Impact of viral hepatitis therapy in multiple myeloma and other monoclonal gammopathies linked to hepatitis B or C viruses

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SUPPLEMENTARY DATA

Supplementary Methods

Purification of monoclonal Igs and evaluation of purity by isoelectric focusing (IEF)

After clotting, blood samples were centrifuged at 2,200g (4°C) and serum aliquots were frozen. Measurement of Ig concentration, purification of non-clonal and monoclonal IgG or IgAs, and verification of the purity of monoclonal Ig preparations were performed as described previously (Supplementary Figure S1)[17-20,27]. Ig concentration in blood serum was measured with an immunonephelemetric assay performed on a Beckman Immage Analyzer (Beckman Coulter, Villepinte, France). After protein separation using electric charge on agarose gel electrophoresis (SAS-MX high resolution, Helena Biosciences, Gateshead, UK), the band corresponding to the monoclonal Ig was carefully cut and proteins were eluted from gels into PBS. The purity of the monoclonal IgG and IgA preparations was verified using IEF. The different bands visible after IEF migration correspond to different levels of sialylation of the purified monoclonal Ig. For patients diagnosed with Bence-Jones MM, light chains were purified using PureProteome™ Protein G and Kappa magnetic beads (Merck Millipore) and the purity was evaluated by conventional native 15% polyacrylamide gel electrophoresis.

GlcSph Immunoblot Assay

Analysis of the presence of polyclonal or monoclonal Ig specific for GlcSph was performed using an immunoblotting assay adapted from Nair et al. [19,21]. Serum (containing polyclonal or non-clonal Igs ± patient's monoclonal Ig) and purified monoclonal IgGs and IgAs were systematically studied in parallel. Polyvinylidene fluoride (PVDF) membranes were incubated for 90 min in 100 µg/mL of GlcSph in 0.1 M sodium bicarbonate, rinsed 3 times in PBS and 0.1% Tween 20 detergent, then blocked for 2 hrs with 5% bovine serum albumin (BSA) in PBS and 0.1% Tween 20. Samples of serum or purified monoclonal IgGs or IgAs were submitted to agarose gel electrophoresis, then the gels were blotted onto the GlcSph-saturated membranes by diffusion blotting during 12 minutes [18,19]. After blocking for 1 hr with 2.5% BSA in PBS and 0.1% Tween 20, membranes were incubated with peroxidase-conjugated AffiniPure donkey anti-human IgG (H+L) antibody (Jackson ImmunoResearch, West Grove, USA) or horseradish peroxidase (HRP)-conjugated goat anti-human IgA α chain antibody (Bethyl Laboratories, Montgomery, TX, USA) for 1 hr, then washed and revealed with Super Signal West Pico chemiluminescent substrate (Thermo Scientific).

MIAA Assay

The MIAA assay allows testing for panels of commercially available antigens (Ag), proteins or/and lysates from 10 infectious pathogens: EBV, HSV-1, HSV-2, cytomegalovirus (CMV), varicella zoster virus (VZV), HBV (recombinant HBcAg (full length protein), HBeAg (full length protein), HBsAg/ad (aa 124-147), and HBx (full length protein)), HCV (recombinant Core, NS-3 and NS-4 proteins), *Helicobacter pylori* (*H. pylori*), *Toxoplasma gondii* (*T. gondii),* and *Borrelia burgdorferi* (*B. burgdorferi)*[17-20,27,28]. Infectious Ag were purchased from Abcam (Cambridge, United Kingdom), Advanced Biotechnologies Inc. (Columbia, MD, USA) and ImmunoDiag (Hämeenlinna, Finland). Lysates were supplied by Advanced Biotechnologies Inc. (Columbia, MD, USA) and EastCoast Bio (North Berwick, USA). The arrays consist of 8×8 matrices that included: (i) 15 Ag: 2 for EBV, 3 for HCV, 3 for HBV, 2 for *T. gondii*, 1 for HSV-1, 1 for HSV-2, 2 for VZV, and 1 for *B. burgdorferi*; (ii) 3 lysates: *H. pylori*, HSV-1, and HSV-2; (iii) 2 mixes: one of 5 Ag for CMV, and one of 2 Ag for *B. burgdorferi*. Infectious proteins, peptides and Ag are spotted in triplicate.

For hybridization, Ig concentrations were adjusted to 400 µg/mL for serum and from 50 to 200 µg/mL for purified monoclonal Igs. 80 µL of samples were incubated for 2 hours at room temperature. After washing, slides were incubated with a labeled secondary antibody (0.4 µg/mL Dylight^{TM}680 Labeled Goat anti-human IgG (H+L), from SeraCare, Milford, MA, USA; Ref. 5230-0342, or DyLight^{TM}680 goat anti-human IgA α chain from Immuno Reagent, Raleigh, NC, USA; Ref. GtxHu-001-E680NHSX). Fluorescence signal, detected with the Odyssey infrared imaging system scanner at 21 µm resolution (LI-COR Biosciences, NE, USA) was quantified using the GenePix® Pro 4 Microarray Acquisition & Analysis Software (Molecular Devices, Sunnyvale, CA, USA).

Dot Blotting Assays used to confirm the Infectious Targets of Monoclonal Igs

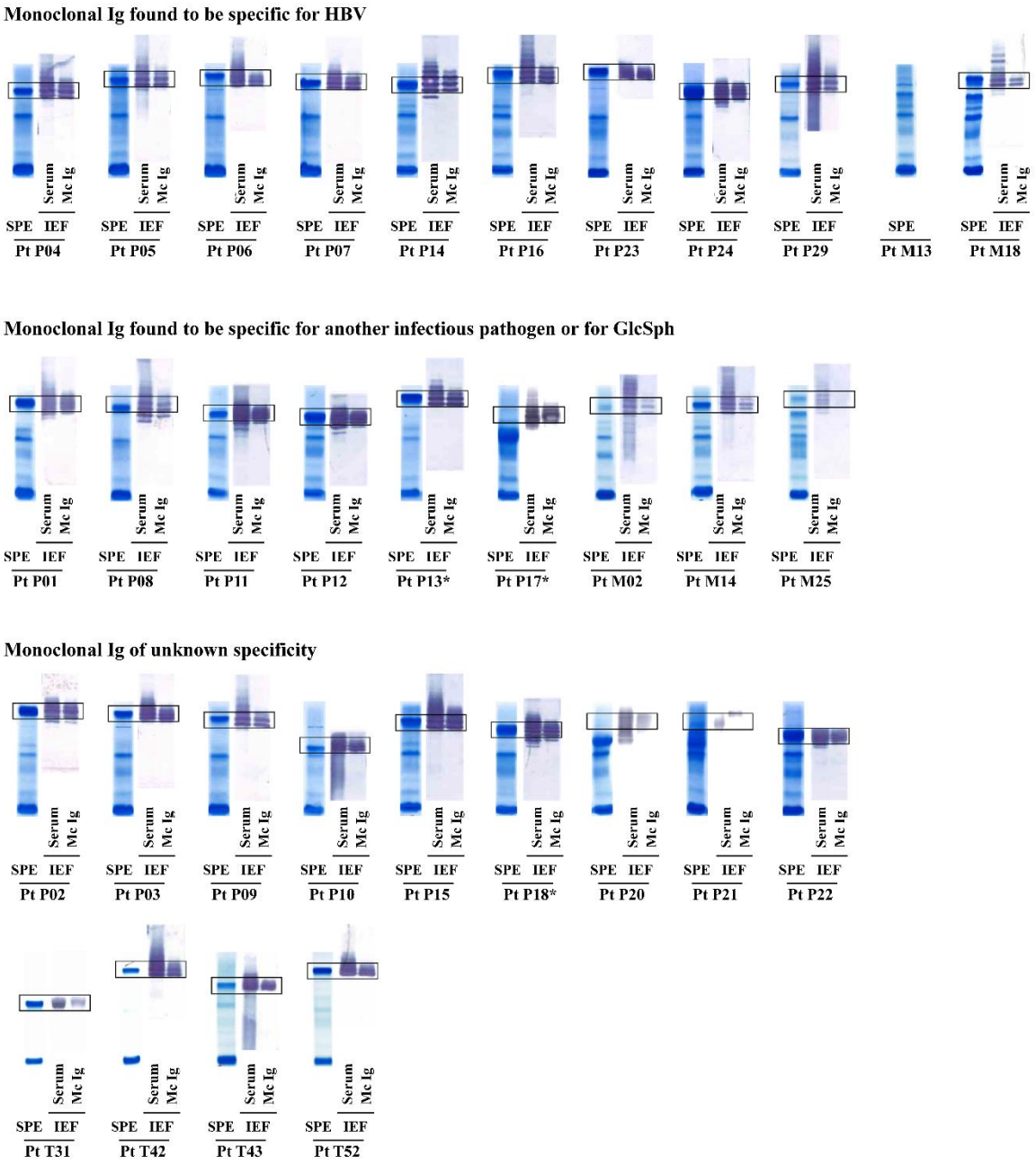
Dot blotting assays with HBV proteins were performed on nitrocellulose membranes (Amersham) spotted with 1 µg of recombinant HBcAg, HBeAg, HBsAg/ad, and HBx (4 spots) (Abcam, Advanced Biotechnologies Inc.). Membranes were then incubated with the patient's serum or with the purified monoclonal IgG or IgA [17-19]. Enterovirus VP1 dot blot assays were performed as published: different peptides (0.9 µg in 1µL, 0.5 nmol) were spotted on an Amersham^{TM} Protran^{TM} 0.45 µm nitrocellulose membrane (GE healthcare Life Sciences, Chicago, IL, USA) and membranes were let to dry[20]. Then membranes were incubated in PBS for 10 min, and saturated with PBST + 5% nonfat dry milk overnight at room temperature (RT).

Fifty µL of serum (0.4 g/L) or 10 µg of purified monoclonal Ig (0.2 g/L) in PBST + 1% BSA were added to the membrane and incubated for 2 hrs at RT. The membrane was washed with PBST, then incubated at RT for 1 hr with peroxidase affinipure donkey anti-human IgG (H+L) (Jackson ImmunoResearch, West Grove, USA) diluted 1:10000 in PBST + 1% BSA for monoclonal IgGs or HRP-labeled goat anti-human IgA (α chain) from Bethyl Laboratories (Montgomery, TX, USA) for monoclonal IgAs, diluted 1:5000 in PBST + 1% BSA. After washes in PBST, membranes were incubated with Super Signal West Pico or Femto chemiluminescence kits (Thermo Scientific, Rockford, IL, USA) and immune complexes were revealed using Camera Azure BioSystems c500 Imager (Azure Biosystems, Dublin, CA, USA).

Statistics

Data analysis was performed by GraphPad Prism 6.01 software. Patient parameters were expressed as medians and ranges, or/and means ± standard error of the mean (SEM). The chi-square ($\chi 2$) test was used for categorical variables. In nonparametric conditions, a Mann-Whitney t-test (2 groups) was performed. The tests used are indicated in the text. A P value below 0.05 was considered statistically significant.

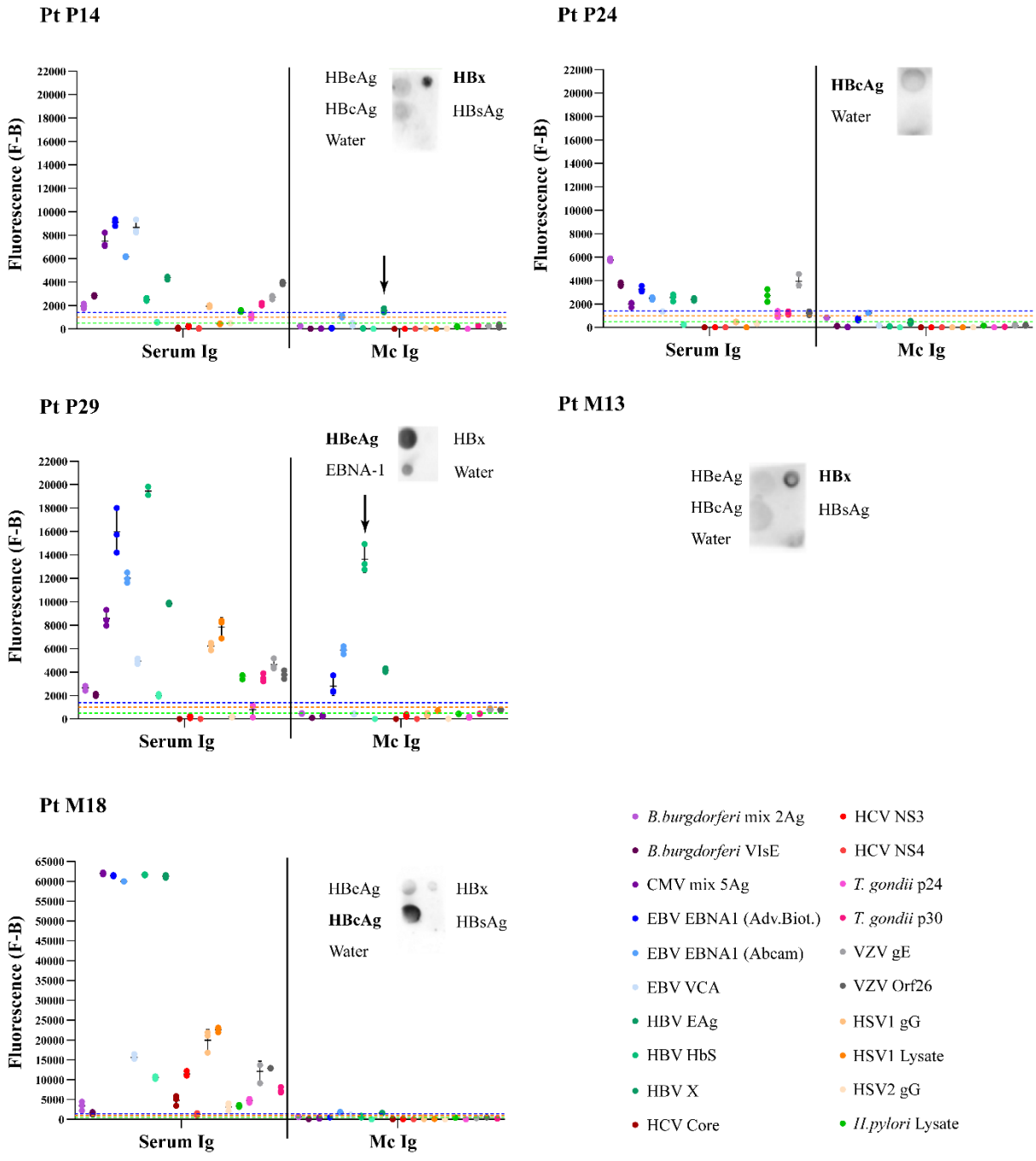
All statistical analysis of the large HBV+ and HCV+ MM cohorts were run with the TriNetX Platform. TriNetX is a custom-developed solution built using Java, for the backend, and R and Python statistical analysis libraries such as Hmisc, lifelines, matplolib, numpy, pandas and scipy. For each of the outcomes analyzed in the study, several metrics of association between cohorts were calculated. The risk difference between cohorts was assessed with a Z-test providing a 95% confidence interval. Risk ratio was also calculated with a 95% confidence interval. Outcomes were also analyzed with Kaplan-Meier method, and the Log-Rank test was used to assess curve differences. For survival studies, patients were included when the time period between the index event (the first MM diagnosis) and the analysis date was at least as long as the time window of the analysis, i.e. 3 years (from 1 month to 1095 days after the diagnosis of MM, the index event). In order to account for the patients who exited the cohort during the analysis period, and therefore should not be included in the analysis, censoring was applied. In this analysis, patients were removed from the analysis (censored) when the last fact in the patient's record is in the time window for analysis, the patient is censored on the day after the last fact in their record.



Supplementary Figure S1. Results of the purification of monoclonal Igs from patients with HBV infection or vaccinated, presenting with monoclonal gammopathy.

Measurement of Ig concentration, separation of the monoclonal Ig (Mc Ig) from other Igs, and verification of the purity of Mc Ig preparations were performed as described above. Purification started with the separation of serum proteins with high resolution agarose gel electrophoresis (SAS-MX high resolution, Helena Biosciences, Gateshead, UK). At the end of the serum protein electrophoresis (SPE), the Mc Ig was cut from the gel and eluted in PBS. The purity of the Mc Ig preparation was verified by isoelectric focusing (IEF) on an agarose gel (pH 3–10) followed

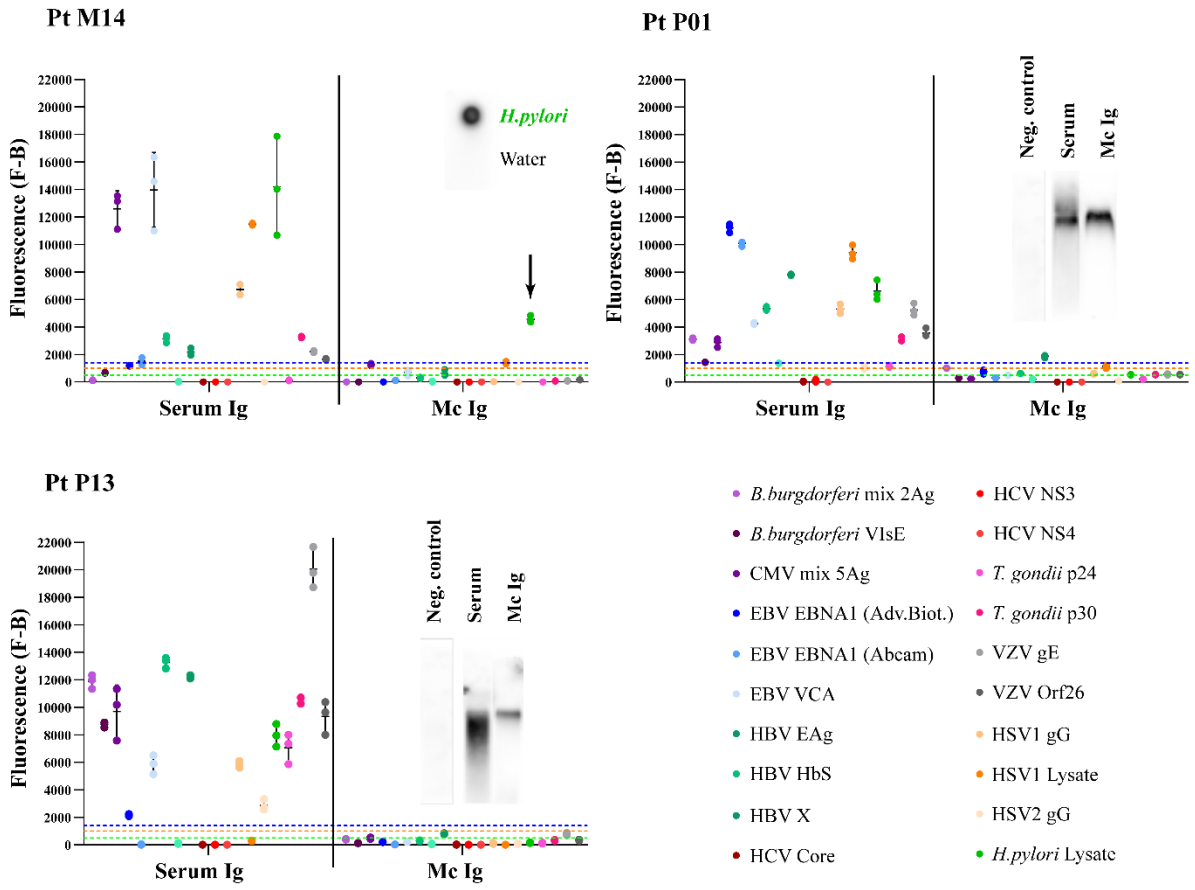
by blotting and immuno-revelation by an anti-human IgG gamma chain (Mc IgG) or anti-IgA alpha chain (Mc IgA) antibodies labeled with peroxidase. Note that the IEF was not possible for Mc IgM (Pt M13). Signals corresponding to the patient's Mc IgG, IgA, or IgM, are encircled in black. The different bands visible after IEF migration correspond to different levels of sialylation of the patient's purified monoclonal Ig. (*) Patients P13, P17 and P18 had been vaccinated against HBV; all other patients had been infected by HBV.



Supplementary Figure S2. Results of the MIAA and dot blot assays of five HBV-specific monoclonal Igs.

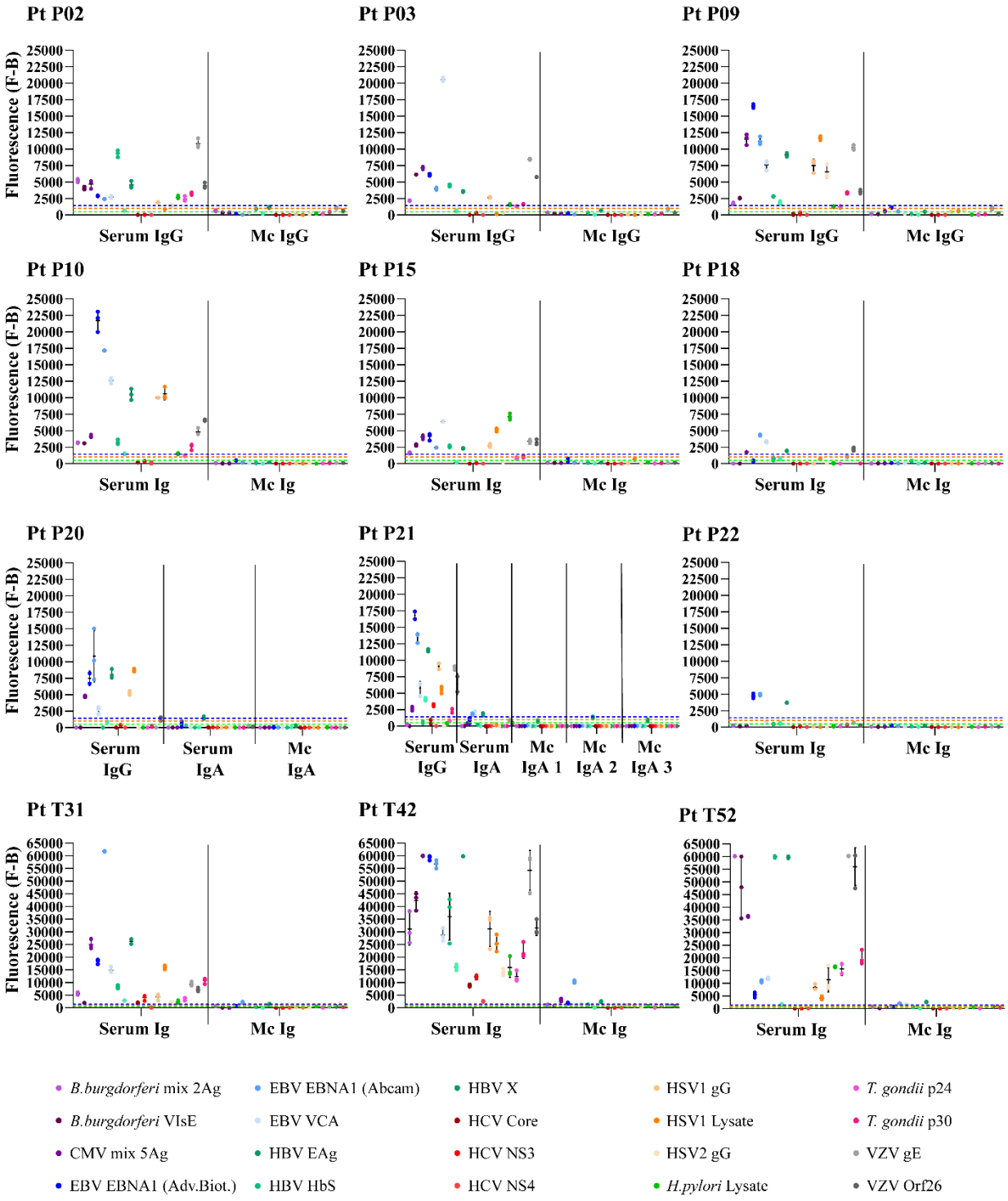
For each patient, results obtained in parallel with unseparated serum IgG or IgA (Serum, left) and the patient's monoclonal Ig (right) using the MIAA assay are represented; results are shown as fluorescent intensity (FI). The FI values shown for each pathogen (protein, lysate) were obtained after subtraction of the fluorescent background (B). For all patients, the serum contained IgG or IgA that recognized Ag, proteins or lysates from at least two infectious pathogens of the MIAA assay (EBV, CMV, HSV-1, HSV-2, VZV, HBV, HCV, *H. pylori*, *T. gondii*, *B.*

burgdorferi), whereas the purified Mc Ig did not recognize any pathogen. The thresholds of specific positivity (dotted lines) were defined for each viral pathogen or protein as 1,400 for EBV, CMV, VZV, HBV and *B. burgdorferi*, blue threshold; 1000 for HSV-1 and HSV-2, orange dotted line; and 500 for HCV, *H. pylori* and *T. gondii*, green threshold. Dots may be superimposed; horizontal bars represent the means of results obtained for a pathogen. Experiments were performed in triplicate, repeated at least once.



Supplementary Figure S3. Results of the MIAA, GlcSph immunoblot and dot blot assays of monoclonal Igs that target *H. pylori* or GlcSph.

For each patient, results obtained in parallel with the unseparated serum IgG (Serum, left) and the patient's purified monoclonal Ig (right) using the MIAA assay are represented; results are shown as fluorescent intensity (FI). The FI values shown for each pathogen, Ag, protein or lysate, were obtained after subtraction of the fluorescent background (B) of each pathogen protein or lysate. The thresholds of specific positivity, shown in dotted lines, were defined for each viral pathogen or protein: 1,400 for EBV, CMV, VZV, HBV and *B. burgdorferi*, blue threshold; 1000 for HSV-1 and HSV-2, orange dotted line; 500 for HCV, *H. pylori* and *T. gondii*, green threshold. Dots may be superimposed; horizontal bars represent the means of results obtained for a pathogen (Ag, lysate). Experiments were performed in triplicate, repeated at least once. Inserts show the results of dot blot assays performed with *H. pylori* lysate or GlcSph, and water or PBS used as control.



Supplementary Figure S4. MIAA assay results of purified monoclonal Igs of unknown target. For each patient, results obtained in parallel with unseparated serum IgG (Serum, left) and the patient's purified monoclonal Ig (right) using the MIAA assay are represented; results are shown as fluorescent intensity (FI). The FI values shown for each pathogen (protein, lysate)

were obtained after subtraction of the fluorescent background (B). For all patients, the serum contained IgG that recognized Ag, proteins or lysates from at least two infectious pathogens of the MIAA assay (EBV, CMV, HSV-1, HSV-2, VZV, HBV, HCV, *H. pylori*, *T. gondii*, *B. burgdorferi*), whereas the purified Mc Ig did not recognize any pathogen. The thresholds of specific positivity (dotted lines) were defined for each viral pathogen or protein as 1,400 for EBV, CMV, VZV, HBV and *B. burgdorferi*, blue threshold; 1000 for HSV-1 and HSV-2, orange dotted line; and 500 for HCV, *H. pylori* and *T. gondii*, green threshold. Dots may be superimposed; horizontal bars represent the means of results obtained for a pathogen. Experiments were performed in triplicate, repeated at least once.

Supplementary Table S1: Characteristics of patients with monoclonal gammopathy from the first cohort of HBV+ patients.

	MGUS/POEMS (n = 15)	**MM/PCL** (n = 30)
Sex		
n	15	30
Male / Female (Male %)	10 / 5 (66.7%)	19 / 13 (59.4%)
Age at diagnosis		
n	15	28
Median (Range)	63 (20-89)	70 (38-91)
Amount of Mc Ig (g/L)		
n	14	30
Median (Range)	9.4 (1.3-17.6)	25.0 (0.6-61)
BM plasma cells (%)		
n	2	22
Median (Range)	3 (2-4)	19.5 (6-90)
β_2-microglobulin (mg/L)		
n	4	18
Median (Range)	2.0 (1.6-2.9)	4.5 (2.1-15.7)
n > 3.5 mg/L (%)	0 (0%)	12 (66.7%)
Leukocytes (10^9/L)		
n	8	23
Median (Range)	6.1 (4.4-14.8)	6.8 (2.2-18.5)
Hemoglobin (g/dL)		
n	8	23
Median (Range)	14.2 (13.6-16.3)	10.0 (7.5-17.6)
Platelets (10^9/L)		
n	8	22
Median	242 (150-589)	194 (59-555)
Calcemia (mmol/L)		
n	6	22
Median (Range)	2.4 (2.2-2.6)	2.3 (2.0-3.0)
Creatinine (µmol/L)		
n	9	24
Median (Range)	70 (58-164)	103 (53-296)
Bone lesions		
n with lesions (%)	1/15 (6.7%)	10/12 (83.3%)

ISS Stage		
n	NA	19
n in Stage I / II / III	NA	4 / 6 / 9

For French patients, the study was promoted by the University Hospital of Nantes (# RC12 0085) with the approval of the local ethical committee and Commission Nationale Informatique et Libertés (CNIL #912335). For Spanish patients, the study was approved by the ethical committee of Hospital 12 de Octubre (Madrid, Spain). *n*, number of patients; Mc Ig, monoclonal Ig; BM, bone marrow; NA, not applicable; ISS, International Staging System; MGUS, monoclonal gammopathy of undetermined significance; POEMS, polyneuropathy, organomegaly, endocrinopathy/edema, monoclonal protein, skin changes; PCL, plasma cell leukemia.

Supplementary Table S2: MM disease characteristics according to the target of the patient's monoclonal Ig.

	Target of Mc Ig from MM Patients		
	HBV (n = 7)	**Other Target** (n = 5)	**Unknown Target** (n = 11)
Sex			
n	4	4	8
Male/Female (Male %)	2 / 2 (50%)	2 / 2 (50%)	6 / 2 (75%)
Age at diagnosis			
n	4	3	8
Median (Range)	67 (63-79)	70 (65-78)	66 (50-91)
Amount of Mc Ig (g/L)			
n	7	4	11
Median (Range)	23.0 (15.8-52.7)	34.0 (8.8-58.2)	25.8 (11.9-61)
BM plasma cells (%)			
n	2	3	6
Median (Range)	13.5 (13-14)	20 (19-90)	26 (4-47)
β_2-microglobulin (mg/L)			
n	2	3	6
Median (Range)	3.1 (2.99-3.3)	3.6 (3.1-4.2)	4.9 (2.1-12.1)
Nbr > 3.5 mg/L (%)	0 (0%)	2 (66.7%)	4 (66.7%)
Leukocytes (10^9/L)			
n	3	3	7
Median (Range)	3.4 (3.0-6.9)	3.6 (3.4-18.5)	4.9 (2.2-11.2)
Hemoglobin (g/dL)			
n	3	3	7
Median (Range)	12.0 (12.0-12.0)	9.8 (9.2-11.5)	10.0 (7.5-13.6)
Platelets (10^9/L)			
n	3	3	7
Median	237 (200-378)	193 (193-194)	206 (162-529)
Calcemia (mmol/L)			
n	2	3	14
Median (Range)	2.5 (2.3-2.64)	2.5 (2.4-3.0)	2.3 (2.0-2.7)
Creatinine (µmol/L)			
n	3	3	7
Median (Range)	88 (53-98)	108 (106-115)	125 (82-253)
Bone lesions			
n	0	2	4

n with lesions (%)	0	2 (100%)	2 (50%)
ISS Stage			
n	2	3	6
Stage I	1	0	2
Stage II	1	2	1
Stage III	0	1	3

n, number of patients; Mc Ig, monoclonal Ig; BM, bone marrow; HBV, hepatitis B virus; ISS, International Staging System.