Journal of Immunological Methods xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

High-throughput allogeneic antibody detection using protein microarrays

Jed Paul [a], Bita Sahaf [a], Spenser Perloff [a], Kelsi Schoenrock [a], Fang Wu [a], Hideki Nakasone [a], John Coller [b], David Miklos [a,*]

[a] Division of Blood and Marrow Transplantation, Stanford University School of Medicine, 269 West Campus Dr., CCSR #2205, Stanford, CA 94305, United States
[b] Stanford Functional Genomics Facility, Stanford University School of Medicine, 269 West Campus Dr., CCSR #0120, Stanford, CA 94305, United States

ARTICLE INFO

Article history:
Received 29 November 2015
Received in revised form 4 February 2016
Accepted 8 February 2016
Available online xxxx

Keywords:
Protein microarray
HY antigen
Hematopoietic cell transplantation
Chronic graft versus host disease
Alloantibody

ABSTRACT

Enzyme-linked immunosorbent assays (ELISAs) have traditionally been used to detect alloantibodies in patient plasma samples post hematopoietic cell transplantation (HCT); however, protein microarrays have the potential to be multiplexed, more sensitive, and higher throughput than ELISAs. Here, we describe the development of a novel and sensitive microarray method for detection of allogeneic antibodies against minor histocompatibility antigens encoded on the Y chromosome, called HY antigens. Six microarray surfaces were tested for their ability to bind recombinant protein and peptide HY antigens. Significant allogeneic immune responses were determined in male patients with female donors by considering normal male donor responses as baseline. HY microarray results were also compared with our previous ELISA results. Our overall goal was to maximize antibody detection for both recombinant protein and peptide epitopes. For detection of HY antigens, the Epoxy (Schott) protein microarray surface was both most sensitive and reliable and has become the standard surface in our microarray platform.

1. Introduction

Following HLA matched hematopoietic cell transplantation (HCT), minor histocompatibility antigen (mHA) presentation to both B and T lymphocytes is critical in both the graft versus leukemia as well as graft versus host responses (Miklos et al., 2005). These mHAs distinguish self from non-self cells and elicit allogeneic B and T cell immune responses. While T cell recognition of mHAs is HLA restricted, the B-lymphocytes express massively diverse B cell receptors, facilitating adaptive immunity to a nearly unlimited number of possible antigens. We have previously demonstrated that allogeneic antibodies develop from donor B cells 3 months post allogeneic HCT in association with chronic graft versus host disease (cGVHD) (Miklos et al., 2004; Miklos et al., 2005; Nakasone et al., 2015b).

cGVHD is a particularly severe problem in male recipients with female donors in which the gender disparity causes a targeted attack on the Y chromosome encoded proteins (Loren et al., 2006; Randolph et al., 2004). As a result, the risk for cGVHD for male patients with a female donor (F→M) is significantly increased compared to any other gender combination in a conditioning-dependent manner. (Nakasone et al., 2015a). F→M patients thus provide an excellent model for studying HY alloimmunity.

In our model for detection of allogeneic antibodies in F→M HCT patients, the male graft recipients develop antibodies against Y-chromosome encoded antigens (HY antigens) that have up to 99% identity with their X-chromosome homologues. These HY antigens, which are ubiquitously expressed in all tissues, include DBY, EIF1AY, RPS4Y, UTY, and ZFY (Popli et al., 2014).

Traditionally, enzyme linked immunosorbent assays (ELISAs) have been used to provide quantitative antibody measurements and were first employed for HY antibody detection as well (Miklos et al., 2004). However, ELISA is only capable of testing a single antigen at a time and requires large amounts of both recombinant antigen and patient plasma samples (Wadia et al., 2011). Moreover, ELISAs are inefficient for studying peptides and specific epitopes, as one protein can be split into hundreds of smaller segments, each requiring its own well. Protein microarrays are a new technology that have promise to overcome these drawbacks.

Protein microarray technology allows for the analysis of ten or more patient samples against many spatially isolated antigens on a single glass slide with higher sensitivity than a conventional ELISA (Robinson et al., 2002; Wilson and Nock, 2003). While protein microarrays have been used for antibody detection in the past, there is disagreement

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HCT, hematopoietic cell transplantation; mHA, minor histocompatibility antigen; cGVHD, chronic graft versus host disease; F→M, female donor into a male recipient; MFI, median fluorescence intensity; IQR, interquartile range.

* Corresponding author at: Division of Blood and Marrow Transplantation, Stanford University School of Medicine, 269 West Campus Dr., CCSR #2205, Stanford, CA 94305, United States.
E-mail addresses: jedpaul@stanford.edu (J. Paul), bsahaf@stanford.edu (B. Sahaf), shperloff@ucdavis.edu (S. Perloff), fangw@stanford.edu (F. Wu), nakasone-tky@umin.ac.jp (H. Nakasone), john.coller@stanford.edu (J. Coller), dmiklos@stanford.edu, dmiklos@stanord.edu (D. Miklos).

http://dx.doi.org/10.1016/j.jim.2016.02.009
0022-1759/

regarding which slide surface chemistry is optimal, and results tend to be antigen-specific (Stoevesandt et al., 2009; Balboni et al., 2008; Guilleaume et al., 2005). We have developed a protein microarray system with high throughput and sensitivity to multiplex the identification of patients with reactivity to HY proteins and their composite epitopes, mimicked by overlapping peptides. To optimize HY protein microarrays, we here compare six commercially available surfaces using both protein and peptide antigens. Each surface was tested for its HY protein and peptide binding, IgG detection signal-to-noise ratio, and reproducibility. We then compared microarray quantifications of anti-HY antibodies to those previously measured by ELISA and their clinical utility. These efforts have maximized HY antibody detection in a high throughput manner, providing proven utility in examining clinical outcomes such as chronic GVHD prediction following allogeneic HCT.

2. Methods

2.1. Patient characteristics and plasma samples

Plasma samples were collected from 32 male patients who had undergone allogeneic HCT with either a related or unrelated HLA matched female donor. In order to test plasma most likely to have allogeneic antibodies against one or more of the HY antigens, we chose patients who had subsequently developed moderate to severe cGVHD. A non-complimentary set of 32 male donor plasma samples was also collected, as healthy males are not expected to have "self" HY antibodies. Patient plasma samples were collected 1 year post transplant and stored at −20 °C until use. HCT patient and donor characteristics are reported in Table 1. Approval for this study was obtained from the Stanford University Institutional Review Board and individual informed consent was obtained from all patients and donors.

2.2. Protein preparation

Five HY antigens and their X homologues were created using cDNA isolated from male peripheral blood mononuclear cells and amplified using polymerase chain reaction as previously reported by Miklos et al. (2005). Each gene was cloned into *Escherichia coli* (pET-Dest42 and pcDNA-Dest40), adding the V5 C-terminus epitope. Inclusion bodies were synthesized in *E. coli* and solubilized in 6 M guanidine. Purification occurred with nickel affinity chromatography in 6 M urea, which was removed over a 12-hour gradient. V5 tagged proteins were eluded using 250 mM imidazole with 20% glycerol for protein stability. Because the recombinant HY protein UTY is prohibitively large, it was divided into three overlapping fragments, UTY1, UTY2, and UTY3. As UTY2 is the most immunogenic of the three (Nakasone et al., 2015a, 2015b), we focused our UTY analysis on this fragment. Five peptides of the DBY protein, including the most immunogenic peptide, DBY2 (Sahaf et al., 2013), were synthesized by New England Peptide Inc. and used to test the platform's peptide detection capabilities.

Two concentrations of IgG, 0.025 and 0.0125, served as positive control antigens with concentrations within the expected range of alloantibodies. As additional positive controls, we printed several infectious antigen products including cytomegalovirus antigen, herpes simplex virus 1, tetanus toxoid, pneumococcus, influenza, Epstein Barr virus nuclear antigen 1 (EBNA1-V5; R&D, Minneapolis, MN), and varicella zoster virus cell extract. We printed 1× PBS and the HIV antigen p24, which was cloned using a C-terminus V5 expression vector and purified in *E. coli* along with the HY antigens, as our negative controls (Wadia et al., 2010). Accordingly, all patient and control sera that were chosen were determined to be HIV negative.

Table 1
Patient Characteristics.

Patient characteristics	n = 32
Median patient age, year (range)	49 (18–64)
Median time since transplant, d (range)	864 (32–3493)
Median donor age, year (range)	46 (12–69)
Primary disease	
Acute lymphoblastic leukemia	5 (16%)
Acute myelogenous leukemia	6 (19%)
Hodgkin disease	1 (3%)
Myelodysplastic syndrome	3 (9%)
Myelofibrosis	3 (9%)
Multiple myeloma	8 (25%)
Non-Hodgkin lymphoma	6 (19%)
Conditioning	
Myeloablative	13 (41%)
Non-myeloablative	19 (59%)
Donor	
Related	26 (81%)
Unrelated	6 (19%)
Graft	
Bone marrow	2 (6%)
Peripheral blood stem cell	30 (94%)
Normal male characteristics	n = 32
Median age, year (range)	50 (12–71)

2.3. ELISA analysis of the HY antigens

The 64 serum samples were analyzed using ELISA for their particular reactivity to the five HY antigens. Each protein was coated to a row of a 96 well NUNC plate with 50 μl/well at 1.0 μg/ml, as previously described by Miklos et al. (2004). The plates were washed using 1× Tris-buffered saline with 0.1% Tween-20 (TBST). Following this wash, the wells were blocked using 2% nonfat milk for one hour and incubated with the patient sera (1:50) at 4 °C overnight. The samples were developed using anti-human IgG conjugated to alkaline phosphatase. The absorbance was measured at 450–550 nm in order to measure the broadest area.

2.4. Microarray printing and slide surfaces

Each protein was printed in quadruplicate at 100 μm using four quill-tip pins on 16 identical subarrays (Fig. 1). The quill-tip pins enabled antigen to be printed on up to 5 slides, providing 100 applications, before requiring reloading. As seen in Fig. 1, each pin printed an entire subarray, decreasing pin-dependent variability within individual subarrays. Before printing a new antigen, the pins were extensively sonicated and washed. This study used a GeneMachines OmniGrid Accent protein microarray printer. After printing, all slides were left to dry in a desiccated environment for two weeks before plasma incubation.

Prior to printing, we stored and prepared each glass slide surface to its manufacturer's specifications. The six slide surfaces were chosen based upon the currently available array of various chemistries. The surfaces consist of two epoxide-based surfaces, Epoxy 2 (ArrayIt Corp., Sunnyvale, CA) and Epoxy (Schott, Tempe, AZ), two nitrocellulose-based surfaces, Gentel Path Plus and Gentel Clear (Grace Bio-Labs, Bend, Oregon), one NHS-ester-based surface (ArrayIt Corp.), and one hydrogel surface (Schott). As all surfaces make use of different methods of binding antigen, examining epitope availability on each surface was especially important, which was accomplished through V5 and IgG antibody detection.

2.5. Slide processing and antibody detection

Before processing, each slide was pre-washed and blocked using 2% casein (EMD) and 0.08% Triton X-100 in PBS for 1 h at 4 °C. Following blocking, we placed each slide in a pre-blocked rubber gasket with 16 wells (ArrayIt Corp., Sunnyvale, CA). We incubated each subarray with patient plasma diluted at 1:50, determined to be the optimal dilution in prior ELISA experiments through a dilution series (Miklos et al., 2005), for 1 h at 4 °C in wash buffer composed of 0.1% Tween-20 and 1% casein (Sigma) in 1× PBS. The subarrays were aspirated and washed ten times with 100 μl of wash buffer per gasket to remove excess

Please cite this article as: Paul, J., et al., High-throughput allogeneic antibody detection using protein microarrays, J. Immunol. Methods (2016), http://dx.doi.org/10.1016/j.jim.2016.02.009