01; FDR < 5%) enriched in the cHL-Treg and Th cell subsets, respectively, in comparison with the corresponding tonsillar T-cell subsets. Three gene sets that reflect a Treg-associated phenotype were enriched in both Treg populations, thus further validating the cell sorting strategy (Table 1). cHL-Th cells also showed enrichment for these gene sets in comparison with tons-Th cells, indicating that cHL-Th cells have acquired features of a Treg cell phenotype (Table 1). The aforementioned high CTLA4 expression in cHL-Th cells supports this finding.

Further GSEA implicated that the cHL-Th cells encompass both Th1 and Th2 polarized Th cells (Fig. 1C). Th2 cell polarization may be linked to IL4 and or/IL6 signaling, as such cytokine-driven signatures were enriched in cHL-Th cells. Elevated levels of prostaglandin E2 (PGE2) impart a CD4+ T-cell activation in the cHL microenvironment (17). A PGE2 response gene set was 2.44-fold enriched in cHL-Th cells, supporting the presence of a prostaglandin signature in cHL-Th cells. We also observed an IL15 signaling signature in cHL-Th cells (Fig. 1C) which supports the finding that HRS cells secrete IL15 (18). Thus, the gene expression patterns of cHL-infiltrating Th cells and Tregs differ from those of the subsets from reactive lymphoid tissues. The changes in gene expression likely reflect responses to signals derived from HRS cells. The cHL-infiltrating Th cell population seems to be heterogeneous, with acquisition of gene expression signatures reflecting those of Th1 and Th2 cells as well as Tregs. From our gene expression analysis, we conclude that immune evasion strategies in cHL include purinergic signaling and the immunosuppressive receptors CD200R and BTLA.

HRS cell lines induce Treg cell features in cocultured CD4+ Th cells

Based on the observation of a partial Treg gene expression pattern among cHL-Th cells, we investigated the potential of HRS cells to induce Treg phenotype and function in CD4+ cells. Either bulk CD4+ T cells or CD25-depleted CD4+ T cells from healthy blood donors were used for coculture experiments. After 3 days of coculture of CD4+CD25+ T cells with HRS cells, a CD4+CD25high subpopulation appeared (Fig. 2A and B). When using bulk CD4+ cells the frequency of CD25+CD127low Tregs was even higher, representing a combination of preexisting (and perhaps expanded) and iTregs (Fig. 2A and B). Cocultures with a DLBCL cell line (SU-DHL1), serving as a non-Hodgkin lymphoma reference, caused no increase of Treg frequencies. Induction of FOXP3 and CTLA4, as measured by qRT-PCR and flow
cytometry, confirmed that the CD4⁺CD25⁺⁺CD127⁻ cells induced from the cocultures that were initially depleted of CD25⁺ T cells were indeed Tregs (Fig. 2C and D). HELIOS (IKZF2), a marker for nTregs (19), was not upregulated in the induced CD4⁺CD25⁺ cells after coculture, further indicating that HRS cells induced the Treg cell phenotype in Th cells (Fig. 2C). Exposure of CD4⁺ T cells to HRS cells also suppressed Th1 differentiation, as reflected by reduced expression of Th1-characteristic cytokines such as TNFα, IL2, and IFNγ after PMA/ionomycin stimulation (Fig. 2E).

The suppressive potential of L428-primed T cells was analyzed with a CFSE-based suppression assay. CD4⁺CD25⁺ T cells were cocultured with L428 cells for 3 days, separated by cell sorting, mixed with CFSE-labeled, autologous CD3⁺ RC. To monitor proliferation of CD8⁺ T cells, RC were stimulated by plate-bound anti-CD3 and anti-CD28 (Fig. 2F). In three
Wein et al.

Table 1. Treg cell gene sets are not only enriched in Treg cell subsets, but also in chL-Th cells in comparison with ton-Th cells

<table>
<thead>
<tr>
<th>Human gene set</th>
<th>CHL-Th vs.</th>
<th>CHL-Treg vs.</th>
<th>ton-Th vs.</th>
<th>CHL-Treg vs.</th>
<th>CHL-Th vs.</th>
<th>ton-Treg vs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSE24634</td>
<td>NES</td>
<td>NES</td>
<td>NES</td>
<td>2.9a</td>
<td>1.03</td>
<td>1.14</td>
</tr>
<tr>
<td>GSE22045</td>
<td>2.8a</td>
<td>2.22</td>
<td>2.36</td>
<td>1.28</td>
<td>1.74</td>
<td>2.0a</td>
</tr>
<tr>
<td>GSE15659</td>
<td>1.14</td>
<td>1.60</td>
<td>1.60</td>
<td>2.0a</td>
<td>1.05</td>
<td>1.05</td>
</tr>
</tbody>
</table>

NOTE: GSE24634, TREG VS TCONV POST DAY7 IL4 CONVERSION UP: Genes upregulated in comparison of CD25+ T cells treated with IL4 at day 7 versus CD25− T cells treated with IL4 at day 7.

GSE22045, TREG VS TCONV UP: Genes upregulated in comparison of regulatory T cells (Treg) versus conventional T cells.

GSE15659, NAIVE_CD4_TCELL VS ACTIVATED_TREG_DN: Genes downregulated in comparison of naive CD4 T cells versus activated Tregs.

*9/q < 0.01. vs., versus.

Wein et al.

independent experiments, we observed reduced proportions of proliferating, CFSE-low CD8+ RC in the condition with cocultured L428-primed T cells (Fig. 2F and G).

These in vitro studies show that HRS cells not only dampen responses typical of Th1 cells but also induce characteristics of Tregs from a population of naive CD4+ T cells.

Checkpoint inhibitors on chL-infiltrating T cells dampen tumor immunity

Treg cell frequencies were on average four times higher in chL lymph nodes than in reactive lymph nodes (rLN) or tonsils (Fig. 3A). Because of the detection of increased BTLA transcript levels in chL-Tregs compared with ton-Tregs, we decided to analyze this factor further. BTLA is an inhibitory receptor; BTLAhigh T cells are inhibited in the presence of its ligand, HVEM (TNFRSF14, CD270; ref. 20). BTLAhigh CD4+ T cells were more common in chL than in rLN or tonsils (Fig. 3B). Flow cytometry, we observed increased frequencies of BTLAhigh cells also among CD4+ CD25+ T cells from chL samples (Fig. 3E and F). As BTLA expression by Tregs has been reported in colitis and HBV infection (22, 23), the immunosuppressive HVEM-BTLA axis might not be exclusive to HRS-T-cell interaction but could also include Treg–T effector cell interaction.

Because of the increased transcription of the inhibitory CD200R (24) in chL-Treg and chL-Th cells in comparison with their reactive tissue counterparts (Fig. 1B), we also analyzed this receptor and its ligand in more detail. Frequencies of CD4+ T cells expressing CD200 were increased among 3/6 chL samples, in comparison with rLN and tonsils (Fig. 3G and H). As CD200 expression by Tregs has been reported in colitis and HBV infection (22, 23), the immunosuppressive HVEM-BTLA axis might not be exclusive to HRS–T effector cell interaction but could also include Treg–T effector cell interaction.

Increased expression of ATX and CD38 and downregulated expression of CD26 in T cells from chL compared with tonsillar T cells (Fig. 1B) implicated purinergic signaling in the chL microenvironment, as did an earlier study showing high expression of ATX by HRS cells (25). eADO-dependent immunosuppression is mediated by the purinergic A2AR receptor (A2AR) on effector T cells. Combined activity of the ectonucleases CD39 and CD73 generates eADO from ATP through hydrolyzation of intermediate products ADP and AMP. Alternative pathways for eADO generation from ATP involve ATX (also known as ENPP2) or CD38 (4A; refs. 26, 27). Removal of eADO is mainly mediated by adenosine deaminase (ADA), which needs CD26 for binding to the cell surface (28).

Purinergic signaling in chL

Increased expression of ATX and CD38 and downregulated expression of CD26 in T cells from chL compared with tonsillar T cells (Fig. 1B) implicated purinergic signaling in the chL microenvironment, as did an earlier study showing high expression of ATX by HRS cells (25). eADO-dependent immunosuppression is mediated by the purinergic A2AR receptor (A2AR) on effector T cells. Combined activity of the ectonucleases CD39 and CD73 generates eADO from ATP through hydrolyzation of intermediate products ADP and AMP. Alternative pathways for eADO generation from ATP involve ATX (also known as ENPP2) or CD38 (4A; refs. 26, 27). Removal of eADO is mainly mediated by adenosine deaminase (ADA), which needs CD26 for binding to the cell surface (28).

Because of the increased expression of ATX and CD38 and the low CD26 mRNA levels in chL-Tregs, revision of published genecip data revealed that ADA is downregulated in HRS cells in comparison with normal GC B cells (Fig. 4B). Flow cytometric analysis revealed that 3/7 chL cases had elevated frequencies of CD39-expressing CD4+ T cells, suggesting that these cells are a source of CD39-mediated eADO (Fig. 4C). Many of these chL CD39+ T cells coexpress CD25, identifying them as Tregs (Fig. 4C). A cellular infiltrate that favors eADO production was also reflected by a histochemical assay to measure 5’-nucleotidase enzymatic activity of CD37. All eight chL cases showed activity (Fig. 4D, Supplementary Fig. S1), as did all five cases of lymphadenitis. Of DLBCL cases, only 2/5 showed any activity.

Finally, we tested effects of eADO on T cells. CD4+ T cells cocultured with a chL cell line showed increased frequencies of CD39+ cells, but cocultures of T cells with a DLBCL cell line did not (Fig. 4E). Four of five chL cell lines expressed CD39 (Fig. 4F). To analyze whether eADO production and purinergic signaling dampen T-cell activation, we cocultured the chL cell line KM-H2 with CD4+ T cells and added ATP with or without the A2AR antagonist SCH-58261. Subsequent PMA/ionomycin-dependent T-cell stimulation triggered a higher proportion of IL2- and TNFα-producing T cells if A2AR signaling was blocked. The effect was ATP-dependent and stronger in cocultures of T cells with KM-H2 than in cocultures without KM-H2 cells, indicating that HRS cells dampened eADO-mediated T-cell effector response (Fig. 4G).
As the cHL microenvironment could promote production and reduce elimination of immunosuppressive eADO, disruption of purinergic signaling might reinstate antitumor immunity in cHL.

Discussion

T cells are the most abundant cell type among the lymphoma-infiltrating immune cells in cHL. The T-cell population is composed of Th cells, Tregs, and CD8^+ T cells. HRS cells attract Th and Tregs into the lymphoma microenvironment to obtain survival and stimulatory signals from them, and Tregs shield HRS cells from attack by CTLs (3, 29). Prior studies reported conflicting results on whether Th2 or Th1 cells predominate among cHL-Th cells (6–8). Our global gene expression study of cHL and tonsillar CD4^+ Th cells and Tregs was not designed to resolve this issue, but we did find stronger gene signatures for both Th1 and Th2 cells among cHL-Th cells than tonsillar Th cells, indicating that both Th cell subsets are present in the cHL microenvironment. Moreover, gene sets that signify IL4-, IL6-, IL15-, and PGE2-induced signaling...
Figure 3.
Increased proportions of CD25-, BTLA-, and CD200R-expressing T cells in cHL lymph nodes. A, Left: Representative examples of CD4⁺ T cells displaying variable frequencies of CD4⁺CD25high T cells in PB mononuclear cells (PBMC, top left), tonsils (ton, top right), rLN (bottom left), and cHL (bottom right). Right: Overview of measurements. Shown is the percentage of CD4⁺CD25high T cells among CD4⁺ T cells. B, Representative examples of tonsillar (top histogram) and cHL (bottom histogram) measurement of BTLAhighCD4⁺ T cells, and summary of the measurements for the frequency of BTLAhigh cells among CD4⁺ T cells. C, Representative histograms of cHL- and NHL-derived cell lines stained for HVEM surface expression. Black line: anti-HVEM staining, gray line, isotype control. D, Diagram displaying the HVEM⁺ proportions of CD25low and CD25high T-cell subsets. Gating strategy is indicated in the bottom graphs. E, Diagram displays proportions of BTLA⁺ cells among the CD4⁺CD25⁺ T cells. F, Exemplary dot plot of CD4⁺ T cells, isolated from cHL lymph nodes that were costained for CD25 and BTLA. G, Representative examples of tonsillar (top histogram) and cHL (bottom histogram) measurements of CD200 expression on CD4⁺ T cells, and the statistical overview of the frequency of CD200⁺ T cells among CD4⁺ T cells in rLN, tonsils (ton), and cHL tissues (right). H, Histograms of cHL cell lines stained for CD200 surface expression. Gray lines: isotype control; black lines: anti-CD200 staining. I, Frequencies of CD4⁺ T cells expressing CD200R. J, Immunohistochemistry for CD200R expression showing a representative example of a lymph node section with CD200R⁺ HRS cells (arrows) and lymphocytes (arrow heads). K, T cells were cocultured with L428 cells and treated or untreated (ctrl) with an anti-CD200R-specific antibody blocking the interaction of CD200 and CD200R. Prior to analysis for intracellular cytokine expression, cells were stimulated with PMA/ionomycin. The frequencies of IL2⁺TNFα⁺ cells among CD4⁺ T cells are depicted. HRS cell-T cell coculture lasted typically for 3 days. *, P < 0.05; paired or unpaired t test. Independent experiments and samples are indicated by dots in diagrams.
Figure 4.
Upregulation of eADO-generating factors by the cHL cellular infiltrate and its immunomodulatory effect on T cells.

A, Sketch of the enzymatic cascade leading to the accumulation and removal of immunosuppressive eADO from ATP. The ectoenzymes CD38, CD39, CD73, and ATX (autotaxin/ENPP2) favor the generation of eADO through intermediate substrates such as ADP, AMP, and cAMP. eADO can be removed by the extracellular ADA that is attached to CD26, catalyzing the deamination of eADO into inosine.

B, Gene chip-based expression intensity of ATX and ADA in primary HRS cells and GC B cells. Data are taken from Tiacci et al. (11).

C, Left: Representative examples of CD39 staining of CD4\(^+\) T cells in tonsils and cHL displaying also a cHL case with a pronounced subpopulation of CD39\(^+\)CD25\(^+\) T cells. Right: The proportion of the CD39\(^+\) population among the CD4\(^+\) T cells in rLN (n = 3), tonsils (n = 3), and cHL (n = 7) cases as indicated in dot plot.

D, Tabular overview of histochemistry assay to measure CD73-specific 5'-nucleotidase enzymatic activity in cHL, DLBCL, and rLN.

E, Flow cytometric analysis of CD39 induction on CD4\(^+\) T cells that were cocultured with L428 cells or SUD-HL4 for 3 days.

F, Histograms of cHL cell lines stained for CD39 surface expression.

G, Single T-cell cultures (\(^*\), P < 0.05) or T cell/KM-H2 cocultures (\(^**\), P = 0.002) were treated with ATP to allow eADO generation for 3 days prior to PMA/ion stimulation.
are enriched in cHL-Th cells. Indeed, these factors can be expressed by HRS cells and may also be expressed by other sources in the micromilieu (17, 18, 30). Our analysis confirms that these factors influence gene expression in infiltrating Th cells.

In comparison with rLN, cHL shows increased Treg numbers (6, 9, 31, 32), as we confirmed by flow cytometry. We assume that these cells prevent elimination of HRS cells by CTLs or NK cells (6, 31, 32). HRS cells secrete several chemokines that attract Tregs (9, 30, 33). Active recruitment of natural Tregs by HRS cells likely promotes accumulation of Tregs in the vicinity of HRS cells. However, HRS cells may also induce Treg differentiation in Th cells, although their suppressive abilities were not validated in vitro (10). cHL-Th cells were enriched for Treg cell-specific signatures and showed upregulation of the Treg cell markers CTLA4 and IRF4 in comparison with ton-Th cells. The observed upregulation of Treg cell-specific genes may reflect the ongoing instruction of Th cells to become iTregs. However, the presence of CD4+/CD25+ CTLA4+ T regulatory type 1 cells among the cHL-Th cells isolated as CD4+/CD25−CD127+ T cells cannot be excluded. Such a population has been postulated to exist in cHL lymph nodes (32, 33). We therefore addressed the potential differentiation of Th cells into iTregs under influence of HRS cells by coculturing experiments of CD4+ T cells with cHL cell lines. As CD4+ T cells were not HLA matched, a potential influence of HLA mismatching on the behavior of the CD4+ T cells must be considered. However, an anti-HRS cell activity of CD4+ T cells against HLR cell lines in coculture becomes detectable only after about 6 days of culture (34). Gene expression profiling revealed that after 5 days of coculture with the cHL cell line L428, HLA-mismatched and -unmatched CD4+ T cells showed similar changes in their gene expression (34). Hence, the influence of HRS cells on cocultured CD4+ T cells is stronger than effects through a mismatched MHC class II. In the majority of cHL cases, HRS cells show downregulation of MHC class I and/or class II expression (35), and in about 40% of cases still expressing MHC class II, it is not functional, as the invariant chain peptide CLIP is presented instead of foreign peptides (36). Thus, cognate MHC/TCR interaction of HRS cells with T cells is impaired in most cHL. In the coculturing experiments, we induced a CD25high T-cell population that expressed FOXP3 and CTLA4 and suppressed activation of CD8+ T cells, thereby showing that the cells acquired functional characteristics of Tregs.

In the cHL CD4+ T-cell subsets, several coidentified receptors were upregulated, potentially mediating inhibitory signals to T effector cells upon engagement. One of these receptors was BTLA, which is structurally related to CTLA4 and PD-1. These three share three conserved immunoreceptor tyrosine-based inhibition motifs (ITIM) for the recruitment of SHP-1/2, consistent with an inhibitory function for BTLA (37). BTLAhigh T cells are inhibited in the presence of its ligand, HVEM, that is expressed on the surface of tumor B cells and by Tregs (20, 21). We observed higher frequencies of CD4+ T cells expressing increased levels of BTLA in cHL than in rLN or tonsils. BTLA-expressing lymphocytes surrounding HRS cells have been reported, but whether these are Treg or Th cells was not specified (38). We observed expression of the BTLA ligand HVEM by cHL-derived cell lines, as did another study that showed that primary HRS cells also express HVEM (21). One study reported recurrent somatic losses of HVEM in primary HRS cells in about 40% of cHL cases, however, all cases and cHL cell lines investigated in that work still expressed HVEM (39). Taken together, HRS cells and cHL-infiltrating Tregs may dampen T effector cell responses through HVEM-BTLA interaction.

A second co-inhibitory receptor that attracted our interest was CD200. In several types of tumors, CD200–CD200R interaction is associated with tumor progression and blockade of this axis can restore antitumor responses (40). CD200R is primarily expressed by myeloid cells, dendritic cells, and T cells, and engagement of CD200R on T cells inhibits their activity (24). This can also induce a shift from a Th1 to a Th2 phenotype in CD4+ T cells or differentiation toward a Treg phenotype. We observed high numbers of CD4+CD200− T cells in 3/6 cHL biopsies. Primary HRS cells consistently and strongly express CD200 (41). CD200R expression was seen at increased frequency among cHL-infiltrating CD4+ T cells and HRS cells. Using an antibody to disrupt CD200–CD200R interaction in coculture of a HRS cell line with CD4+ T cells, we observed an increased percentage of IL2−TNFα−-activated T cells upon in vitro stimulation. Whether this activation is due to interruption of the CD200–CD200R interaction between CD200+ HRS cells and CD200− T cells or between CD200+ T cells and CD200+ T cells was unclear, as T cells coexpress CD200 and CD200R following TCR activation (42).

Regarding purinergic signaling in cHL, we showed expression of factors generating immunosuppressive eADO from ATP. These factors are also expressed by several cell types other than CD4+ T cells. The presence of sufficient eATP in the cHL microenvironment for eADO generation could not be evaluated but seems probable, considering the inflammatory microenvironment densely packed with apoptotic and necrotic cells, conditions known to favor ATP release (3, 43–45). One factor favoring eADO production in cHL may be high CD39 expression on some Tregs, as we observed. Removal of eADO by extracellular ADA requires CD26, and cHL-Tregs express little CD26 (46). Therefore, the reported location of CD4+CD26+ T cells close to HRS cells supports the accumulation of eADO in the proximity of HRS cell-rosetting T cells. A role of purinergic signaling in cHL is further supported by our demonstration of CD73 activity in the cHL tissue, and of an influence of eADO on T-cell stimulation in cHL cell lines cocultured with CD4+ T cells. We observed increased mRNA for CD38, which can also mediate eADO generation, in cHL-Treg and -Th cells, in comparison with these T-cell subsets from tonsils (Fig. 1B). CD4+CD26−CD38+ T cells are frequent in the cHL microenvironment (38). Hence, cHL-infiltrating T cells can contribute to eADO production through CD38 expression. Similarities between immunosuppression triggered by eADO and established checkpoint pathways suggest that blockade of A2AR, as shown for solid cancers, may be attractive (47, 48). Local cellular stress, such as hypoxia, may be the common ground by which solid tumors and cHL promote eADO (3).

In conclusion, we found that immune evasion strategies in cHL include induction of Treg cell functions in Th cells in the microenvironment, expression of the inhibitory CD200R and BTLA receptors on cHL-infiltrating T cells, and expression of their ligands on HRS cells and/or other immune cells. We showed that purinergic signaling in the microenvironment may be a further immunosuppressive strategy. These complex immune evasion strategies in cHL are likely required for its pathophysiology. Indeed, targeting the inhibitory checkpoints CTLA4 and PD-1 has already shown therapeutic potential for treatment of cHL (49).
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: F. Wein, R. Küppers
Development of methodology: F. Wein
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Wein, B. Höing, J. Arnolds, A. Hüttermann, M.-L. Hansmann, S. Hartmann
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): F. Wein, M.A. Weniger, M.-L. Hansmann, S. Hartmann, R. Küppers
Writing, review, and/or revision of the manuscript: F. Wein, M.A. Weniger, B. Höing, S. Hartmann, R. Küppers

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Wein, M.A. Weniger, M.-L. Hansmann, S. Hartmann

References

Study supervision: F. Wein, R. Küppers
Other (performed research): F. Wein

Acknowledgments
This work was supported by the Deutsche Forschungsgemeinschaft (KL1131/7-1). We thank Philip Abstoß and Kerstin Heise for expert technical assistance, and Jens Stanelle for isolation of several T-cell samples. We also thank Eva Gau and Florian Windsdorfer for their contribution and advice on purinergic signaling.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 23, 2017; revised August 15, 2017; accepted October 13, 2017; published OnlineFirst October 25, 2017.
Complex Immune Evasion Strategies in Classical Hodgkin Lymphoma

Frederik Wein, Marc A. Weniger, Benedikt Höing, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-17-0325

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2017/10/25/2326-6066.CIR-17-0325.DC1

Cited articles
This article cites 49 articles, 18 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/5/12/1122.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/5/12/1122.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link:
http://cancerimmunolres.aacrjournals.org/content/5/12/1122.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.