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LETTER TO THE EDITOR

Hodgkin/Reed-Sternberg cells induce GPNMB expression and release from macrophages to suppress T-cell responses to the Epstein-Barr virus-encoded LMP2A protein

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Contributions: NM, TAP, KV, PK, GT and PGM designed the study; NM, TAP and AH performed the experiments; NM, TAP, KV, EF, WW and PGM analyzed the data; MP provided pathology review and interpretation of immunohistochemistry results; KB was involved in sample collection; NM, KV, GT and PGM wrote the manuscript. All authors approved the final version of the manuscript.

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Classical Hodgkin lymphoma (cHL) is characterized by the presence of Hodgkin-Reed-Sternberg (HRS) cells surrounded by a prominent inflammatory tumor microenvironment (TME). Although the TME is thought to prevent immune recognition of HRS cells by tumor-specific T-cells, the mechanisms responsible are poorly understood. Here, we show that tumor-associated macrophages (TAM) in the TME of cHL strongly express glycoprotein non-metastatic B (GPNMB). Co-culture with cHL cell lines induced the M2 polarization of macrophages, which was accompanied by increased surface expression of GPNMB and its release as a soluble form. Importantly, soluble recombinant GPNMB (rGPNMB) inhibited CD8+ T-cell recognition of Epstein-Barr virus (EBV)-derived tumor epitopes in cHL cells, suggesting that inhibiting GPNMB in the cHL TME could enhance anti-tumor immune responses.

Classical Hodgkin lymphoma (cHL) is characterized by single malignant Hodgkin-Reed-Sternberg (HRS) cells surrounded by a pro-inflammatory tumor microenvironment (TME) that supports HRS cell survival, growth and immune escape. The EBV genome is present in HRS cells in 30-50% of cHL and expresses the immunologically subdominant EBV latent proteins, Epstein-Barr nuclear antigen 1 and latent membrane proteins, LMP1 and LMP2A.[1] Cells expressing LMP1 and LMP2A are sensitive to lysis by EBV-specific cytotoxic CD8+ T-cells *in vitro*.[1, 2] Moreover, EBV-specific CD8+ T-cells have been shown to be present in the cHL TME.[3] These data suggest that immune suppressive mechanisms operate in the TME of EBV+ cHL.

Macrophages are broadly classified into M1 (classically-activated) and M2 (alternatively-activated) macrophages depending on their anti/pro-inflammatory properties and their polarization fluctuates in response to different stimuli/signals received from their environment. Tumor-associated macrophages (TAM) infiltrating cHL tissues have M2-like characteristics and a higher frequency of these cells is associated with inferior survival of cHL patients.[4] Multiplex immunofluorescence has revealed that the majority of PD-L1-expressing cells in the TME of cHL are macrophages, which are in close proximity to PD-1-expressing CD4+ T-cells, suggesting that macrophages contribute to the dysregulation of anti-tumor T-cell responses in cHL.[5] Importantly, this dysfunctional T-cell phenotype is reversible, as evidenced by the success of PD-1 blockade therapy in cHL patients with relapsed or refractory disease.[6]

Glycoprotein non-metastatic B (GPNMB), also known as DC-HIL receptor, is a transmembrane protein that is known to be over-expressed in numerous cancer types, and in some of these has been shown to promote a more metastatic phenotype.[7] GPNMB was also shown to function as a novel immune checkpoint that can bind to its ligand, syndecan-4, and inhibit T-cell activation.[8-12] Accordingly, blocking GPNMB was shown to exacerbate autoimmune responses, inhibit wound healing, and potentiate anti-tumor immunity in melanoma-bearing hosts.[10, 11, 13, 14] The secreted form of GPNMB (sGPNMB) has also been shown to be functional and can exclude T-cells from pre-metastatic niches to promote tumor progression and reduce the trans-endothelial migration of T-cells.[15] Moreover, elevated plasma levels of sGPNMB are associated with resistance to PDL-1 inhibitor monotherapy in patients with advanced non-small cell lung carcinoma.[16] Notably, GPNMB expression was shown to define a subset of mononuclear phagocytes associated with inferior survival of patients with colorectal cancer.[17] In glioblastoma, these GPNMB expressing macrophages were unable to activate T-cells.[9] In this study, we have explored the expression and potential role of GPNMB as a novel immune checkpoint in cHL.

We first investigated GPNMB expression in 86 cases of histologically confirmed cHL of known EBV status using GPNMB-specific antibodies. The cases were obtained with ethical approval from West Midlands - Black Country Research Ethics Committee, UK (REC:16/WM/0037, IRAS project ID:181189). Immunohistochemistry revealed the expression of GPNMB in morphologically characteristic tumor-associated macrophages (TAM) in all cases, but only rarely in HRS cells (Figure 1A). There were no significant differences in the number of GPNMB-positive cells between cases based on subtype, age, EBV status or CD8 counts (data not shown). GPNMB expression was associated with a shorter progression free survival and overall survival although these differences were only of borderline significance (Supplementary Figure 1A). We also interrogated our unpublished Nanostring GeoMx data which revealed an inverse correlation between PD-L1 expression and GPNMB expression in the macrophage enriched regions of interest (Supplementary Figure 1B). As expected, macrophages were also positive for GPNMB in normal tonsil (Figure 1A). We used multiplex immunofluorescence to confirm the expression of GPNMB in CD68-positive TAM, and as expected, it's absence from CD30-positive tumor cells in most cases (Figure 1B). We also observed very low or undetectable levels of GPNMB expression

(compared to GC B cells) in a panel of cHL cell lines using qRT-PCR (data not shown), further supporting the observation that HRS cells do not generally express GPNMB.

We reasoned that HRS cells might mediate the high-level expression of GPNMB observed in TAM. To test this, we exposed M1 and M2 macrophages to cHL-derived conditioned media (CM) or cultured them in direct contact with either EBV-negative (L1236, L428) or EBV-positive (L591) cHL cell lines at different cell ratios. Leukocyte cones were obtained with ethical approval from the National Blood Service (Birmingham, UK; REC_RG_15_165). M1 and M2 macrophages were generated by differentiation of CD14+ peripheral blood-derived monocytes with GM-CSF or M-CSF, respectively. In keeping with previous reports, GM-CSF polarized M1-like macrophages were CD68+CD163- and moderately CD206+, whereas M-CSF polarized M2-like macrophages were CD68+CD163+CD206+ (data not shown). To confirm the phenotype of polarized cells, we treated them with LPS for 24h and measured cytokine levels in the conditioned media. As expected, GM-CSF polarized macrophages had a characteristic M1 cytokine profile with low IL-10 and high IL-12(p70) secretion, and M-CSF polarized macrophages, a typical M2 macrophage cytokine profile with high IL-10 and low IL-12(p70) (data not shown). Treatment of M1 and M2 macrophages with cHL-derived CM significantly increased the number of macrophages expressing surface GPNMB. Co-culture with each of the cHL cell lines also increased the numbers of M1 and M2 macrophages expressing surface GPNMB, an effect apparent at all cell ratios (Figure 2A, Supplementary Figure 2A). These effects were accompanied by increased expression of CD163 and CD206, indicating polarization of M1 macrophages to an M2-like phenotype and the further polarization of M2 macrophages (Figure 2B, Supplementary Figure 2B).

Membrane-bound GPNMB can be cleaved by metalloproteinases, such as ADAM10, to generate the soluble isoform, sGPNMB.[9] We next studied if sGPNMB could be released by macrophages polarized by HRS cells. We repeated the co-culture experiments using macrophages differentiated from the blood monocytes of three new donors, but this time measuring sGPNMB in the supernatant by ELISA. As before, CM was as effective as direct co-culture in inducing surface GPNMB expression (data not shown). However, sGPNMB levels in cell supernatants were substantially higher after co-culture compared with exposure of

macrophages to CM alone (Figure 2C, Supplementary Figure 2C). Thus, while soluble factors released by HRS cells are effective in inducing GPNMB surface expression, optimal sGPNMB release is dependent upon cell-cell contact. One explanation for this result is that cell-cell contact triggers the activation and/or upregulation of proteases that cleave GPNMB.

We next assessed the influence of GPNMB on cytotoxic T-lymphocyte (CTL) recognition of EBV-derived epitopes in cHL cell lines. cHL cell lines have been shown to efficiently process and present epitopes from EBV proteins to HLA-class I-restricted EBV-specific CTL clones.[1, 2] EBV antigens are expressed in the tumor cells of 30-50% of cHL and are therefore ideal targets to assess the effects of GPNMB on T-cell recognition. Two EBV negative cHL cell lines (KMH2 or L1236) were used as targets. These cell lines were infected with either a modified vaccinia Ankara (MVA) virus expressing the EBV LMP2A protein (MVA-LMP2A) at two different multiplicity of infections (MOI; 10, 1) or MVA-pSC11 (empty vector, control). We also pulsed cells with epitope peptides from LMP2A or the DMSO solvent (control); these peptides bind to HLA class I on the cell surface and are presented to T-cells. Two different LMP2A specific CD8+ T-cell clones recognizing distinct LMP2A peptides, HLA-A24-restricted TYG and HLA-A2-restricted CLG, were used as effector cells and interferon-γ release was used to measure CD8+ T-cell recognition. As expected, neither T-cell clone recognized the EBV-negative cHL lines infected with control virus or pulsed with DMSO. However, both T-cell clones recognized the cHL cells infected with MVA-LMP2A or pulsed with their cognate peptide, producing interferon-γ (Figure 3A, B). We found that recognition was significantly decreased in the presence of soluble recombinant GPNMB (rGPNMB). Interestingly, this inhibition was strongest at the lowest dose of rGPNMB and diminished at the highest dose tested (Figure 3A, B). We repeated this experiment using the optimal inhibitory dose of rGPNMB (0.04µg/ml) and included two further T-cell clones specific for additional LMP2A epitopes, HLA-A2-restricted FLY and HLA-A11-restricted SSC, and an additional EBV-negative HLA-A11-positive cHL line, L540. In each case, recognition of MVA-LMP2A infected cHL cells was decreased in the presence of rGPNMB (Supplementary Figure 3A). Finally, to confirm the effects of rGPNMB on T-cell activation in primary cells, we activated PBMCs with a range of different concentrations of soluble CD3/CD28

activators and measured interferon-γ release in the presence/absence of rGPNMB (0.04µg/ml). As expected, rGPNMB significantly reduced the activation of T-cells (Supplementary Figure 3B).

In summary, we have shown that TAM in the cHL TME strongly express GPNMB. Levels of surface GPNMB were increased upon *in vitro* exposure of macrophages to cHL CM or following direct co-culture with cHL cells. These effects were associated with M2 polarization. Optimal release of the soluble isoform, sGPNMB, by macrophages was achieved only following direct co-culture with cHL cells. Importantly, low levels of soluble recombinant rGPNMB inhibited the recognition of cHL cells by CD8+ T-cells specific for epitopes from the LMP2A protein, a well-known tumor antigen expressed in around 30-50% of all cases of cHL.[1] Our results indicate that blocking GPNMB in the TME of cHL could enhance tumor-specific CD8+ T-cell recognition.

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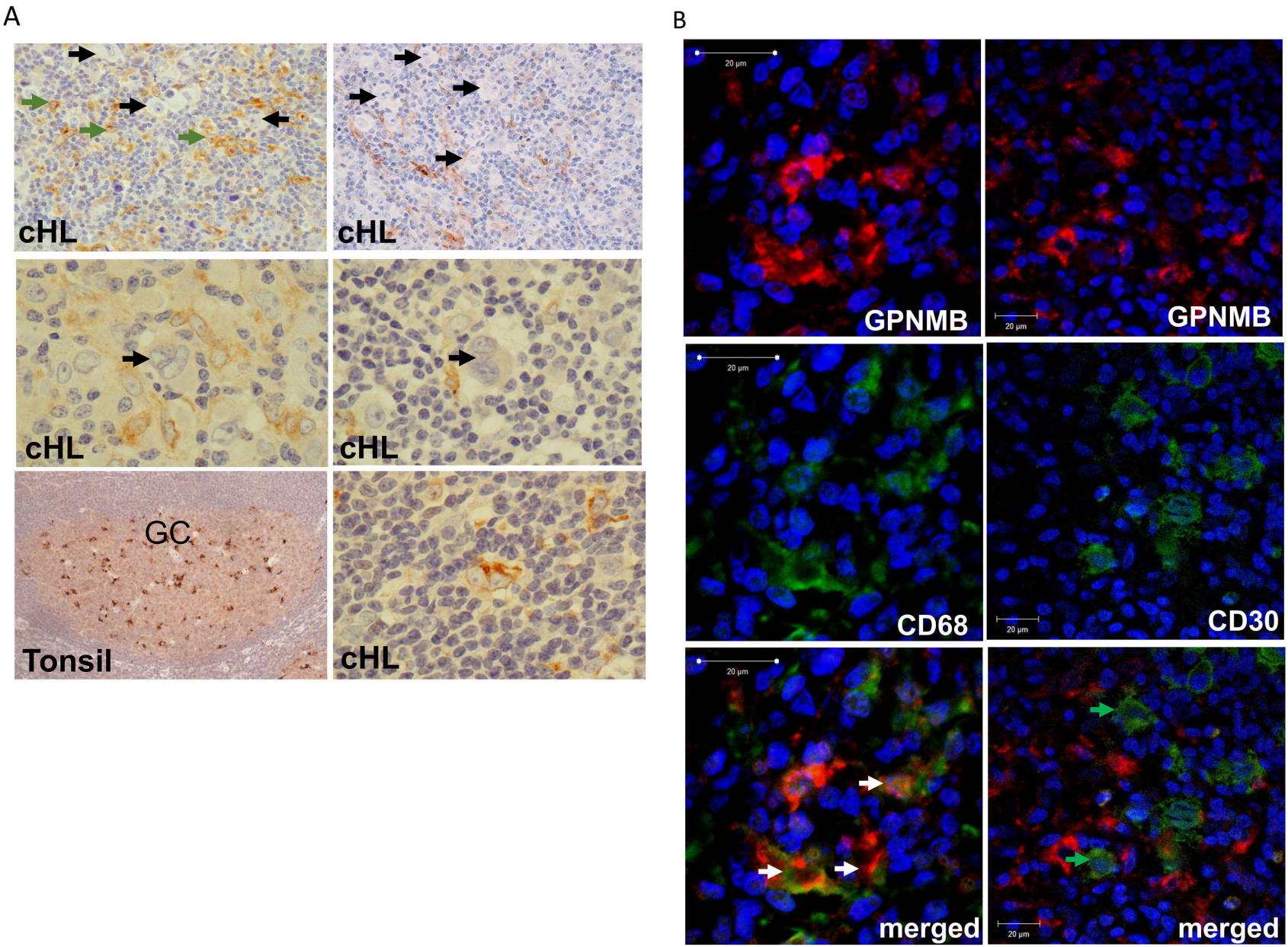
FIGURE LEGENDS

Figure 1: GPNMB expression in primary cHL A) Immunohistochemistry of representative cases of cHL. Upper and middle panels: Prominent expression of GPNMB in the tumor associated macrophages (TAM) (green arrows, ab175427, Abcam, Cambridge, UK). Black arrows indicate GPNMB-negative HRS cells. Lower right panel: a rare case of cHL with HRS cell expression of GPNMB. Lower left panel: GPNMB expression in macrophages within normal germinal centers (GC) of tonsil (ab125898, Abcam). Images were taken on an Olympus BX-51WI microscope with 10x magnification (bottom left), 20x (upper panels) and 40x (center and bottom right panels). The number of GPNMB-positive cells in three high-power fields (HPF; 40x; area 230mm^2) per case were counted and the mean calculated. **B)** Multiplex immunofluorescence shows strong expression of GPNMB in CD68-positive TAM (left columns, white arrows), and its absence in CD30-positive HRS cells (right columns, green arrows). Anti-GPNMB (ab175427, Abcam), anti-CD68 (clone PG-M1; Agilent Dako, Santa Clara, CA, USA), anti-CD30 (clone Ber-H2; Agilent Dako) antibodies and Opal 4-plex kit (Perkin Elmer, Shelton, CT, USA) were used.

Figure 2: cHL cells induce GPNMB expression and release from macrophages and their polarization to M2 phenotype. A) Flow cytometry for GPNMB expression on M1 or M2 macrophages following their culture in L1236 conditioned media (+CM) or their direct co-culture with L1236 cells at different macrophage:HL cell ratios for 24h. Macrophages generated from at least nine different individuals were tested per condition. Anti-GPNMB-PE antibody (HOST5DS) and CD68/PE-Texas Red (Thermo Fisher, eBioscience, Waltham, MA, USA) were used. **B)** Flow cytometry for CD163+CD206+ M2 marker expression on M1 or M2 macrophages following their culture in L1236 CM or by their co-culture with L1236 cells as in A). CD163/APC and CD206/PE-Cy7 (Thermo Fisher) antibodies were used. Macrophages generated from at least nine different individuals were tested per condition. **C)** ELISA measurement of GPNMB release by M1 and M2 macrophages exposed to L1236 CM or directly co-cultured with L1236 cells for 24h. GPNMB Duoset ELISA kit (R&D Systems, Minneapolis, MN, USA) was used. Shown are the results of three separate donors. Means (solid bars) for all experiments were compared by

Student's t-test. The results for additional two cHL-derived cell lines are shown in Supplementary Figure 2A-C.

Figure 3: Low dose soluble recombinant rGPNMB inhibits T-cell recognition of cHL lines *in vitro*. A) ELISA measurement of interferon-γ release by T-cells co-cultured with cHL cell lines for 18h. CD8+ T-cell clone specific for the HLA-A24-restricted LMP2A epitope TYG (LMP2 amino acids 419-427) was exposed to HLA-A24-positive KMH2 cHL cells infected with MVA LMP2A (MOI of 1 or 10) or negative control virus, MVA-pSC11 (MOI 10), in the presence of varying concentrations of soluble recombinant GPNMB (2550-AC, R&D Systems, left panel). In parallel, KMH2 cells pulsed with synthetic TYG epitope peptide or DMSO solvent (negative control) were also used as T-cell targets (right panel). **B)** Similar experimental design using a CD8+ T-cell clone specific for the HLA-A2-restricted LMP2A epitope CLG (LMP2 amino acids 426-434) and the HLA-A2-positive (left panel) or TYG-epitope pulsed (right panel) cHL line L1236 as the target cells. Means (solid bars) were compared by Student's t-test.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cHL
cHL
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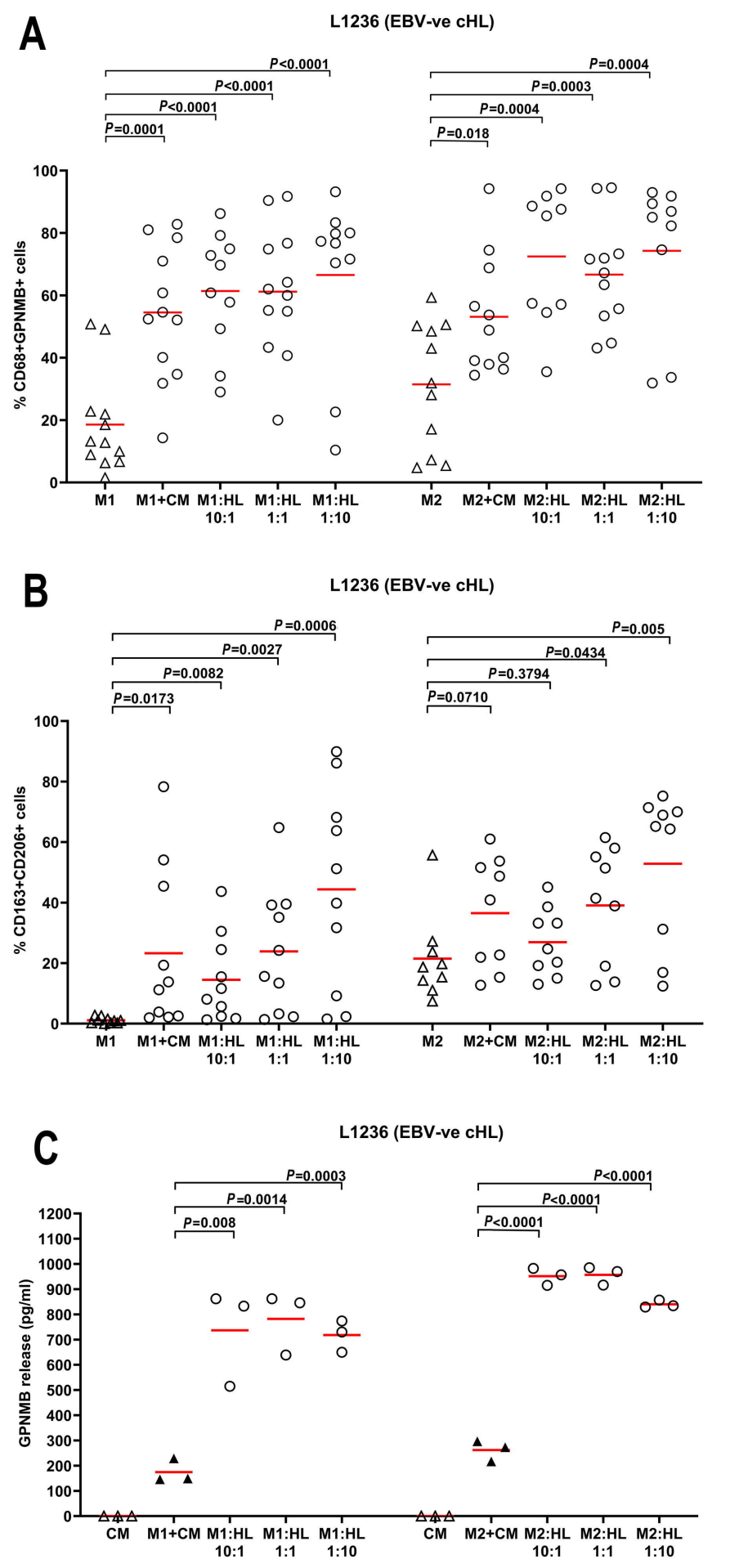
A
L1236 (EBV-ve cHL)
P<0.0001
P<0.0001
P<0.0001
P=0.0001
P=0.0004
P=0.0003
P=0.0004
P=0.018
% CD68+GPNMB+ cells
100
80
60
40
20
0
M1
M1+CM
M1:HL 10:1
M1:HL 1:1
M1:HL 1:10
M2
M2+CM
M2:HL 10:1
M2:HL 1:1
M2:HL 1:10
B
L1236 (EBV-ve cHL)
P=0.0006
P=0.0027
P=0.0082
P=0.0173
P=0.005
P=0.0434
P=0.3794
P=0.0710
% CD163+CD206+ cells
100
80
60
40
20
0
M1
M1+CM
M1:HL 10:1
M1:HL 1:1
M1:HL 1:10
M2
M2+CM
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M2:HL 1:1
M2:HL 1:10
C
L1236 (EBV-ve cHL)
P=0.0003
P=0.0014
P=0.008
P<0.0001
P<0.0001
P<0.0001
GPNMB release (pg/ml)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
CM
M1+CM
M1:HL 10:1
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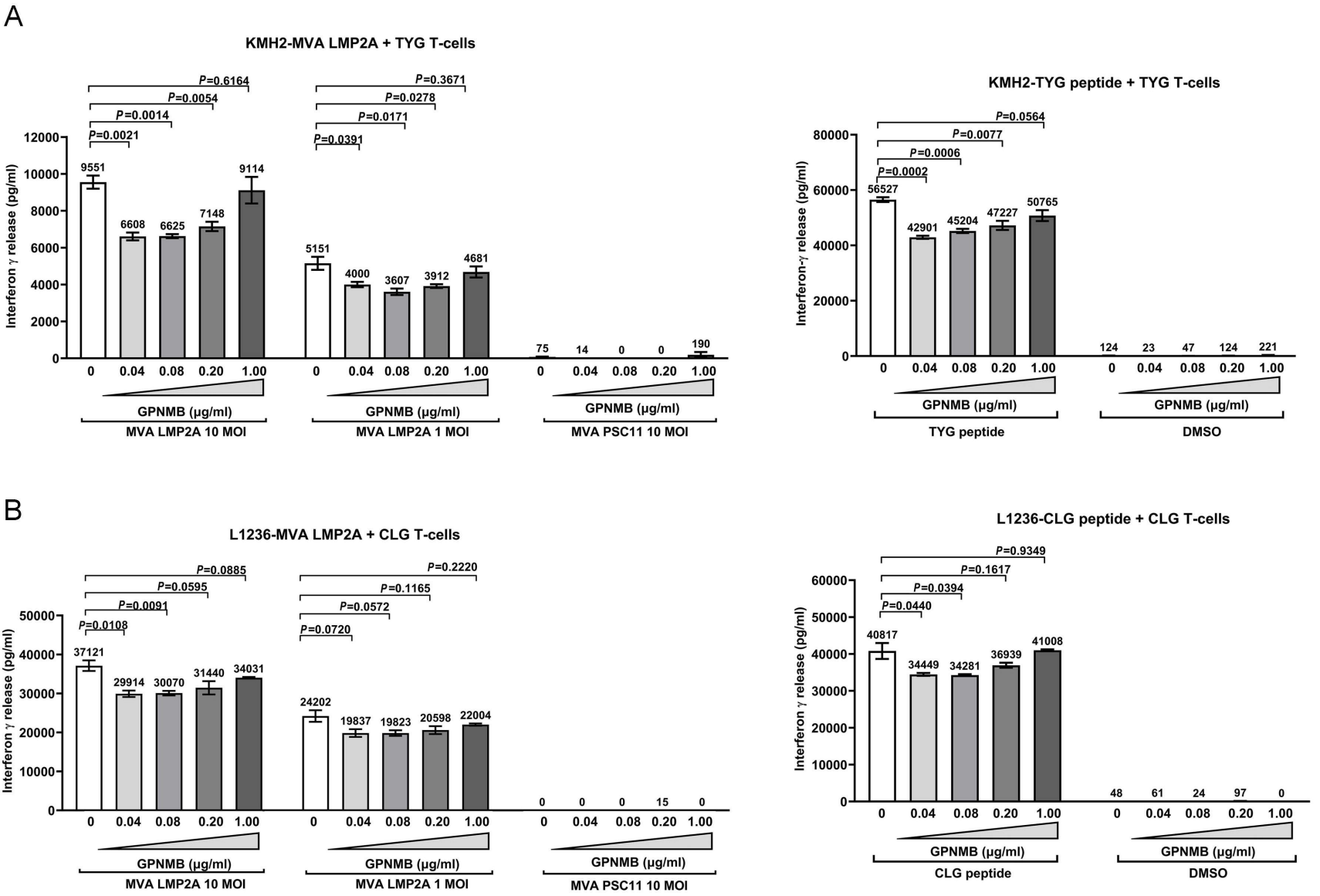
M2:HL 1:1
M2:HL 1:10


A
KMH2-MVA LMP2A + TYG T-cells
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P=0.0054
P=0.0014
P=0.0021
P=0.3671
P=0.0278
P=0.0171
P=0.0391
9551
6608
6625
7148
9114
5151
4000
3607
3912
4681
75
14
0
0
190
0 0.04 0.08 0.20 1.00
GPNMB (µg/ml)
MVA LMP2A 10 MOI
MVA LMP2A 1 MOI
MVA PSC11 10 MOI
KMH2-TYG peptide + TYG T-cells
Interferon-γ release (pg/ml)
P=0.0564
P=0.0077
P=0.0006
P=0.0002
56527
42901
45204
47227
50765
124
23
47
124
221
TYG peptide
DMSO
B
L1236-MVA LMP2A + CLG T-cells
Interferon γ release (pg/ml)
P=0.0885
P=0.0595
P=0.0091
P=0.0108
P=0.2220
P=0.1165
P=0.0572
P=0.0720
37121
29914
30070
31440
34031
24202
19837
19823
20598
22004
0
0
0
15
0
MVA LMP2A 10 MOI
MVA LMP2A 1 MOI
MVA PSC11 10 MOI
L1236-CLG peptide + CLG T-cells
Interferon γ release (pg/ml)
P=0.9349
P=0.1617
P=0.0394
P=0.0440
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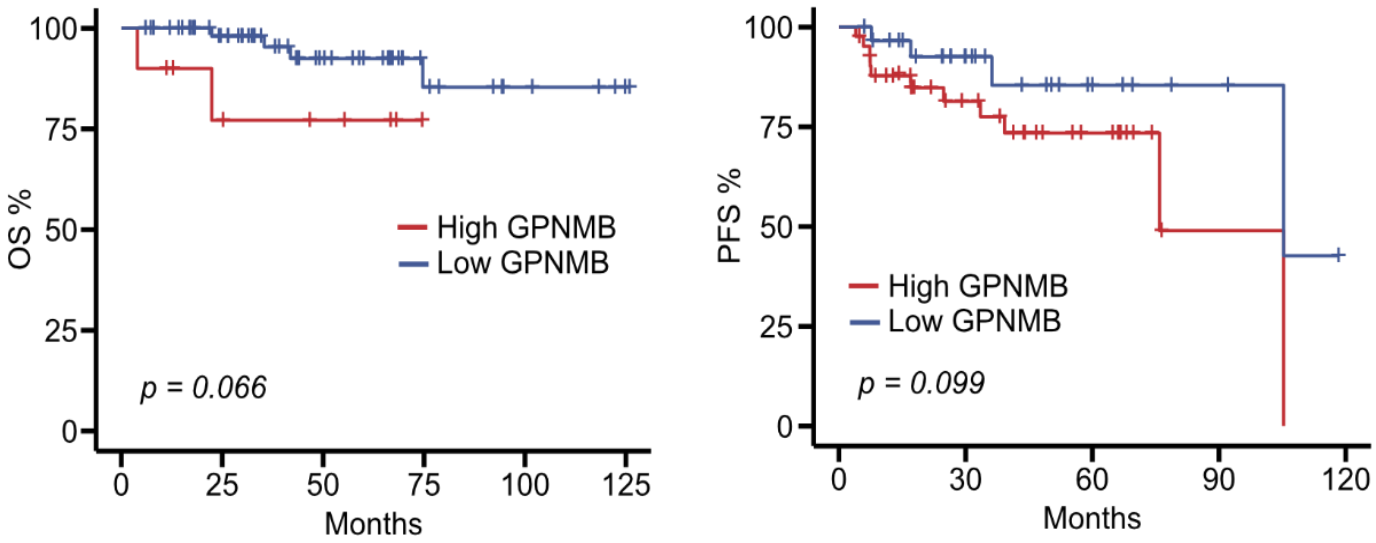
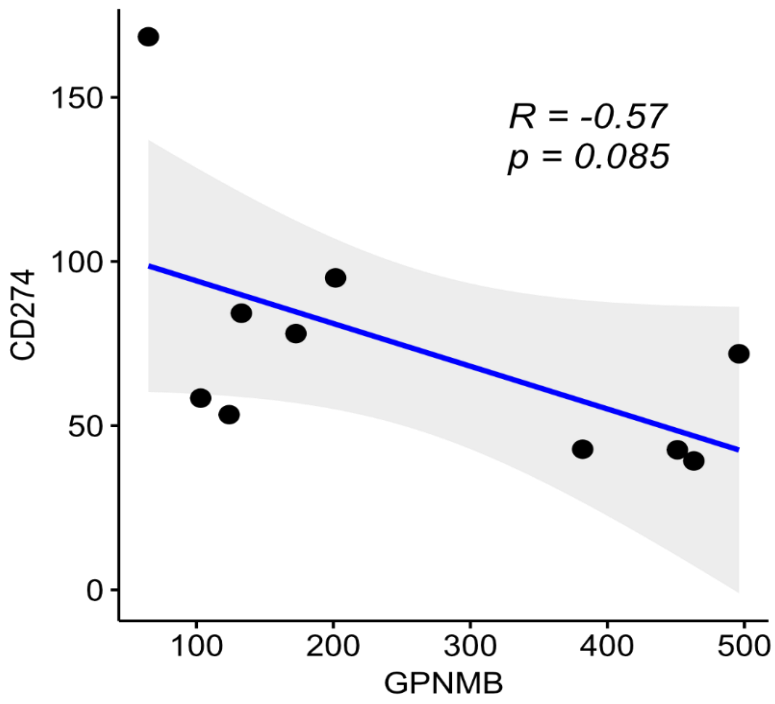
36939
41008
48
61
24
97
0
CLG peptide
DMSO

Supplementary Figure 1

A

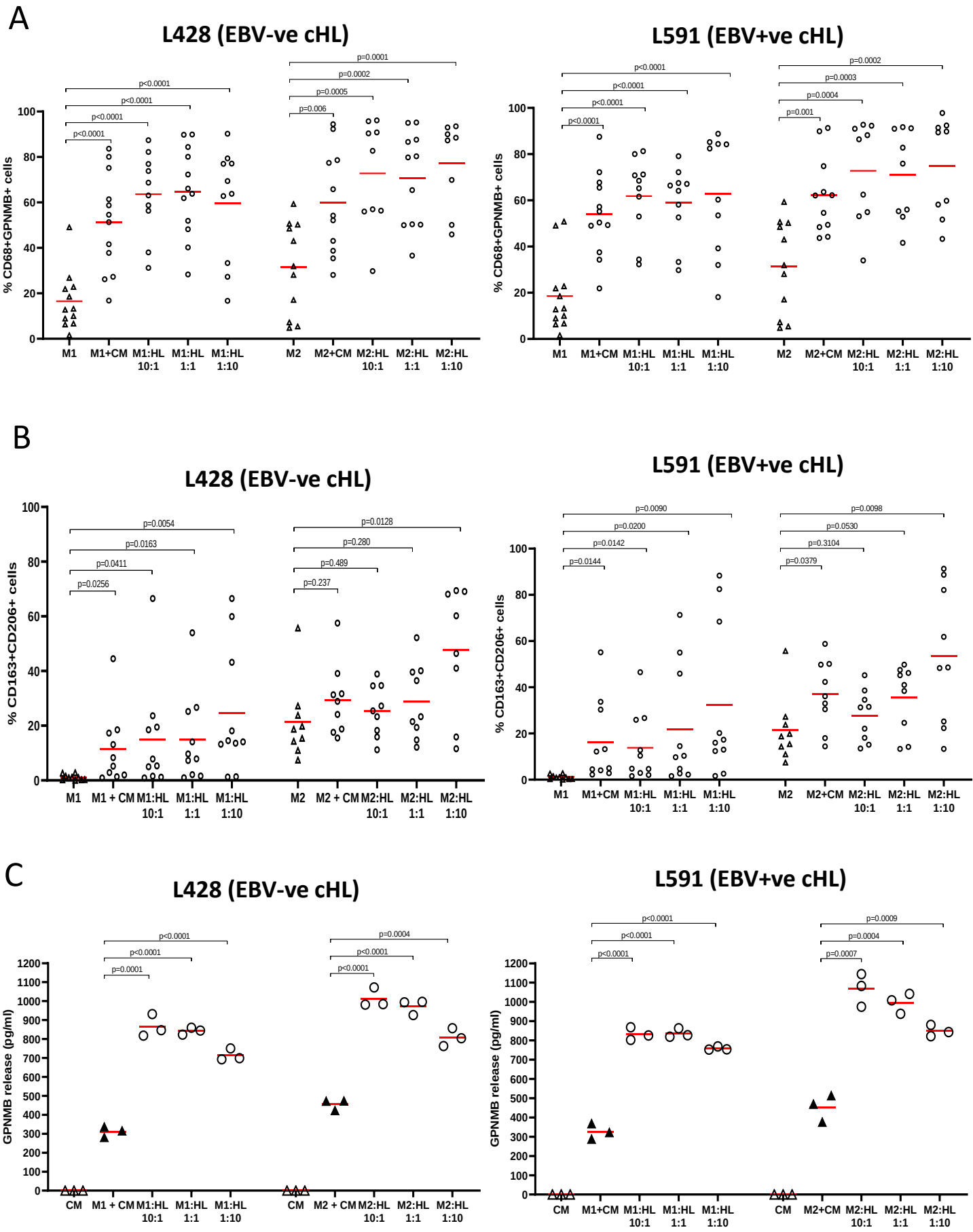


B



Supplementary Figure 1: GPNMB expression in primary cHL A) Survival analysis comparing GPNMB high versus GPNMB low tumors by Kaplan-Meier curves. Overall survival (OS) and progression free survival (PFS) were reduced in GPNMB high tumors compared to low tumors, but these differences were only of borderline significance. **B)** Correlation between PD-L1 (CD274) expression and GPNMB expression in cHL determined by Nanostring GeoMx in the macrophage enriched compartment (defined by CD68 expression).

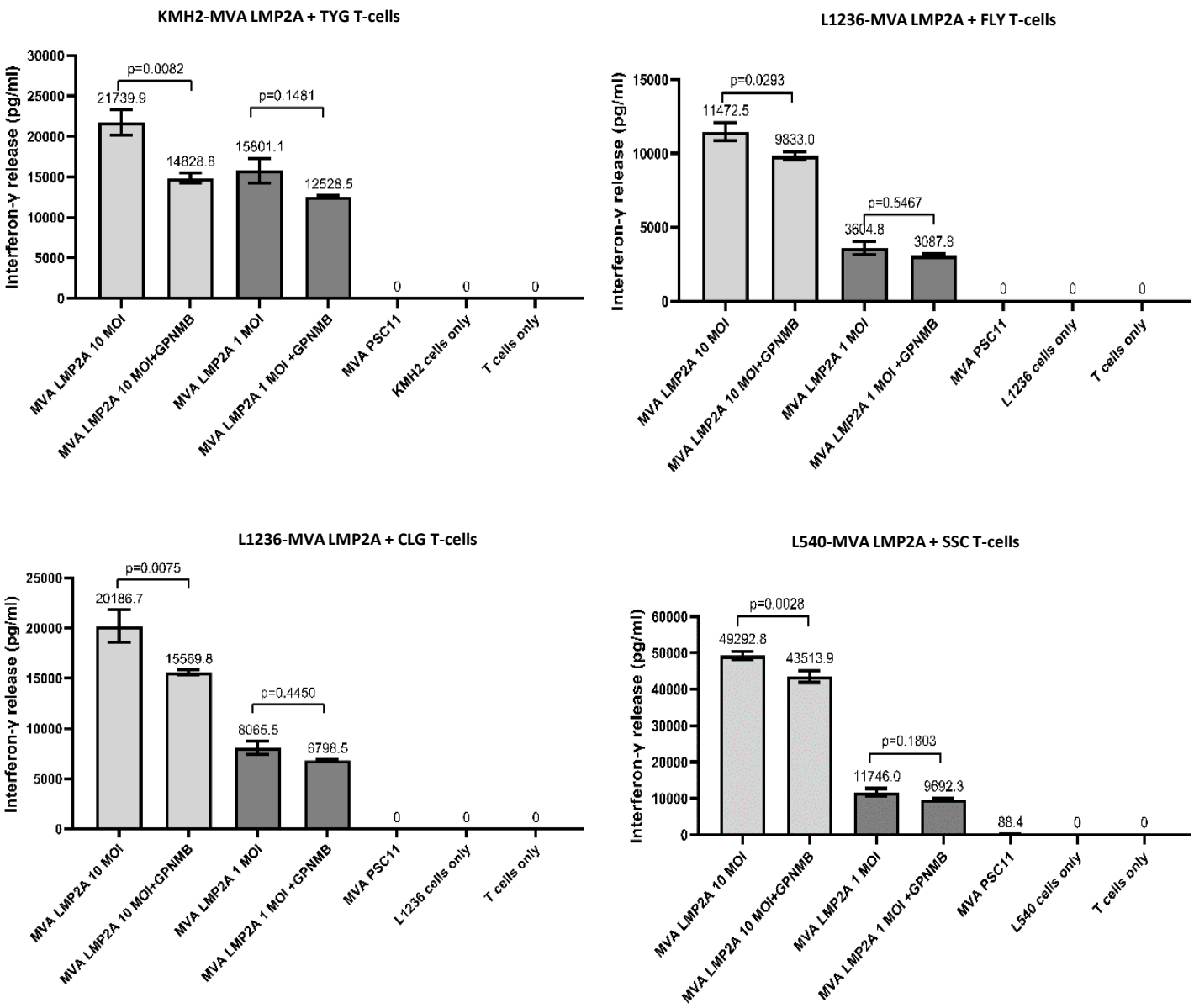
Supplementary Figure 2



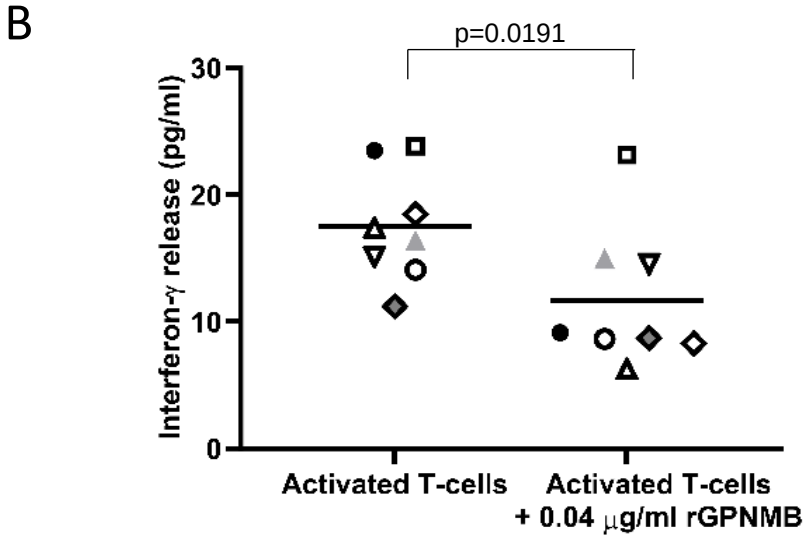
Supplementary Figure 2: cHL cells induce GPNMB expression and release from macrophages and their polarization to M2 phenotype. A) Flow cytometry for GPNMB expression on M1 or M2 macrophages following their culture in L428 or L591 cHL-derived conditioned media (+CM) or their direct co-culture with L428 and L591 cells at different macrophage:HL cell ratios for 24h. Macrophages generated from at least nine different individuals were tested per condition. Anti-GPNMB-PE antibody (HOST5DS) and CD68/PE-Texas Red (Thermo Fisher, eBioscience, Waltham, MA, USA) were used. **B)** Flow cytometry for CD163+CD206+ M2 marker expression on M1 or M2 macrophages following their culture in L428 and L591 CM or by their co-culture with L428 or L591 cells as in A). CD163/APC and CD206/PE-Cy7 (Thermo Fisher) antibodies were used. Macrophages generated from at least nine different individuals were tested per condition. **C)** ELISA measurement of GPNMB release by M1 and M2 macrophages exposed to L428 or L591 CM or directly co-cultured with L428 or L591 cells for 24h. GPNMB Duoset ELISA kit (R&D Systems, Minneapolis, MN, USA) was used. Shown are the results of three separate donors. Means (solid bars) for all experiments were compared by Student's t-test.

Supplementary Figure 3

A



B



Supplementary Figure 3: Low dose soluble recombinant rGPNMB inhibits T-cell recognition of cHL lines *in vitro*. A) ELISA measurement of interferon-γ release by T-cells co-cultured with cHL cell lines for 18h. cHL cells were infected with MVA LMP2A (MOI of 1 or 10) or negative control virus, MVA-pSC11 (MOI 10). An optimal dose of soluble recombinant GPNMB (rGPNMB, 0.04μg/ml) was tested using the same TYG and CLG-specific CD8+ T-cell clones (as in Figure 3) and additional CD8+ T-cell clones specific for the HLA-A2-restricted epitope FLY (LMP2 amino acids 356-364) and HLA-A11-restricted epitope SSC (LMP2 amino acids 340-350). For the latter, HLA-A11-positive L540 HL line was also used as the target cells. Means (solid bars) were compared by Student's t-test. **B)** ELISA measurement of interferon-γ release by primary PBMCs from eight independent donors in the presence/absence of 0.04μg/ml rGPNMB. PBMCs were activated using a range of six different concentration of soluble CD3/CD28 activator (Stemcell technologies, Vancouver, Canada, 10971; 0.125, 0.25, 0.5, 1, 2, 5μl/ml). All CD3/CD28 concentrations tested had a similar effect on T-cell activation (data not shown). The data shown are means (solid bars) of means of all CD3/CD28 activators contractions tested for all eight donors and compared by Student's t-test.