Expression of LAG-3 by Tumor-infiltrating Lymphocytes is Co-incident with the Suppression of Latent Membrane Antigen-specific CD8+ T-cell Function in Hodgkin Lymphoma Patients

Maher K. Gandhi1,3*, Eleanore Lambley1*, Jaikumar Duraiswamy1, Ujjwal Dua1, Corey Smith1, Suzanne Elliott1, Devinder Gill3, Paula Marlton3, John F. Seymour2 and Rajiv Khanna1**

1Tumor Immunology Laboratory, Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research, Brisbane, Australia, 2Haematology Service, Peter MacCallum Cancer Centre, and University of Melbourne, Melbourne, Australia, 3Department of Haematology, Princess Alexandra Hospital, Brisbane, Australia.

*These authors have contributed equally to this work and their order should be considered arbitrary

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**Address for correspondence: Dr. R. Khanna, Queensland Institute of Medical Research, Bancroft Centre, 300 Herston Rd, Brisbane, Australia 4029

Tel: 61-7-3362 0385; Fax: 61-7-3845 3510; E mail: rajivK@qimr.edu.au

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Abstract

In Hodgkin’s Lymphoma (HL) the malignant Hodgkin Reed-Sternberg (HRS) cells comprise only 0.5-10% of the diseased tissue. The surrounding cellular infiltrate in enriched with T-cells which are hypothesized to modulate anti-tumor immunity. We show that a marker of regulatory T-cells, LAG-3 is strongly expressed on infiltrating lymphocytes present in proximity to HRS cells. Circulating regulatory T-cells (CD4+CD25hiCD45ROhi, CD4+CTLA4hi and CD4+LAG-3hi) were elevated in HL patients with active disease when compared to remission. Longitudinal profiling of EBV-specific CD8+ T-cell responses in 94 HL patients revealed a selective loss of interferon-γ expression by CD8+ T-cells specific for latent membrane proteins (LMP) 1 and 2, irrespective of EBV tissue status. Intra-tumoral LAG-3 expression was associated with EBV tissue positivity, whereas FOXP3 was linked with neither LAG-3 nor EBV tissue status. The level of LAG-3 and FOXP3 expression on the tumor-infiltrating lymphocytes was co-incident with impairment of LMP1/2-specific T-cell function. In vitro pre-exposure of peripheral blood mononuclear cells to HRS cell-line supernatant significantly increased the expansion of regulatory T-cells and suppressed LMP-specific T-cell responses. Deletion of CD4+ LAG-3+ T-cells enhanced LMP-specific reactivity. These findings indicate a pivotal role for regulatory T-cells and LAG-3 in the suppression of EBV-specific cell-mediated immunity in HL.
Introduction

There is now compelling evidence that latent Epstein-Barr virus (EBV) infection is controlled by a population of virus-specific (largely CD8+) cytotoxic T lymphocytes (CTL). These CTLs recognize epitopes frequently derived from EBV nuclear antigens (EBNA) 2, 3, 4 and 6\(^1\). Although the mechanisms involved in controlling intermittent virus reactivation from latency have not yet been completely defined, it is clear that the interaction between specific CTL and their targets form a key element in preventing unchecked viral proliferation.

EBV-associated malignancies can arise in both immunosuppressed and immunocompetent individuals. These malignancies are of variable cellular origin and while some involve specific genetic lesions, all involve the expression of either some or all of the EBV latent proteins (reviewed in\(^1\)). In developed countries EBV may be implicated in the pathogenesis of 30-50% of cases of Hodgkin's lymphoma (HL)\(^{1,3-5}\) and cell-free EBV-DNA can be used as a biomarker for EBV-positive HL such that serial monitoring can predict response to therapy\(^6\). In EBV-positive HL, the majority of viral genome positive cells are monoclonal, indicating that infection of the malignant B-cells occurred before clonal expansion\(^7\). Serologically confirmed infectious mononucleosis-related EBV infection is associated with an increased risk of EBV-positive HL in young adults\(^8\). Like many other EBV-associated malignancies, the malignant B-cells (referred to as Hodgkin Reed-Sternberg, HRS cells) in HL are characterized by their unique viral and cellular phenotype. HRS cells display a type II form of latency with viral antigen expression limited to EBNA1, latent membrane proteins 1 and 2 (LMP1/2), as well as the EBER1, EBER2 and BamHIA transcripts\(^9\). Recent data describes a specific HLA class I association with EBV-positive HL, suggesting that deficient antigenic presentation of LMP1/2
peptides may be involved in the pathogenesis of EBV-positive HL\textsuperscript{10}. Furthermore, HRS cells have evolved multiple strategies to evade the potent EBV-specific CTL response and these have been proposed to be linked to the cell-mediated immune deficiency that is present in the early phase of the disease.

Although the majority of patients with HL can be cured with currently available therapies, up to 30\% of patients with advanced HL will progress or relapse\textsuperscript{11}. New, more intensive chemotherapy regimens have significantly improved outcome\textsuperscript{12}, however, less than half of relapsed and refractory patients will respond to conventional salvage strategies. These data have raised the possibility of combining immunotherapy with chemo/radiotherapy particularly in patients with relapsed/refractory EBV-positive HL. Indeed, a number of attempts have been made to expand EBV-specific T-cell immunity \textit{in vitro} in the absence of an immunosuppressive environment, and to transfer the resulting expanded T-cells to treat patients with HL\textsuperscript{13,14}. However, an objective analysis of disease outcome highlights the need to explore alternate strategies to break tumor evasion mechanisms that preclude the development of a robust anti-tumor response. To enable further development of these strategies, it is important to understand the mechanism(s) by which the existing immune response to EBV-encoded antigens fails to block the outgrowth of malignant cells. Previous studies on HL patients have indicated that EBV-specific T-cells can be detected in the peripheral circulation that recognize EBV-specific antigens which are expressed in HRS cells, yet this rarely corresponds with effective tumor eradication\textsuperscript{15,16}. Recent studies on human malignancies have suggested that the lack of an effective anti-tumor response may be due to activation of unique subsets of CD4\textsuperscript{+} Treg cells which specifically inhibit antigen-specific CD8\textsuperscript{+} T-
cell responses\textsuperscript{17}. HL is a unique clinico-pathological entity in which the malignant HRS cells typically comprise only 0.1-10\% of the diseased tissue\textsuperscript{18}. Immunohistochemical and flow cytometric analysis of the surrounding cellular infiltrate from HL lymph nodes shows enrichment with cells that express the typical phenotypic markers of Treg cells including FOXP3, TGF-\(\beta\) and CTLA-4\textsuperscript{19-21}. Functional assays confirm that these infiltrating cells are capable of suppressing IFN-\(\gamma\) production and proliferation of autologous PBMC to mitogens and recall antigens\textsuperscript{19}. However, a role for Treg cells in modulating EBV-specific T-cell immunity against the EBV latent antigens expressed in HL has not previously been explored.

Here, in a collaborative study conducted under the auspices of the Australasian Leukaemia & Lymphoma Group (ALLG), we have prospectively profiled intra-tumoral Lymphocyte activation gene-3 (LAG-3) expression, which has recently been shown to be selectively up-regulated on Treg cells\textsuperscript{22}. Our aim was to investigate an association between Treg cells, the LAG-3 protein and the EBV peptide-specific T-cell responses in HL patients. Having demonstrated that the level of LAG-3 expression on the tumor-infiltrating lymphocytes was co-incident with the loss of LMP1/2-specific T-cell function, we then went on to establish that CD4\+ LAG-3 T-cells had regulatory properties. The data presented here firmly establishes the importance of CD8\+ T-cell responses to LMP1/2 antigens and has important implications for understanding the pathogenesis of HL.
Materials and Methods

Study Participants

A total of 94 newly diagnosed (ND), relapsed (RL) and remission (> 2 years from diagnosis) patients with histologically confirmed HL were included in this study. Table 1 provides details of the patient characteristics. One patient withdrew prior to providing samples. Data regarding age, gender, date of diagnosis and histology were collected in all patients. Mean age of the total group was 32 years (range 5-76 years) and 38% were female. In newly-diagnosed and relapsed patients, additional prognostic information was obtained to enable the prognostic score to be calculated. To serve as controls, ELISPOT analysis was performed on blood from 29 healthy subjects (12 female: 17 male, mean age 43 years, range 23-63). There was no significant difference in gender mix between the four groups, however, both RL patients and healthy subjects were significantly older than ND patients (p=0.0497 and p=0.0006 respectively) and remission patients (p=0.0113 and p<0.0001 respectively). This study conformed to the tenets of the Declaration of Helsinki and informed consent was provided as per each participating sites research ethics process. This study was formally approved by the Human Ethics committees of QIMR, PeterMac Cancer Institute and PA Hospital.

Tissue Staining

All assays were carried out on sections of routinely fixed, paraffin-embedded material. LMP1 and EBER assays were performed as per published guidelines. The distribution of HRS cells was assessed by a morphologist on matching haematoxylin and eosin slides prior to interpreting the LMP1 and EBER staining. Following antigen retrieval with trypsin, the anti-LMP IgG1 or isotype control (Dako, CA, USA) was used to detect the presence of LMP1.
Positive reactivity was detected as per manufacturer’s instructions incorporating DAB as the chromogenic substrate (Envision kit, Dako, CA, USA). The EBER in situ hybridisation assay utilised a commercially available hybridisation kit (Dako, CA, USA) and custom-made probes derived from EBER-1 and EBER-2. A fluoresceinated poly-dT oligonucleotide probe was used to hybridize to polyA mRNA in the tissue sections to serve as a control for tissue and mRNA integrity. For all stains, a known case of EBV-positive post-transplant lymphoproliferative disorder (responsive to EBV-specific CTL therapy) was used as a positive control.

LAG-3 and FOXP3 immunohistochemistry were performed as per manufacturer’s guidelines (Novacastra, Newcastle-Upon-Tyne, UK and Abcam, Cambridge, UK respectively). For both proteins, tonsillar tissue was used as a positive control and a characteristic interfollicular pattern was seen.

**Blood Samples**

In ND and RL cases, 30ml of EDTA blood was taken at diagnosis (prior to initiation of therapy), and then at six monthly intervals for 1 year. With healthy subjects and remission cases, one 30ml blood sample was taken, and in the latter group the time interval from sample to diagnosis recorded. PBMC were prepared by gradient centrifugation (Ficoll-Paque; Amersham Biosciences, Sweden). PBMC were stored by controlled rate freezing in liquid nitrogen, and plasma samples were stored at –70°C until further processing.

**Synthetic peptides.**

Twenty nine EBV-epitope synthetic peptides presented by a wide range of HLA class I alleles covering >90% of Caucasians were used in this study (HLA restriction in brackets): latent cycle protein LMP1 peptides YLLEMLWRL (A2), YLQPNWWTL (A2) and ALLVYLSFA (A2) and IALYLQQNW (B57)
 latent cycle protein LMP2A peptides FLYALALLL (A2), CLGGGLTMV (A2), LTAGFIFL (A2), SSSSCPLSDKI (A11), PYLFWLAAI (A23/24), TYGPVFMC (A24), VMSNTLLSAW (A25), FTASVSTVV (A28), and IEDPPFNSL (B60) \textsuperscript{27,16}; latent cycle protein EBNA3 peptides RLRSEAQVK (A3), RYSIFFDY (A24), RPPIFRRL (B7), FLRGRAYGL (B8), QAKWRLQTL (B8), YPLHEQHGM (B35) \textsuperscript{28-31}, latent cycle protein EBNA4 peptides AVFDKSKAK (A11), IVTDFSVIK (A11), AVLLHEESM (B35), GQGGSPATAM (B62) \textsuperscript{32}; latent cycle protein EBNA6 peptides LLDVRFGMV (B37), EENLLDFVRF (B44.02), EGGVGWRHW (B44.03) \textsuperscript{31,33,34}; lytic cycle protein peptides GLCTLVAML (A2), RAKFKQLL (B8), DYCNYLNKEF (A24) \textsuperscript{35-37}, derived from BMLF1, BZLF1, and BRLF1 respectively. All epitope peptides will be subsequently abbreviated to the first three amino acids throughout this text. The peptides were purchased from Mimotopes Pty Ltd., Melbourne, Australia and dissolved in 20% dimethyl sulfoxide at 2 mg/ml and diluted in RPMI 1640 to a final concentration of 10\mu g/ml.

**Peptide-specific T-cell cytokine secretion by IFN-\gamma ELISPOT assay.**

The IFN-\gamma ELISPOT assay has been described in detail elsewhere\textsuperscript{26}. Spots were counted automatically using image analysis software (ImagePro) and were expressed as spot-forming cells (SFC) per 10\textsuperscript{6} PBMCs. The number of IFN-\gamma secreting T-cells was calculated by subtracting the negative control value from the SFC count.

**Flow cytometry**

Monoclonal antibodies (mAb) conjugated to Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), or TriColour (TC) were specific for CD3, CD4, CD8, CD28, CD25, CD45RO and CTLA-4, (TCS Biologicals, Burlingdale, CA, USA), GITR (R&D Systems, Minneapolis, USA), LAG-3 (Alexis Biochemicals, CA,
USA) and relevant isotype controls. Intracellular expression of CTLA-4 and GITR, was performed using as per manufacturer's instructions (Cytofix/Cytoperm kit; BD PharMingen and ebioscience inc., San Diego, CA, USA respectively). We used peptide-MHC class I pentamers (Proimmune, Oxford, UK) to visualize T-cells recognizing the EBV peptides YLL, YLQ, FLY, IED, CLG, FLR, RPP, GLC and RAK. Staining of lymphocytes was undertaken by incubating the cells with a pre-titrated concentration of pentamer at room temperature for 20 minutes. The cells then were then washed in staining buffer, and stained for surface markers by incubation at 4°C for 15 minutes. Flow cytometry was performed on a FACS Canto (Becton Dickinson) cytometer and results analysed using FlowJo (Tree Star, Inc., Stanford, U.S.A.) software.

**In vitro exposure of PBMC to cell line supernatants**

To determine the effect of exposure of PBMC to HL cell line supernatants, 2 x 10^6 PBMC were incubated in either serum-free culture media (SFCM) or supernatants from HRS cell lines (L1236, HDLM2, L428, L540^{38-40}). These cell lines had been previously cultured in SFCM. In some experiments, CD4+ and LAG-3+ T-cells were depleted from these PBMC. This depletion was carried out using a MoFlo high performance cell sorter (DakoCytomation, Carpinteria, CA). Prior to cell sorting, PBMC were stained with PE-labelled anti-CD4 and FITC-labelled anti-LAG-3 antibodies. After incubation, these cells were analysed for CTLA-4 expression and ELISPOT assays as described above.

**Statistics**

Comparison of patient gender and age between groups was performed using the non-paired T test. For *ex vivo* studies using IFN-γ ELISPOT, flow cytometry, and peptide-HLA class I pentamers, the Mann Whitney non-parametric test for
un-paired groups was used to analyse samples from different time-points. Associations between EBV tissue status, % LAG-3, % FOXP3 and histology were performed using the Chi-squared test. The Wilcoxon matched pairs test was used to compare paired T-cell subsets generated within PBMC following exposure to HRS cell supernatants and for analysis of pre and post-therapy ELISPOT data. All p values were 2 sided, with values <0.05 considered significant. All statistical analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA.
Results

Intra-tumoral and peripheral blood lymphocytes from HL patients express high levels of LAG-3

Recent studies in a murine model have identified LAG-3 as a cell surface molecule which is selectively up-regulated on Treg cells that may be directly involved in mediating Treg function\textsuperscript{22}. To explore the potential role of LAG-3 in HL, we have investigated the expression of this marker on both tumor infiltrating and peripheral blood lymphocytes. In the first instance, tissue sections from 45 patients with histologically confirmed HL (Table 1) were stained for LAG-3. Patients were chosen solely on the basis of availability of tissue. There were no significant differences in age, gender or EBV tissue status between this subgroup and the total group of patients: mean 32 years (subgroup) versus 32 years (total); 40\% females versus 38\%; and 37\% EBV-positive HL versus 37\% respectively. LAG-3 staining on tumor-infiltrating lymphocytes was both at the cell surface and cytoplasmic. LAG-3 predominately stained lymphocytes present in those areas that were rich in HRS cells (see Figure. 1A). For each tissue section, the mean percentage of LAG-3 expressing cells as a proportion of all cells was calculated from five representative areas visualized using a 40x objective. Results were then graded in a semi-quantitative manner as a percentage of non-HRS cells within these areas that were positive for LAG-3. A summary of this analysis is presented in Table 2. Intra-tumoral LAG-3 staining was observed in classical and nodular lymphocyte predominant HL. We also noticed an association between LAG-3 expression and HL histology. HL with mixed cellularity (MC) and lymphocyte rich (LR) histology showed significantly higher frequency of LAG-3-positive tumor-infiltrating lymphocytes than nodular sclerosing (NS) HL (p=0.013). In agreement with the known association
between EBV status and HL histology\textsuperscript{1,3-5}, we also observed an association between LAG-3 and EBV gene expression in tumor tissues (p=0.023). In patients with remaining tissue samples (n=33), immunohistochemistry for the forkhead transcription factor FOXP3 was performed. In agreement with the findings of Alvaro and colleagues (22), we observed distinct intra-nuclear staining within lymphocytes, with variation of expression between patient samples. Lymphocytes more frequently expressed LAG-3 than FOXP3 (p=0.0003 Wilcoxon matched pairs test) (Table 2). Dual-staining for FOXP3 and LAG-3 showed that the majority of tumor-infiltrating lymphocytes did not express both proteins (Figure 1B). No association was found between FOXP3 expression and histology (p=0.489), EBV status (p=0.833) or LAG-3 (p=0.404).

Circulating CD4+LAG-3\textsuperscript{hi} are elevated in HL patients with active disease.

Having demonstrated that LAG-3 expressing tumor-infiltrating lymphocytes were prominent within HL lymph nodes, we next determined whether cells with the phenotype of Treg cells and LAG-3 were also present in the peripheral blood from patients with HL. Peripheral blood mononuclear cells (PBMC) from patients with HL were co-stained for CD3, CD4, CD8, CD25, CD45RO and LAG-3. This allowed identification of CD4+ CD3+ and CD8+ CD3+ T-cell subsets, and CD4+ CD25\textsuperscript{hi} CD45RO\textsuperscript{hi} Treg cells. In addition, PBMC were also stained for intracellular CTLA-4 (cytolytic T lymphocyte antigen-4), FOXP3 and GITR (glucocorticoid–induced tumor necrosis factor receptor family–related gene), which are expressed by various subsets of Treg cells\textsuperscript{41,42}. A representative flow cytometric plot showing LAG-3-positive CD4+ T-cells from one patient in remission is presented in Figure 2, panel A. To determine the distribution of LAG-3-positive CD4+ T-cells within the conventional Treg cell population, we analysed PBMC samples from a total of
12 newly diagnosed/relapsed (ND/RL) HL (prior to therapy) and 2 patients in long-term remission (for CTLA-4 and GITR), and 4 ND/RL (prior to therapy) and 10 remission HL patients (for FOXP3). A summary of data from these patients is presented in Figure 2, panel B. We observed that LAG-3-positive cells were enriched within intracellular CTLA-4$^{hi}$, and GITR$^{hi}$ CD4$^+$ T-cells but not within FOXP3 CD4$^+$ T-cells. This data strongly suggests that LAG-3 is expressed differentially within different subsets of Treg cells and is in accordance with our observation that tumor-infiltrating lymphocytes do not co-express LAG-3 and FOXP3. We analysed CD4$^+$ CD3$^+$ and CD8$^+$ CD3$^+$ T-cell subsets in 16 patients with ND/RL and 28 in long-term remission. The proportions of these subsets did not vary between patients with ND/RL HL and those in long-term remission (data not shown).

In the next set of analyses we compared the frequency of different Treg cell subsets in the peripheral blood from patients with ND/RL HL and those in long-term remission. A summary of this analysis is presented in Figure 3. The most striking observation was that all different subsets of Treg cells expressing conventional markers or CD4$^+$ LAG-3$^+$ T-cells were consistently elevated in patients with ND/RL HL when compared to the PBMC from patients in long-term remission and to 11 randomly selected healthy laboratory subjects (gender 7F:4M, mean age 37 years, range 22-47). Taken together these data strongly suggest that during active HL there is a significant expansion of Treg cells both at the tumor site and as well in the peripheral blood. Based on these observations, we hypothesized that this expansion may significantly impair antigen-specific T-cell responses.
**Ex-vivo** Latent Membrane Protein epitope-specific T-cell functional responses are impaired at diagnosis in HL patients.

To explore the potential effect of Treg cells on the antigen-specific CD8+ memory T-cell response, we longitudinally monitored EBV-specific T-cell responses in a large cohort of HL patients and healthy virus-carriers using interferon-γ (IFN-γ) ELISPOT and MHC-peptide pentamer assays. These cohorts included 94 HL patients (newly diagnosed/relapsed and long-term remission, Table 1) and 29 healthy virus-carriers. For ELISPOT assays, we used a panel of HLA class I restricted CD8+ T-cell epitopes derived from either latent (LMP1/2, EBNA3/4/6) and lytic (BZLF1 and BMLF1) proteins. The detailed data from these ELISPOT analyses is presented in Table 3 and Figure 4, Panel A-D). Table 3 and Figure 4, Panel A represent a cross-sectional analysis allowing comparison between patients with ND/RL HL at different time-points, and long-term remission and healthy EBV-seropositive subjects. The number of assays performed at each time-point reflects the HLA class I type and availability of blood from each patient. These analyses revealed that ND/RL HL patients with active disease (i.e. prior to therapy) displayed a selective loss of IFN-γ expression by CD8+ T-cells specific for LMP1/2 epitopes when compared to CD8+ T-cell responses from patients in long-term remission and healthy virus-carriers (p<0.0001, p=0.0016 respectively). These results were confirmed by sequentially following a given LMP1/2 peptide-specific CD8+ T-cell response in individual ND/RL HL patients from pre-therapy to post-therapy. (Figure 4, Panel C). In this matched-pair analysis, a significant increase in the LMP1/2 epitope-specific CD8+ T-cell response was observed in these patients following recovery from active HL (p<0.0001). In contrast, T-cell responses to the epitopes derived from EBNA3/4/6 and lytic antigens showed no impairment...
during active HL when compared to the responses observed following completion of therapy, or from patients in long-term remission or healthy virus-carriers (Table 3 and Figure 4, Panel C). The overall hierarchy of T-cell responses towards latent and lytic antigens in HL patients was quite similar to those seen in healthy virus-carriers (Lytic>EBNA3/4/6>LMP1/2; Figure 4; Panel B).

Intriguingly, the loss of CD8+ T-cell responses to LMP1/2 epitopes during active HL was not associated with EBV tumor status (Figure 4; Panel D). Patients with EBV-negative and EBV-positive HL showed comparable loss of LMP1/2 T-cell responses. Furthermore, the loss of LMP1 and LMP2 CD8+ T-cell responses also showed no association with the clinical staging or prognosis score of the patients (Figure 4; Panel D). For both ND/RL patients prior to therapy, and patients in remission, no difference was seen when ELISPOT responses against LMP1 were compared to LMP2 (data not shown). Unsurprisingly, similar results were seen in patients with active disease irrespective of whether they were newly diagnosed or relapsed (data not shown).

**Ex-vivo enumeration of EBV-specific CD8+ T-cells from HL patients shows no difference between active disease and remission.**

To determine whether the loss of LMP1 and LMP2 T-cell function was due to the deletion of T-cell precursors, we enumerated antigen-specific T-cells in patients with HL using MHC-peptide pentamers. A summary of this analysis based on a cohort of patients with ND/RL HL, in long-term remission and healthy virus-carriers is presented in Figure 5. In contrast to the ELISPOT assays, HL patients with acute disease showed normal precursor frequencies of CD8+ T-cells specific for LMP1/2 epitopes when compared to patients in long-
term remission and healthy virus-carriers (Figure 5). In agreement with the ELISPOT data, there was no significant difference in frequencies observed in EBV-positive and EBV-negative HL cases.

The level of LAG-3 and FOXP3 expression on the tumor-infiltrating lymphocytes is co-incident with the loss of LMP1/2-specific T-cell function.

Taken together our data suggest that the loss of IFN-γ production by LMP1 and LMP2-specific CD8+ T-cells during active HL is due to a functional impairment and this impairment might be related to the increased Treg cells in these patients. To explore this possibility we conducted an analysis to determine whether the level of LAG-3 and/or FOXP3 expression within HL lymph nodes is associated with loss of ex-vivo IFN-γ production by LMP1 and LMP2-specific CD8+ T-cells. This analysis revealed that IFN-γ production by LMP1 and LMP2-specific CD8+ T-cells in the peripheral blood was significantly impaired in HL patients whose tumor tissues showed high levels of LAG-3 and/or FOXP3 expression by tumor-infiltrating lymphocytes (26-100%) when compared to the tissues with low LAG-3 and/or FOXP3 expression (0-25%) (p=0.0151, and p=0.0266 respectively, Mann Whitney test; Figure 6).

In vitro pre-exposure of PBMC to supernatants from HRS cell lines induces expansion of Treg cells.

In the next set of experiments, we assessed the effect of supernatants on the HRS cells on the expansion of Treg cells in vitro. Data presented in Figure 7A and 7B show that incubation of PBMC with pooled supernatants from HRS cells resulted in 1.5-2 fold enhanced expansion of CD4+ CD3+ T-cells staining with intracellular CTLA-4 and surface LAG-3 when compared to the PBMC incubated with control culture medium. Following incubation for 3 days, overall
cell numbers (as determined by trypan blue exclusion assay) and CD3+ CD8+ / CD3+ CD4+ ratios remained unchanged (data not shown).

**Depletion of CD4+ LAG-3 T-cells from PBMC pre-exposed to HRS cell line supernatant results in enhancement of LMP1/2 -specific T-cell function.**

Previous studies have suggested that immunosuppressive cytokines secreted by HRS cells may contribute towards the suppression of virus-specific T-cell function in HL patients. Consistent with this, data obtained from experiments from 3 healthy EBV seropositive subjects showed that LMP-specific IFN-γ responses to LMP2 peptides by ELISPOT assay in PBMC exposed to HL line supernatants were significantly reduced as compared to matched controls (p=0.0078) (Figure 7C). To determine whether this effect is in part mediated by CD4+ LAG-3+ T-cells, we depleted CD4+ LAG-3+ T-cells from fresh PBMC and from the enriched CD4+ LAG-3+ T-cell population that had been generated following incubation with HRS supernatant PBMC. Purity of FACS sorted cells ranged between 98% and 100%. As can be seen in Figure 7D, removal of CD4+ LAG-3+ T-cells resulted in enhancement of LMP2-specific IFN-γ responses to LMP2 peptides.
Discussion

A number of phenotypic markers including intracellular CTLA-4, GITR, CD25 and FOXP3 have been identified as Treg cell selective receptors\textsuperscript{41-43}. The categorization between regulatory T-cells expressing different phenotypic markers may prove arbitrary, with the functional relationship between populations requiring clarification. Further complexity is generated by the expression of some of these markers in activated non-regulatory T-cell subsets. Recently, the CD4 homologue LAG-3 has been recognized as a marker of Treg cells and a mediator of their suppressive activity in a murine model\textsuperscript{22}. In this study, activated Treg cells had a 20-50 fold increase in LAG-3 mRNA expression, compared to only modest (1.5-4 fold) increases in FOXP3, GITR and CTLA-4. More importantly, CD4+CD25\textsuperscript{hi} Treg cells from LAG-3\textsuperscript{−/−} mice exhibited reduced regulatory activity and ectopic expression of LAG-3 on CD4+ T-cells conferred suppressor activity towards antigen-specific T-cells. Recent studies have also indicated that FOXP3 may be essential but is not sufficient for the Treg cell like suppressive activity.

We found LAG-3 was strongly expressed on tumor-infiltrating lymphocytes present in proximity to the HRS cells and the proportion of LAG-3 expressing lymphocytes correlated with the EBV status of the tumor. However, this may simply reflect the tendency of LAG-3 to be more highly expressed in MC and LR histological subtypes than in NS, as the latter was less frequently shown to be EBV-positive. A higher proportion of lymphocytes stained for LAG-3 than for FOXP3. This is consistent with the findings of Huang and colleagues (16) in which activated Treg cells had a 20-50 fold increase in LAG-3 mRNA expression, compared to only modest (1.5-4 fold) increases in FOXP3. \textit{Ex vivo} analysis of PBMC from HL patients revealed that LAG-3-positive cells were
enriched within CD4+ T-cells expressing high levels of intracellular CTLA-4<sup>hi</sup> and GITR<sup>hi</sup> but not FOXP3<sup>hi</sup>, and that the number of Treg cells were elevated in ND/RL patients prior to therapy but returned to normal levels following attainment of remission. Furthermore, we also found that pre-exposure of PBMC to the supernatants from HRS cell lines significantly increased the expansion of CD4+ CTLA<sub>4</sub><sup>hi</sup> and CD4+ LAG-3<sup>hi</sup> Treg cells. Pre-exposed PBMC displayed functional impairment of LMP1/2-peptide specific T-cells as assessed by IFN-γ ELISPOT assay. These results are consistent with the hypothesis that soluble product(s) from the HRS cells are released into the microenvironment of the diseased lymph node and may contribute to the enrichment of Treg cells within the lymph node and peripheral blood.

IFN-γ ELISPOT assays were performed on PBMC from HL patients. Although this analysis was restricted to those patients with informative alleles, we minimized this limitation by using 29 different EBV peptides presented by a wide-array of MHC class I restricted alleles, such that 97% of our patient population had at least one informative allele (with a median of seven informative HLA class I EBV-specific peptides per patient). An alternate approach would be to use pooled peptides spanning each individual EBV latent protein, thus enabling us to determine effector T-cell function irrespective of HLA restriction<sup>44</sup>. Indeed, this methodology was used to map many of the LMP1/2 peptides used in this study<sup>26</sup>.

In line with our observation of elevated Treg cells prior to therapy, we also noted a selective impairment of LMP1/2-specific effector T-cell function in newly diagnosed or relapsed HL patients when compared to patients in remission and healthy EBV-seropositive individuals. In striking contrast, responses to the immunodominant EBNA3/4/6 and lytic proteins (which are not
expressed by HRS cells) were unimpaired. Although the precise mechanism for this selective loss of T-cell function is unknown, it is possible that the presence of LMP-specific CD4+ Treg cells within diseased lymph nodes of HL patients may suppress the antigen-specific CD8+ T-cell function. Although impairment in LMP1/2 effector T-cell function was reduced in EBV-positive cases as compared to EBV-negative, this difference did not reach statistical significance. Paradoxically we showed that LMP1/2 effector T-cell functional impairment is proportional to the degree of both LAG-3 and FOXP3 staining in HL tissues, but only the former was associated with EBV tissue status. It should be emphasized that our analysis of LMP1/2 specific effector T-cell function was performed on the peripheral blood. This may not reflect LMP1/2 effector T-cell function at the tumor site, which may indeed be suppressed within EBV-positive lymph nodes to a greater extent than EBV-negative tumors. Interestingly, previous groups have been able to generate LMP1/2 specific CTL from EBV-negative tumor biopsies(10, 11), whereas generation from EBV-positive tumors has been consistently unsuccessful. Their findings suggest that a local inhibition of EBV-specific CTL response is involved in EBV-positive HL cases.

Ineffective immunity against HRS cells is most probably linked to the well established cell-mediated immune deficiency present early in the disease (reviewed in ref 45. It remains uncertain whether this immune deficiency predates oncogenesis and results in an increased susceptibility to HL, or conversely is a consequence of malignancy itself. Our finding that HRS cells are implicated in the enrichment of Treg cells within the diseased lymph nodes (and most likely the peripheral circulation), implies that the drive to generate excess Treg cells is reversed once HRS cells are eradicated, and is inconsistent with immune suppression mediated by Treg cells predating establishment of the
tumor. Previous studies by Marshall and colleagues demonstrated distinct variability between HL patients in the extent to which HL infiltrating cells induced inhibition of PBMC responses. These observations are consistent with our finding that the loss of LMP-specific T-cell function was co-incident with the increased intra-tumoral LAG-3 expression, and is suggestive of a specific suppressive activity generated by Treg cells within the vicinity of HRS cells. Accumulating in vivo evidence suggests that antigen-specific Treg cells control the intensity of T-cell responses and influence the magnitude of memory to a variety of human persistent viruses including HSV, HIV and HCV. For EBV, it has been suggested that LMP1 and EBNA1-specific HLA class II-restricted peptide epitopes can selectively recruit regulatory T-cells and impair antigen-induced IFN-γ production. The N-terminal sequence of LMP1 encodes a number of potential MHC class II-restricted epitopes that suppress T-cell responses in vitro. Similarly, CD4+ T-cell lines specific for EBNA1 epitopes suppress IL-2 secretion by EBNA1-specific effector T-cells.

Human in vitro experiments have shown that LAG-3 has high affinity for MHC class II molecules and down-regulates CD3-T-cell receptor mediated signalling and blockade of LAG-3 mediated signalling induces enhanced activation of human CD8 T-cells. The recognition that CD4+ LAG-3+ Treg cells and LMP1/2-specific CD8+ effector T-cells appear to play pivotal but opposing roles in regulating host EBV-specific cell-mediated immune responses in HL patients, has important implications for understanding the pathogenesis of EBV-positive HL. Preliminary results of EBV-specific CTL therapy in relapsed/refractory EBV-positive HL patients are encouraging, and taken together, our findings have important implications in the improved design of immunotherapeutic strategies to boost LMP1/2-specific CTL activity.
Acknowledgements

This study was performed under the auspices of the ALLG. We would like to thank our colleagues from the referring centres whose patients are included in this analysis. We also wish to thank Ms. Laurie Kear for her help in the coordinating the clinical aspects of this study.
References


34. Morgan SM, Wilkinson GW, Floettmann E, Blake N, Rickinson AB. A recombinant adenovirus expressing an Epstein-Barr virus (EBV) target antigen


Table 1. Patient Characteristics

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<th>Newly Diagnosed</th>
<th>Relapsed</th>
<th>Long-term Remission</th>
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<tr>
<td><strong>Total (Gender)</strong></td>
<td>41 (14F:27M)</td>
<td>9 (5F:4M)</td>
<td>43 (17F:26M)</td>
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<tr>
<td><strong>Mean age at Diagnosis</strong></td>
<td>31 yrs</td>
<td>41 yrs</td>
<td>30 yrs</td>
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<tr>
<td><strong>Clinical Stage: IIB/III/IV</strong></td>
<td>20(49%)</td>
<td>5(66%)</td>
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<tr>
<td>*Prognostic Score ≥ 3</td>
<td>12(29%)</td>
<td>4(44%)</td>
<td>Not applicable</td>
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<tr>
<td><strong>Timing of blood sample(s)</strong></td>
<td>Prior to therapy 6 months</td>
<td>Prior to therapy 6 months</td>
<td>Mean 5 (2-33) yrs post-diagnosis</td>
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<td><strong>Histology:</strong>**</td>
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<tr>
<td>Nodular Sclerosing</td>
<td>27/41 (66%)</td>
<td>7/9 (78%)</td>
<td>18/36 (50%)</td>
</tr>
<tr>
<td>Mixed Cellularity</td>
<td>7/41 (17%)</td>
<td>1/9 (11%)</td>
<td>6/36 (17%)</td>
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<tr>
<td>Lymphocyte Rich</td>
<td>4/41 (10%)</td>
<td>0/9 (0%)</td>
<td>4/36 (11%)</td>
</tr>
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<td>Lymphocyte Depleted</td>
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<td>0/9 (0%)</td>
<td>1/36 (3%)</td>
</tr>
<tr>
<td>Nodular Lymphocyte Predominant</td>
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<td>1/9 (11%)</td>
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<td>Hodgkin’s Unclassified</td>
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<td>0/9 (0%)</td>
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</tr>
<tr>
<td>Positive EBV serology***</td>
<td>39/40 (98%)</td>
<td>1/9 (89%)</td>
<td>40/43 (93%)</td>
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<tr>
<td>Positive EBV tissue status****</td>
<td>15/41 (36%)</td>
<td>1/9 11%</td>
<td>9/27 (33%)</td>
</tr>
</tbody>
</table>

*Hasenclever/Diehl International Prognostic Score
**Histology available in all Newly Diagnosed and Relapsed cases, and 36 of 43 (84%) remission cases.
***Serology undetermined in one newly diagnosed case.
****Biopsy available for EBV testing in all Newly Diagnosed and Relapsed cases, and 27 of 43 (63%) remission cases. EBV tissue status correlated with histology, with 55% of mixed cellularity or lymphocyte rich cases positive for EBV, against 27% of nodular sclerosing cases (r=0.38, p=0.0127). In 2/67 cases the LMP1 and EBER stains were discordant (EBER positive / LMP1 negative). Both cases were classified as EBV tissue positive.
Table 2: Expression of LAG-3 and FOXP3 by tumour infiltrating lymphocytes in malignant HL lymph nodes

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Disease Status</th>
<th>Age at diagnosis (yrs) / Gender</th>
<th>Histology</th>
<th>LAG-3+ cells within HL nodes</th>
<th>FOXP3+ cells within HL nodes</th>
<th>EBV tissue status</th>
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<tr>
<td>32</td>
<td>ND</td>
<td>30/M</td>
<td>NS</td>
<td>++</td>
<td>+</td>
<td>+ve</td>
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<tr>
<td>35</td>
<td>ND</td>
<td>48/F</td>
<td>NS</td>
<td>++</td>
<td>-</td>
<td>+ve</td>
</tr>
<tr>
<td>36</td>
<td>RL</td>
<td>34/F</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>+ve</td>
</tr>
<tr>
<td>38</td>
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<td>+ve</td>
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<td>-/+</td>
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<tr>
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<td>35/F</td>
<td>MC</td>
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<td>-</td>
<td>+ve</td>
</tr>
<tr>
<td>53</td>
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<td>-/+</td>
<td>+ve</td>
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<tr>
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<td>MC</td>
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<td>19/F</td>
<td>NS</td>
<td>-/+</td>
<td>-/+</td>
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</table>

+/− respectively indicates % of non-HRS cells within HRS rich areas that are positive for LAG-3. ++++ indicates 100-75%, +++ 74-50%, ++ 49-25%, + 24-10%, /+ 10-2%, - <1%. ND, RL and Remission indicate newly diagnosed, relapsed and Long-term Remission respectively. NA signifies tissue not available for FOXP3 testing. Histology subtypes are NS (nodular sclerosing), MC (mixed cellularity), LR (lymphocyte rich), LD (lymphocyte depleted) and Nodular Lymphocyte Predominant (NLP). There was a significant association between % LAG-3 staining and MC and/or LR histology versus NS (p=0.013) and between LAG3 and positive EBV status (p=0.023). There was no association between % FOXP3 and histology (p=0.489), with EBV status (p=0.833), or with % LAG-3 (p=0.404). % LAG-3 staining in tumor-infiltrating lymphocytes was higher than % FOXP3 (p=0.003).
<table>
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<tr>
<th>EBV antigen</th>
<th>Patient group</th>
<th>Number of assays</th>
<th>Mean (SE)* SFC</th>
<th>Median (IQR)* SFC</th>
<th>P value (vs prior to therapy)</th>
<th>P value (vs 6 month)</th>
<th>P value (vs 12 month)</th>
<th>P value (vs long-term remission)</th>
</tr>
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<td>LMP1 &amp; 2</td>
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<td>87</td>
<td>30 (8)</td>
<td>0 (0-24)</td>
<td>-</td>
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<tr>
<td></td>
<td>ND/RL 6 months</td>
<td>45</td>
<td>87 (24)</td>
<td>0 (0-106)</td>
<td>0.0907</td>
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<tr>
<td></td>
<td>ND/RL 12 months</td>
<td>41</td>
<td>101 (25)</td>
<td>27 (0-169)</td>
<td>**0.0136</td>
<td>0.4942</td>
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<td>Long-term remission</td>
<td>136</td>
<td>153 (33)</td>
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<td>**&lt;0.0001</td>
<td>0.0695</td>
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<td>102 (40)</td>
<td>32 (0-94)</td>
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<td>0.3288</td>
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<td>58 (0-187)</td>
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<td>-</td>
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<td>21</td>
<td>149 (42)</td>
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<td>351 (93)</td>
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<td>290 (74)</td>
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<td>10</td>
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<td>ND/RL 12 months</td>
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<td>631 (161)</td>
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<td>0.4923</td>
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*Arithmetic mean and median values of γ-interferon producing cells (referred to as spot forming cells, SFC/10⁶ PBMC) in response to HLA class I-restricted LMP1 & 2, EBNA3/4/6 and lytic antigen peptide epitopes.

SE indicates standard error
IQR is inter-quartile range
** indicates a significant p value.
Figure Legends

**Figure 1:** A: Representative photomicrographs of HL tumor biopsy samples from different histological subtypes (MC, NS and NLP, a-c respectively). The EBV status of tumor tissues was confirmed by EBER *in situ* hybridization (d-f) and LMP1 immunohistochemistry (h-j). Tissue samples shown in panels d, e, h and i were positive, while f and j were negative for EBV. Panels l-n shows LAG-3 protein on tumor-infiltrating lymphocytes in HL biopsies. The numbers of LAG-3-positive cells in each tissue section were graded as described in Table 2. The tissue sections were graded +++ (panel l), ++ (panel n) and –ve (panel m). The relevant isotype controls are shown in panels g, k and o. Panels a-c: original magnification x10, panels d-o: original magnification x40. B: Panels a-c show representative lymph node sections from a patient with lymphocyte rich EBV-positive classical HL. Panels a and b show LAG-3 and FOXP3 protein on tumor-infiltrating lymphocytes respectively, and dual-staining in panel c illustrates that the nuclear FOXP3 protein (red) and surface/cytoplasmic LAG-3 protein (brown) do not generally co-localize within the same lymphocyte.

**Figure 2:** LAG-3 is enriched within the CD4+ T-cells expressing intracellular CTLA-4^hi^ and GITR^hi^. Panel A: Representative data from an HL patient in remission showing LAG-3
staining; Panel B: HL patient PBMC samples (n=14) showing mean fluorescence intensity for LAG-3 on CD4+ T-cells with intracellular CTLA4<sup>lo</sup> and CTLA4<sup>hi</sup>, and GITR<sup>lo</sup> and GITR<sup>hi</sup> cells. P values were generated using the Wilcoxon matched pairs test.

**Figure 3:** Cross-sectional analysis of Treg cell subsets in HL patients. Box and whisker plots summarizing the percentages of the mean (horizontal bar), standard error (box) and standard deviation (whiskers) of Treg cells in HL patients at diagnosis, in remission and in randomly chosen healthy laboratory controls. The percentage of positive cells with each phenotype is shown on the vertical axis, and the p value and number of samples is indicated above and below each plot respectively. P values were generated using the Mann-Whitney test.

**Figure 4:** Mean and standard error of *ex-vivo* EBV-specific IFN-γ spot forming cells following stimulation with peptide epitopes (SFC/10<sup>6</sup> PBMC). Responses were assessed pre-therapy (Pre-Rx), 6m and 12m (refers to the number of months following diagnosis). 6m and 12m columns include only data from newly diagnosed patients who have gone on to attain remission. These responses were compared to HL patients in long-term remission, and healthy seropositive individuals. Panels A-C show data irrespective of EBV-
tumor status. In Panels A, B & D: n refers to the number of assays performed with the number of patients shown in brackets. Panel A shows a cross-sectional analysis of LMP1/2-specific ELISPOT responses in HL patients at different time-points and in healthy virus-carriers. Panel B shows a comparison of T-cell responses directed towards LMP1/2, EBNA3/4/6 and lytic epitopes in ND/RL HL patients and healthy virus-carriers. Panel C shows a matched pair analysis of LMP1/2, EBNA3/4/6 and lytic epitope-specific CD8+ T-cell responses in HL patients both and after receiving chemotherapy (Pre-Rx and Post-Rx). Panel D shows LMP1/2-specific CD8+ T-cell responses in ND/RL EBV-positive and EBV-negative HL patients. This panel also shows ELISPOT responses in ND/RL HL patients with respect to clinical stage and Hasenclever/Diehl International Prognostic Score. *denotes statistical significance.

**Figure 5:** *Ex-vivo* enumeration of EBV-specific CD8+ T-cells using MHC-peptide pentamers in PBMC from HL patients (Pre-Rx and long-term remission). The values shown on the logarithmic axis indicate the percentage CD8+ T-cells positive for HLA class I-peptide pentamers. There was no significant difference in pentamer frequencies between EBV-positive HL and EBV-negative HL cases.
Figure 6: Histograms (showing mean and standard error) demonstrating the association of intra-tumoral LAG-3 expression and LMP1/2-specific CD8+ T-cell responses in ND/RL HL patients prior to therapy. ELISPOT responses were compared in patients categorized as either low (0-25%) or high (26-100%) LAG-3 expressing tumor-infiltrating lymphocytes within HL lymph nodes.

Figure 7: Effect of HRS cell line supernatant on LMP effector T-cell function and expansion of Treg cells in vitro. PBMC from five different healthy subjects were cultured in supernatants from HRS cell lines (L1236, HDLM2, L428, L540) for 3 days (panels A and B). A fibroblast line supernatant and serum free culture media (SFCM) alone were used as negative controls. Panel C shows an LMP2A peptide-specific IFN-γ ELISPOT assay following 3 day incubation of PBMC (from a healthy HLA A2 EBV-seropositive subject) with supernatant from the L540 HL cell line. Results are representative of 3 separate experiments. Panel D shows T cell reactivity towards CLG epitope (HLA A2-restricted, LMP2A) in PBMC from two different HLA A2-positive individuals (referred to as D1 and D2) with or without depletion of CD4+ LAG-3+ T-cells. Data from fresh PBMC and PBMC cultured in HRS cell supernatant are shown.
Fig. 1
Fig. 2
Pre-Rx Remission

% +ve cells

CD4/CD25hi CD45ROhi

p=0.019

p=0.003

p<0.001

p=0.003

p=0.004

P<0.001

n=16; 31; 10

n=14; 21; 10

n=22; 19; 11

n=13; 9; 11

Fig. 3
**Fig. 4**

A. SFC/10⁶ PBMC

- Pre-Rx: n=87 (28)
- 6m: n=45 (14)
- 12m: n=41 (14)
- Remission: n=136 (37)
- Healthy: n=41 (20)

B. SFC/10⁶ PBMC

- LMP: n=87 (28)
- EBNA3/4/6: n=33 (20)
- LMP-lytic: n=43 (12)
- EBNA3/4/6-lytic: n=6 (6)

C. SFC/10⁶ PBMC

- LMP1 & 2 (p<0.0001)
- EBNA3/4/6 (p=0.3575)
- Lytic (p=0.083)

D. SFC/10⁶ PBMC

- EBV tissue status
- Clinical stage
- Prognostic score

For personal use only.
Fig. 5

- Pre-Rx (n=15) vs Remission (n=14)
- LMP1 & 2
- Pre-Rx (n=6) vs Remission (n=15)
- EBNA3,4,6 & lytic

p=ns

% CD8+/MHC-Pentamer

0.01 0.1 1 10

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% LAG3 positive cells in HRS rich areas

SFC per 10^6 PBMC

0-25% n=16
26-100% n=20

p=0.0151

Fig. 6
Fig. 7
Expression of LAG-3 by tumor-infiltrating lymphocytes is co-incident with the suppression of latent membrane antigen-specific CD8+ T-cell function in Hodgkin lymphoma patients

Maher K Gandhi, Eleanore Lambley, Jaikumar Duraiswamy, Ujjwal Dua, Corey Smith, Suzanne Elliott, Devinder Gill, Paula Marlton, John Seymour and Rajiv Khanna