A simple method to distinguish residual elotuzumab from monoclonal paraprotein in immunofixation assays for multiple myeloma patients

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Abbreviations:

antigen-specific therapeutic monoclonal antibody depletion assay (ASADA); bone marrow (BM); complete response (CR); free light chain (FLC); immunofixation electrophoresis (IFE); minimal residual disease (MRD); monoclonal antibodies (mAbs); monoclonal immunoglobulin protein (M-protein); multiple myeloma (MM); signaling lymphocyte activation molecule F7 (SLAM7).

Abstract

Negative immunofixation electrophoresis (IFE) of serum and/or urine is a diagnostic marker for determining a complete response (CR) after immunotherapy for multiple myeloma (MM). However, residual therapeutic antibodies such as elotuzumab (IgG-κ), can compromise IFE evaluation when the affected immunoglobulins belong to the same IgG-κ subclass. We thus sought to develop a simple and rapid method to treat patient serum before IFE to distinguish the residual elotuzumab. Serum samples from patients receiving elotuzumab were treated with a predetermined amount of soluble signaling lymphocyte activation molecule F7 (SLAMF7) protein and then subjected to conventional IFE testing. We tested our method in samples from 12 patients. The IgG-κ band in IFE disappeared or shifted after elotuzumab treatment in 4 patients with negative bone marrow minimal residual disease and a normalized free light chain whereas seven patients with any sign of residual MM showed a remaining IgG-κ band after treatment. One-hour incubation of samples with 6–9 molar excess soluble SLAMF7 before IFE was sufficient to distinguish residual elotuzumab in 11 of 12 samples. This simple method does not require special reagents, can be performed in most clinical laboratories, and enables differentiation between patients with a CR and those requiring further treatment.

Keywords:

elotuzumab; immunofixation electrophoresis; monoclonal antibody; multiple myeloma; therapeutic

1. INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy wherein plasma cells secrete a large amount of monoclonal immunoglobulin protein (M-protein) [1, 2]. Treatments for MM include therapeutic monoclonal antibodies (mAbs) such as elotuzumab and daratumumab. A complete response (CR) should achieve the following standard (according to the international Myeloma Working Group): negative immunofixation electrophoresis (IFE) of serum and urine, disappearance of any soft tissue plasmacytomas, and < 5% plasma cells in the bone marrow whereas a very good partial response (VGPR) is defined as serum and urine M-protein detectable by immunofixation but not on electrophoresis or > 90% reduction in serum M-protein plus a urine M-protein level < 100 mg/24 h [3]. IFE is a qualitative analysis of M-protein and is about 10 times more sensitive than conventional serum protein electrophoresis. The migration of M-protein depends on their electrical charge but not on their molecular weight as the pores of agarose gel used in IFE are too small to sieve proteins unlike DNA.

Elotuzumab is a humanized IgG-κ mAb specific to the signaling lymphocyte activation molecule F7 (SLAMF7), which is highly expressed in MM cells [4], and induces antibody-dependent cellular cytotoxicity targeted to MM cells [5]. Elotuzumab is administered at 10 mg/kg per week for the first two cycles, followed by a dose every two weeks [6]. Serum elotuzumab levels reach peak concentrations of around 0.3 g/L [7], or steady-state trough concentrations of 0.194 g/L [8]. An obstacle in evaluating the efficacy of this mAb is that it can also be detected by IFE (detection limit is 0.1 to 0.3 g/L) owing to co-migration with the patient's M-protein [9, 10]. Therefore, it is critical to distinguish the residual mAb from patient's M-protein. In case of daratuzumab, Hydrashift 2/4 Daratuzumab has been developed to shift the residual daratumumab in the alpha-1 zone on an IFE gel by the creation of a daratumumab/anti-daratumumab immune complex [11-13]. In case of elotuzumab, the antigen-specific therapeutic monoclonal antibody depletion assay (ASADA), which was recently reported, involves incubation of the patient serum with magnetic beads coated with SLAMF7 protein to deplete the coexisting elotuzumab

[14]. Based on the fact that soluble SLAMF7 binds elotuzumab [15], we sought to develop a simpler method to eliminate it from patient samples.

2. METHODS

2.1 Patient Samples

This study was performed in accordance with the Declaration of Helsinki, and the research protocol was approved by the Ethics Committee of Nagoya University and that of the participating institutions. Waste clinical serum samples were collected and stored at -20°C before being treated with soluble SLAMF7 and subjected to IFE testing at Bio Medical Laboratories (BML), Inc. (Kawagoe, Japan). Patients with insufficient serum samples were excluded from the analysis.

2.2 Recombinant SLAMF7 Protein

Recombinant cDNA consisting of the codon-modified human interleukin-2 signal sequence, the extracellular part of human SLAMF7 (aa 23-226), and a polyhistidine tag (6 x His) was synthesized (Thermo Fisher Scientific, MA, USA) and cloned into pcDNA3.1(+) plasmid. CHO-S cells (Thermo Fisher Scientific) were transfected with the plasmid, and recombinant SLAMF7 was expressed using the EpiCHO expression system (Thermo Fisher Scientific). The protein was purified and concentrated to 20 g/L using His GraviTrap (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden), verified by SDS-PAGE, and stored in working aliquots at -80ºC. The yield of SLAMF7 was 5.15 g from 256 mL CHO-S cell culture.

2.3 Serum Treatment and IFE

Five hundred microliters of patient serum samples were split into two paired 1.5 mL microcentrifuge tubes, and the SLAMF7 solution was then added (1.52 %(v/v), final concentration, 0.2 or 0.3 g/L) to one of the tubes. The tubes were rocked on a hand-motion shaker (at ~120 rpm for 1 hour at room temperature). As model experiments, pooled human

AB serum (Access Biologicals LLC, CA, USA) diluted with the same volume of saline (50 %) was aliquoted and pulsed with elotuzumab, cetuximab, or bevacizumab (all IgG-κ, final concentration, 0.3 g/L), and then treated with graded amounts of SLAMF7 (final concentrations: 0, 0.1, 0.2, and 0.3 g/L) were also used. The treated samples were chilled and directly shipped to BML Inc., where IFE was performed on the Helena SPIFE 4000 analyzer (Texas, USA) as per the manufacturer's instruction. Briefly, patient serum samples were diluted 1:3 with 0.85% saline for serum protein (SP) lanes and 1:5 for IgG, IgA, IgM, κ and λ lanes, then loaded into the destined slots of an agarose gel. The dilution factor with saline may be further adjusted according to the M-protein concentration. Following electrophoresis in pH 9.0 buffer, a fixative was added to the SP lanes and antisera were added to the respective lanes. Finally, unreacted proteins other than respective precipitated immunoglobulins were removed by washing and the antigen-antibody complexes were visualized by Acid Violet staining. Thus, the SP lane indicated serum protein electrophoresis patterns of individual samples such as albumin (Supplementary Fig. 1a) at the bottom (close to the anode side), preceded by α1-, α2-, β-, and γ-globulins as a reflection of differences in their electrical charge. In the buffer with pH 9.0, most serum proteins charge negatively, and thus migrate toward the anode located at the bottom of the gel.

3. RESULTS

3.1 Removal of Elotuzumab from Model Samples by Soluble SLAMF7 Treatment

The molecular weight and theoretical isoelectric point (pI) of the expressed SLAMF7 are 23.3 Kd and 6.77, whereas those of elotuzumab are 145 Kd [8], and 7.92 (calculated using Compute pI/Mw software [16] at https://web.expasy.org/compute_pi/), respectively. As IgG has two antigen binding sites, 1 mole of elotuzumab binds 2 moles of SLAMF7; thus the pI of the resultant trimer is calculated as 7.64 suggesting that the trimer should migrate faster toward the bottom of the gel [16]. We titrated the soluble SLAMF7 (0, 0.1, 0.2, and 0.3 g/L) against 0.3 g/L elotuzumab in normal 50% AB serum. Here, 50% AB serum was used to make the M-protein band clearer otherwise the γ-globulin showed a

dense smear pattern. To facilitate the exact comparison of band positions obtained from gel pictures run on different electrophoresis apparatus or different dates, a white line passing just below the band corresponding to β-globin band was added throughout all the gel pictures. A band corresponding to elotuzumab without SLAMF7 appeared in IFE, which was still visible in the presence of 0.1 g/L SLAMF7, but became invisible in the presence of 0.2 g/L SLAMF7 (Fig. 1a, solid arrow). However, in the presence of 0.3 g/L SLAMF7, a faint band was weakly detected at a lower position (Fig. 1a, dashed arrow) but this phenomenon was not always observed (Supplementary Fig. 1b). A similar experiment conducted in the absence of AB serum showed an unexpected presence of a shifted band in the presence of 0.3 g/L SLAMF7 as well as 0.2 d/L SLAMF7 (Supplementary Fig. 2). In contrast, a band corresponding to cetuximab (Fig. 1b) and bevacizumab (Fig. 1c) remained unchanged after SLAMF7 treatments. In summary, only bands of elotuzumab were either specifically disappeared or shifted by the change in pI due to the elotuzumab/SLAMF7 complex formation.

3.2 Clinical Sample Analysis

We collected waste serum from 12 patients with MM, on elotuzumab therapy, who were in VGPR or better according to the IMWG Uniform Response Criteria for Multiple Myeloma on elotuzumab therapy (Table 1), and evaluated the performance of our simple approach to examine whether our method can help diagnose patients who might have achieved CR or better but are forced to remain in VGPR due to the presence of monoclonal band(s) in the IFE test. All except one sample from Pt-12 were collected on day 14 after the last elotuzumab therapy when its serum concentration was at the trough timing. First, we tested the serum from 3 patients with MM other than the IgG-κ type because the presence of IgG-κ band in IFE should strongly suggest the residual elotuzumab. Indeed, Pt-2 was IFE negative for IgG-κ prior to elotuzumab treatment. As shown in Fig. 2, the IgG-κ band in Pt-1 and Pt-3 disappeared after SLAMF7 treatment, whereas that in Pt-2 showed a faint band shifted to the anode side as observed in the model experiment (Fig. 1a). Because these

3 patients were negative for bone marrow (BM) minimal residual disease (MRD) and had a normalized free light chain (FLC) ratio, it was possible to determine that they were in stringent CR (sCR) at the time of the study. The remaining 9 patients were all diagnosed with IgG-κ MM. Pt-4 was also negative for BM-MRD, had a normalized FLC ratio, and showed a negative IFE after SLAMF7 treatment, indicating that Pt-4 was in sCR. Pt-5 showed negative IFE after SLAMF7 treatment; however, due to the lack of BM aspiration the disease status after the study remained VGPR. Both Pt-6 and Pt-7 with two distinct IgG-κ bands showed a remaining upper band but either a disappeared lower band (Pt-6) or a shifted band (Pt-7) after SLAMF7 treatment, suggesting residual MM at VGPR status. Finally, the IgG-κ bands of Pt-8 through Pt-12 (Fig. 2, right column) were much denser than those of residual elotuzumab as above and remained unchanged even after SLAMF7 treatment. Indeed, two patients (Pt-8 and Pt-9) had an abnormal FLC ratio, three patients (Pt 10 to Pt-12) were positive for BM-MRD, and the residual IgG-κ after SLAMF7 treatment was thought to be caused by residual MM in these patients. Interestingly, a band suggestive of residual elotuzumab was not observed in these 5 patients (see Discussion). Collectively, the results of paired IFE tests before and after SLAMF7 treatment matched the clinical diagnosis made by circumstantial findings such as FLC and BM-MRD status.

4. DISCUSSION

Elotuzumab and daratumumab are IgG-κ mAbs currently used for the treatment of MM, and the co-migration of these mAbs with the patient's M-protein near the CR stage compromises the evaluation of their therapeutic efficacy [9]. Although elotuzumab is frequently reported to be undetectable by IFE, whose detection limit is 0.1 to 0.3 g/L, due to its lower Cmax of ~0.22 g/L [14, 8] compared with daratumumab (~0.9 g/L) [17], we found that 7 of 12 patients showed an IgG-k band suggestive of residual elotuzumab in our cohort. The presence of these bands was specifically distinguished by our assay in all 7 samples, although this distinction was made by either disappearance of the band or a combination of decreased intensity and shift toward the anodal side (lower position). The

reason for why two patterns were observed is to be determined in a future study. Theoretically, we expected to see a shifted band only when an observed IgG-κ band was the reflection of elotuzumab after SLAMF7 treatment due to pI change from 7.92 to 7.64 caused by the binding of SLAMF7 to elotuzumab. As IFE relies on the precipitation of insoluble immune complexes of immunoglobulins and their respective detection antibodies, it is speculated that the elotuzumab precipitation with both anti-IgG and anti-κ antibodies might be inhibited by the presence of some serum proteins depending on various samples. This possibility may be supported by the observation that this disappearance was not observed when elotuzumab was mixed with SLAMF7 in saline. In addition, presence of soluble SLAMF7 in MM patient serum [18] may also affect the effect of SLAMF7 treatment in IFE. Finally, destruction of elotuzumab by SLAMF7 is unlikely because elotuzumab administration results in the transition of free SLAMF7 to stable elotuzumab-bound SLAMF7 both of which are detectable by ELISA [15]. Accordingly, the finding of no band suggestive of residual elotuzumab in Pt-8 through Pt-12 might also be partly explained by the presence of soluble SLAMF7 in patients with active MM; however, there is another possibility that the two bands of M-protein and elotuzumab might be overlapped with each other.

The shift of daratumumab in the case of the Hydrashift assay is remarkable [13, 12, 11] wherein the daratumumab and anti-daratumumab antibody complex is observed at the position of the α1-globin fraction with a pI range of 5–6. This major shift is an advantage of the Hydrashift assay. One potential improvement to be considered is to add acidic amino acids such as aspartic acid or glutamic acid to the C terminus of SLAMF7 to make the protein's pI more acidic, resulting in a remarkable shift when bound to elotuzumab.

The recently reported method, ASADA [14], can potentially deplete any therapeutic mAbs by overnight incubation of serum with magnetic beads coated with antigens of the respective mAbs. Here, we directly added the concentrated antigen (SLAMF7) to the samples to minimize their dilution and incubated them for only 1 hour before analysis. It is not clear whether both approaches are similarly efficient without a direct parallel

comparison. However, our data show that it is feasible to apply this method in the clinical setting if a paired sample (i.e. with or without SLAMF7 treatment) is analyzed in parallel on the same gel. In addition, we found that SLAMF7 was stable at least for 2 years when stored at -80°C. Overall, we surmise that this method may be applied to other mAbs so long as a stable soluble antigen, with the necessary post-translational modifications critical for mAb recognition, can be produced *in vitro*.

5. CONCLUSIONS

We describe a simple, rapid, and efficient method for discriminating residual elotuzumab in samples from patients with MM. This method can be performed in most clinical laboratories and enables differentiation between patients with a CR and those who require further treatment. It can also be adapted to other therapeutic mAbs so long as their cognate target antigen can be prepared as a stable concentrated protein. However, the mechanism underlying the disappearance of this complex remains to be determined. Finally, further efforts to increase band resolution are warranted.

CONFLICTS OF INTEREST

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Figure Legends

Fig. 1. Titration of SLAMF7 against elotuzumab in immunofixation electrophoresis
a Elotuzumab (IgG-κ) was added to 50% normal human AB serum (0.3 g/L) and treated with mock saline or graded concentrations of SLAMF7 for 1 hour with shaking at room temperature, and was then subjected to regular immunofixation electrophoresis. To facilitate the exact comparison of band positions obtained from gel pictures run on different electrophoresis apparatus or on different dates, a white line passing just below the band corresponding to β-globin band was added to all gel pictures. **b, c** Cetuximab (IgG-κ) or bevacizumab was added to 50% normal human AB serum (0.3 g/L) and treated with mock saline or SLAMF7 at a final concentration of 0.3 g/L as in **a**. Solid arrow: a band corresponding to therapeutic monoclonal antibodies; dashed arrow. a shifted band after SLAMF7 treatment. SP, serum protein; G, IgG heavy chain; A, IgA heavy chain; M, IgM heavy chain; κ, kappa light chain; λ, lambda light chain.

Fig. 2. Immunofixation electrophoresis of patient samples
Serum samples from patients were treated with mock saline or SLAMF7 (right column) for 1 hour with shaking at room temperature, and then subjected to regular immunofixation electrophoresis. Pt-1 to Pt-3, samples from patients with non-IgG-κ type multiple myeloma. Pt-4 to Pt-12, samples from patients with IgG-κ multiple myeloma. Solid arrow, IgG-κ bands observed in all samples before and/or after the addition of SLAMF7; dashed arrow. a shifted band after SLAMF7 treatment as in Figure 1a. SP, serum protein; G, IgG heavy chain; A, IgA heavy chain; M, IgM heavy chain; κ, kappa light chain; λ, lambda light chain.

Figure 1

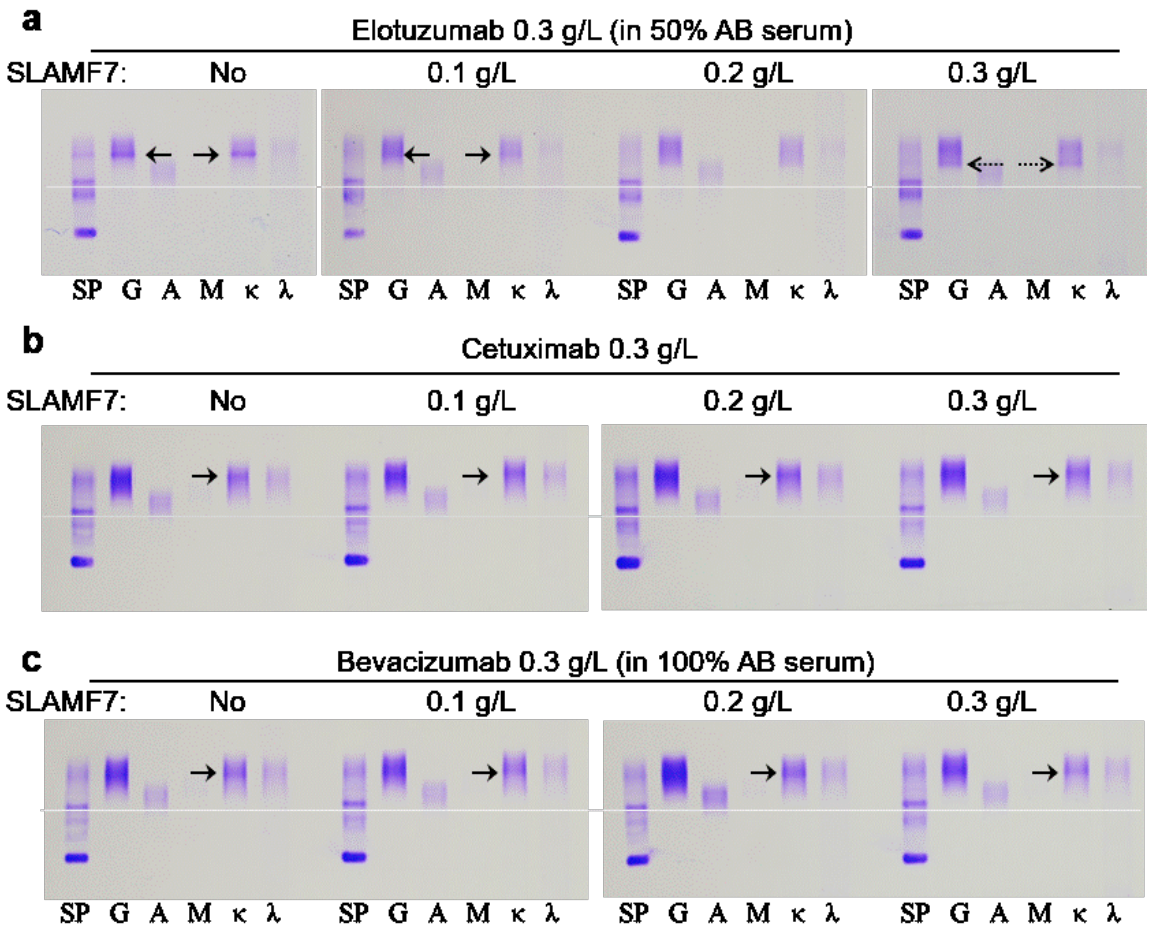
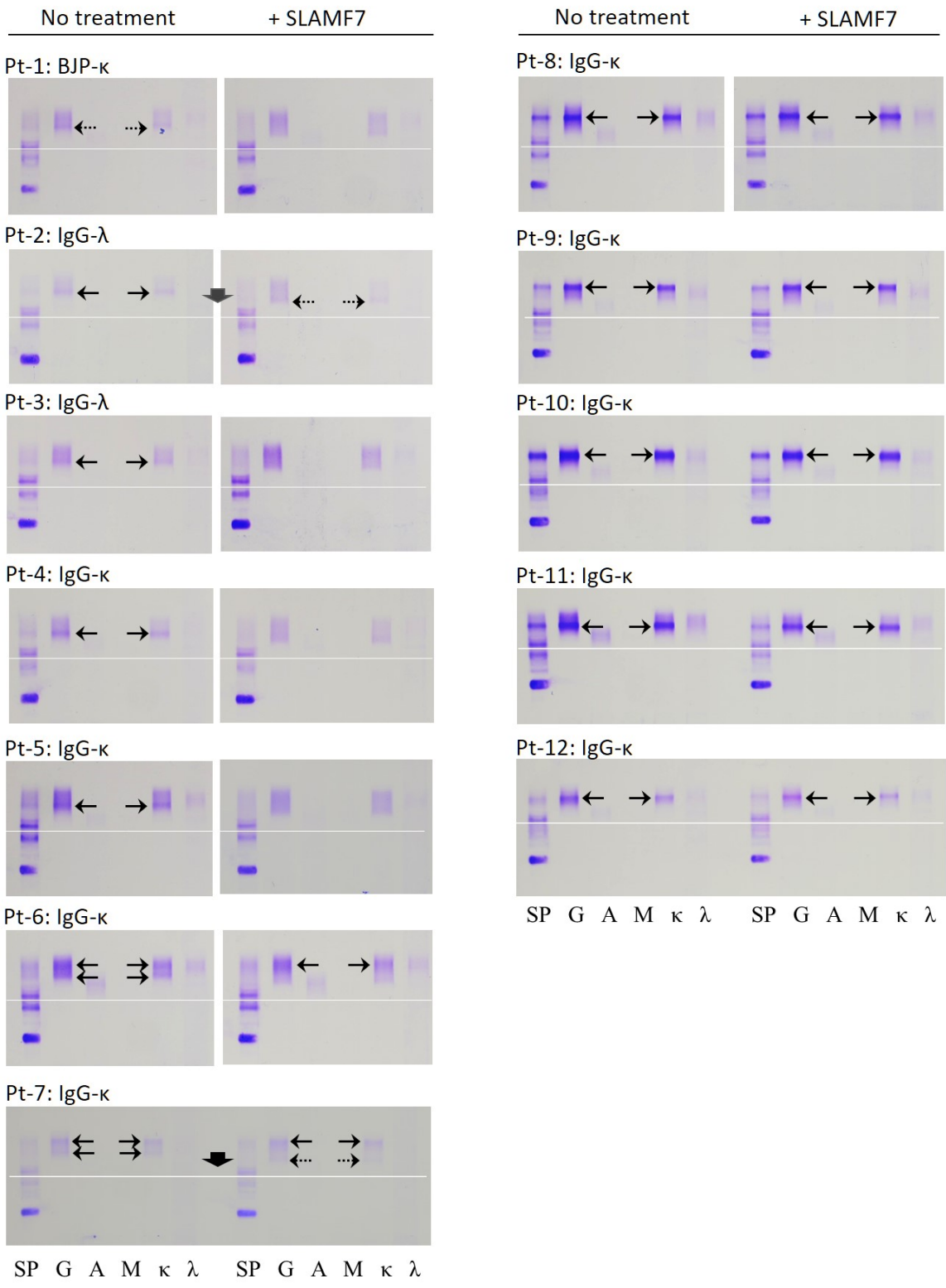

a
Elotuzumab 0.3 g/L (in 50% AB serum)
SLAMF7:
No
0.1 g/L
0.2 g/L
0.3 g/L
SP G A M κ λ
b
Cetuximab 0.3 g/L
c
Bevacizumab 0.3 g/L (in 100% AB serum)

Figure 2


No treatment
+ SLAMF7
Pt-1: BJP-κ
Pt-2: IgG-λ
Pt-3: IgG-λ
Pt-4: IgG-κ
Pt-5: IgG-κ
Pt-6: IgG-κ
Pt-7: IgG-κ
SP G A M κ λ SP G A M κ λ
No treatment
+ SLAMF7
Pt-8: IgG-κ
Pt-9: IgG-κ
Pt-10: IgG-κ
Pt-11: IgG-κ
Pt-12: IgG-κ
SP G A M κ λ SP G A M κ λ